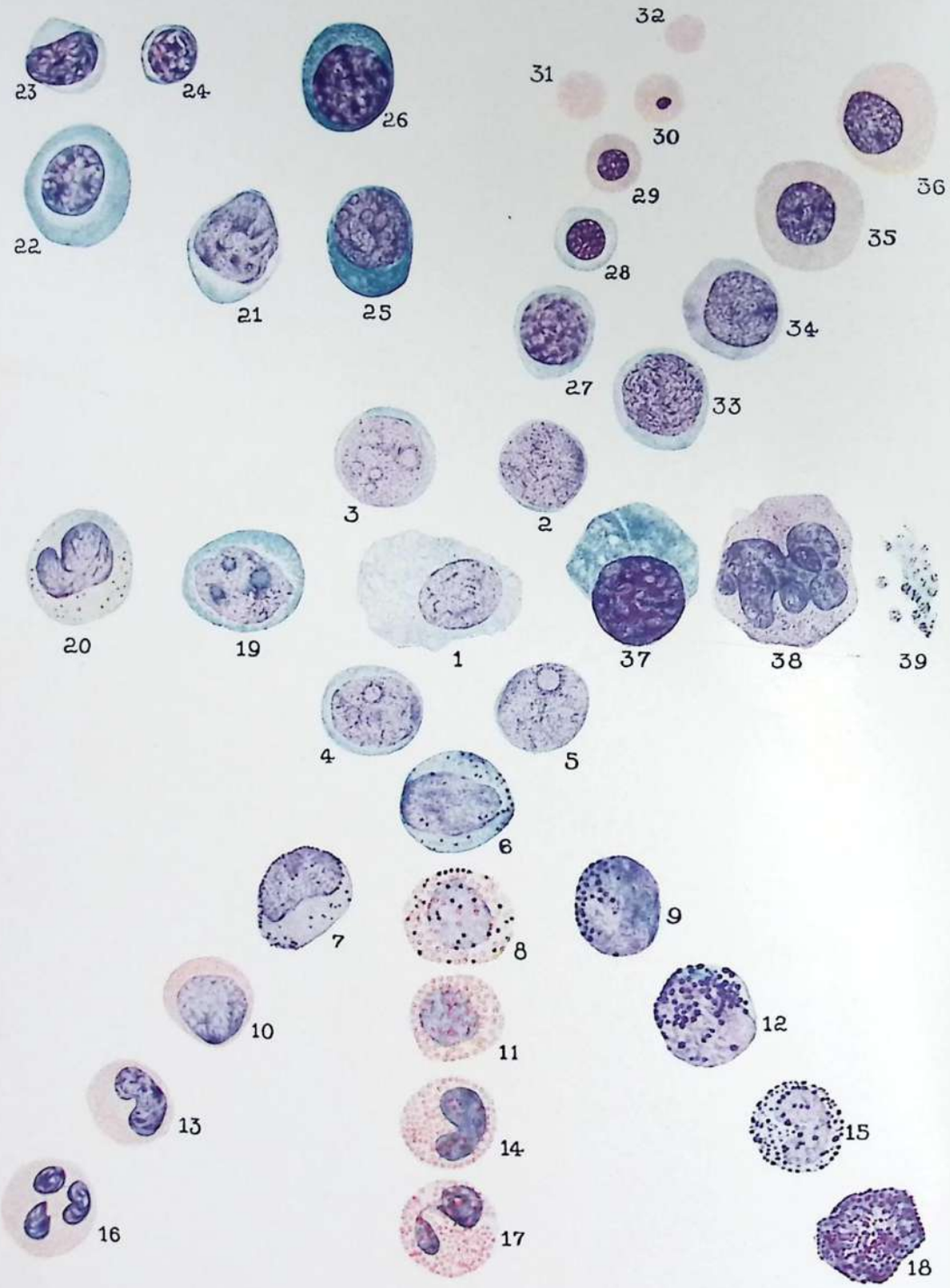
PLATE I



Cells of normal and pathologic blood. (Elaine Limbert, pinx.)

Description of Plate I

The cells of normal and pathologic blood reproduced from actual cells. Wright's stain (× 1000: 1 mm. = 1 μ). Zeiss lamp; ammonia-cupric oxide filter.

1, Reticulo-endothelial cell from normal lymph node. 2, Stem cell, (megaloblast) from bone marrow. 3, Stem cell, (lymphoblast) from acute lymphatic leukemia. 4, Stem cell, (monoblast) from monocytic leukemia. 5, Stem cell, (myeloblast) from myelogenous leukemia. 6–15, Cells from myelogenous leukemia. 6, Leukoblast. 7, 8, 9, Promyelocytes. 10, 11, 12, Myelocytes. 13, 14, 15, Metamyelocytes. 16, 17, 18, Leukocytes, normal blood cells. 19, Immature monocyte, from monocytic leukemia. 20, Monocyte, normal blood cell. 21, Immature lymphocyte, chronic lymphatic leukemia. 22, 23, 24, Lymphocytes, from normal blood. 25, Türk's irritation leukocyte, from chronic myelogenous leukemia. 26, Plasma cell, from chronic myelogenous leukemia. 27–31, Cells from bone marrow. 27, Pronormoblast, or late megaloblast. 28, Basophilic normoblast, or early erythroblast. 29, Polychromic normoblast, or late erythroblast. 30, Orthochromic normoblast. 31, Polychromic erythrocyte, from pernicious anemia. 32, Erythrocyte, from normal blood. 33, 34, 35, 36, Megaloblasts from bone marrow of pernicious anemia. 37, Megakaryoblast, from myelogenous leukemia. 38, Megakaryocyte (× 500: 1 mm. = 2 μ), from bone marrow. 39, Platelets, or thrombocytes from normal blood. For a discussion of Plate I, and theories of hematopoiesis see page 287.

CLINICAL DIAGNOSIS

BY LABORATORY METHODS

A WORKING MANUAL OF CLINICAL PATHOLOGY

By

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TENTH EDITION, THOROUGHLY REVISED
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PREFACE TO THE TENTH EDITION

It is thirty-five years since the appearance of the first edition of this book. Since that time clinical pathology has undergone marvelous development and the book has of necessity grown in size and scope with each new edition. The steadily increasing favor with which the successive editions have been received by students, teachers and laboratory workers indicates that the purpose of the original author was worth while. Ever since my association with Dr. James C. Todd in the preparation of the sixth edition, it has been my desire to maintain his high standards. It is hoped that this tenth edition will meet the needs of teachers, students, medical technologists and physicians.

Clinical pathology has become a recognized specialty in medicine. Every year new methods that have been developed in research laboratories are added to the facilities of the clinical laboratories; consequently, the worker in this field must constantly grow in his knowledge of diagnostic procedures, but old and tried methods must not be forgotten or discarded unless they become obsolete.

Although a few obsolete methods have been dropped, the chief changes are in the inclusion of much new material and a large number of illustrations. Mention may be made of the fluorescent dye method for staining tubercle bacilli. There is a discussion of the significance of porphyrins in the urine. Methods for determining the quantity of sulfonamides and sulfones in the blood and for the identification of sulfonamide crystals in the urine are included. There is a description of the Quick prothrombin test. The importance of hemoconcentration is briefly discussed. The significance of various inclusion bodies is given consideration. There is a new arrangement of the material on rickettsial diseases. Diseases due to fungi are given brief mention, with a number of illustrations. Brewer's new methods for growing anaerobic organisms are described.

I wish to thank a number of my colleagues for helpful suggestions in the preparation of this edition. I would especially mention Dr. Joseph Berkson for the preparation of the material on the normal error of the erythrocyte count, and also Dr. L. H. Beizer for the use of his photographs arranged in plate VIII and for the use of his thesis material in the description of his method for the examination of bone

marrow. Dr. Luther Thompson and Dr. Fordyce Heilman have been consulted frequently concerning bacteriologic methods.

There have been a number of new methods added to the chapter on clinical chemistry, and I wish to express my appreciation to Dr. A. E. Osterberg and to Dr. M. H. Power for their advice in the preparation of this chapter, and to Dr. H. L. Mason in connection with the section on porphyrinuria. In the chapter on clinical chemistry, there has been added the technic for the Haskins and Osgood titration method for the determination of the alkaline reserve figure. Brief discussions of various photelometric methods have been included. The phosphatase method of King and Armstrong, the Kramer and Gittleman method for the determination of sodium, and the Kramer and Tisdall method for the determination of potassium are all given in full. The Ågren and Lagerlöf methods for the determination of trypsin and amylase and the Somogyi technic for the determination of serum amylase have been added. For the determination of sulfonamides, the method of choice is now the technic of Bratton and Marshall, although the older Marshall modified method with slight modification is also included for the determination of sulfones.

Dr. E. R. Mugrage of the University of Colorado Medical School, Denver, has offered many helpful suggestions in the preparation of this and many previous editions. I am especially grateful to Dr. I. Davidsohn of Mt. Sinai Hospital, Chicago, for careful criticisms and information on the section on blood groups. The importance of the Rh factor is emphasized.

The section on serodiagnostic tests for syphilis was completely revised in the ninth edition. The reports of the committee appointed by the Surgeon General of the United States Public Health Service for the purpose of evaluating serodiagnostic methods indicate the desirability of performing more than one test on the same sample of serum. At the time of the last evaluation study in 1941, it became evident that the Mazzini test, which has been used for a number of years in Indiana, is an excellent serodiagnostic method. It has, therefore, been included with the Kline, Kahn, Hinton, Eagle, and Kolmer technics. All technics are given as described by the originators of these methods. I wish to thank Dr. R. A. Vonderlehr, editor of "Venereal Disease Information," published by the United States Public Health Service, for permission to include these technics as published in that bulletin.

There have been added four new plates, three of these in color. I wish to thank Dr. E. T. Bell and Lea and Febiger, publishers of his "Textbook of Pathology," for the use of two colored plates of blood

corpuscles, and also Dr. Phillips Thygeson for the use of his beautiful colored plate of inclusion bodies in inclusion blennorrhea. There are seventy-two new illustrations, in addition, while some of the more obsolete cuts have been deleted. As usual, I am especially appreciative of the invaluable assistance of the Editorial Department of the Mayo Clinic, and of the prompt and efficient cooperation of the publishers, W. B. Saunders Company, in the preparation of this edition.

ARTHUR HAWLEY SANFORD.

ROCHESTER, MINNESOTA,
February, 1943.

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CLINICAL DIAGNOSIS

BY LABORATORY METHODS

INTRODUCTION

USE OF THE MICROSCOPE

THERE is probably no laboratory instrument whose usefulness depends so much upon proper manipulation as the microscope, and

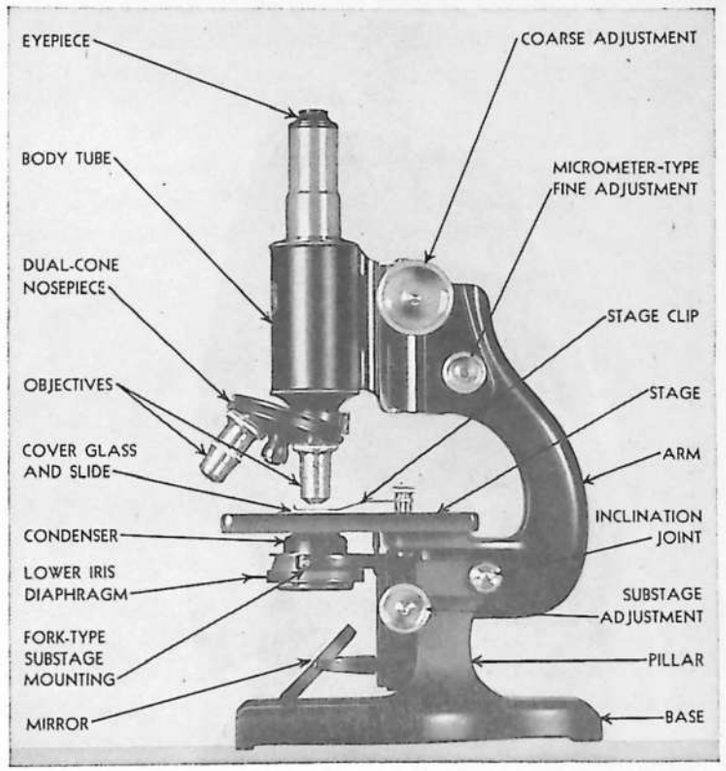


Fig. 1.—The microscope.

none is so frequently misused by beginners. Some suggestions as to its proper use are, therefore, given at this place. It is presumed that the reader is already familiar with its general construction (Fig. 1).

A careful and frequent study should be made of the booklet on the microscope, its use, and care that is furnished with each new microscope by the manufacturer.

Fig. 2.—Lamp for use with the microscope. It should be placed close to the microscope so that the shade protects the eyes from the glare. A blue glass disk is placed in the ring beneath the condenser.

Source of Light.—Good work cannot be done without proper illumination, and this is, therefore, the first and most important consideration for one who wishes to use the microscope effectively.

The light which is generally recommended as best is that from a white cloud, the microscope being placed by preference at a north

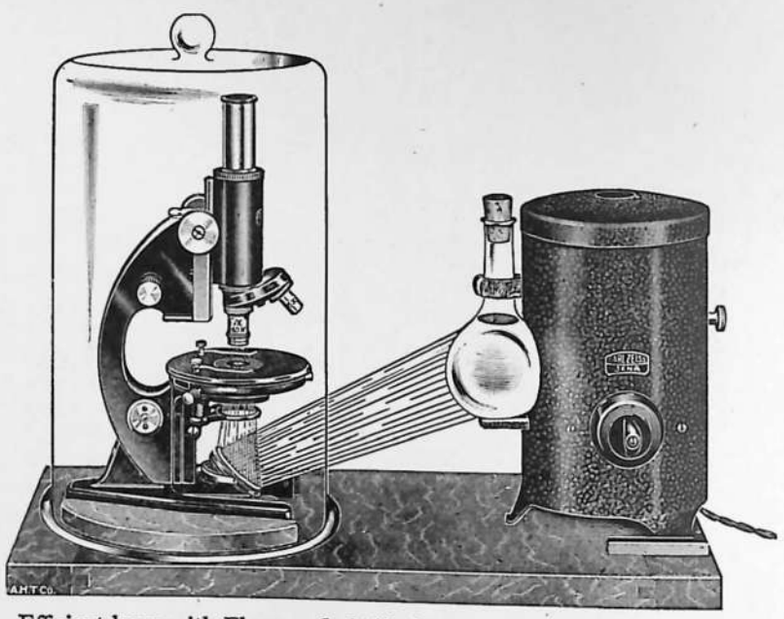


Fig. 3.—Efficient lamp with Florence flask filled with copper sulfate used as a condenser.

window. to avoid direct sunlight. At any other window a white window-shade is desirable. Such light is satisfactory for all ordinary work. Artificial light is, however, imperative for those who must work at night, and is a great convenience at all times. Properly regulated artificial light, moreover, offers decided advantages over day-

light for critical work. Almost any strong light which is diffused through a frosted globe will give fair results. Such a bulb may conveniently be inclosed within a tin or paste-board box, with small openings in the back for ventilation and a circular window in the front to transmit the light. An inexpensive lamp, shown in Fig. 2, is

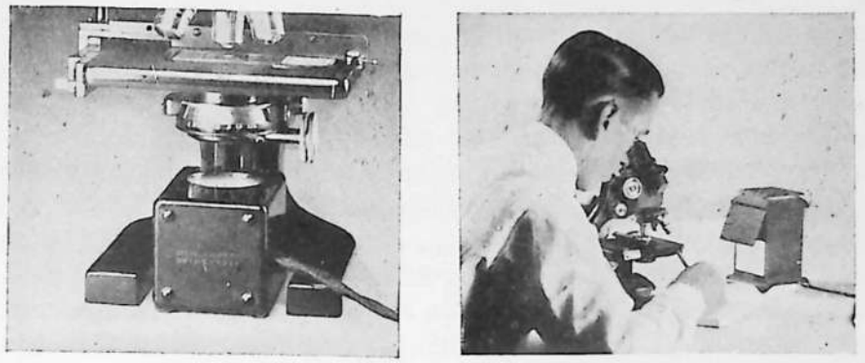


Fig. 4.—Small microscope lamps with daylight-glass filters.

often used. It has the advantage that the eyes are shaded from the glare, while at the same time there is abundant light for drawing or writing upon the table beside the microscope. All such lights have a yellow tinge, and to counteract this a blue glass disk, usually supplied with the microscope, is placed in a supporting ring beneath the

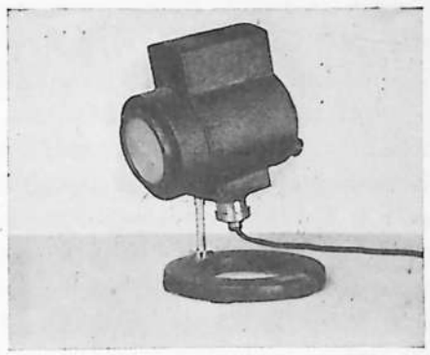


Fig. 5.—An excellent type of microscope lamp suitable both for ordinary work and for dark-field illumination.

condenser. A very effective lamp is shown in Fig. 3. A projection lamp is used for the light; in front of this is placed a piece of frosted glass. In the circular opening in the metal housing, there is placed a Florence flask. In order to filter out the yellow rays the flask is filled with water, to which have been added a few crystals of copper sulfate and a little ammonia.

Two good types of lamps are shown in Fig. 4. Both can be fitted with light filters made of "daylight glass," which, when used with the nitrogen-filled tungsten lamp, transmits a light practically indistinguishable from daylight. Figure 5 illustrates a more expensive type of lamp, but one which has a wide range of usefulness.

The microscope lamp should not stand at so great a distance from the microscope that its image fails to fill the aperture of the condenser—a condition which one can readily detect by removing the ocular and looking down the tube.

Forms of Illumination.—After one has arranged the microscope in proper relation to the source of light, whether this be daylight or any of the artificial sources mentioned above, the next problem is to secure an evenly illuminated field of view without mottling or any trace of shadows. This is accomplished by manipulating the mirror and the condenser. Following this, the direction and the amount of light must be considered in relation to the character of the object under examination as is indicated in the following paragraphs.

Illumination may be either *central* or *oblique*, depending upon the direction in which the light enters the microscope. To obtain **central illumination** the mirror should be so adjusted that the light from the source selected is reflected directly up the tube of the microscope. This is easily done by removing the eyepiece and looking down the tube while adjusting the mirror. The eyepiece is then replaced, and the light reduced as much as desired by means of the diaphragm.

Oblique illumination is obtained in the more simple instruments by swinging the mirror to one side, so that the light enters the microscope obliquely. The more complicated instruments obtain it by means of a rack and pinion, which moves the diaphragm laterally. Beginners frequently use oblique illumination without recognizing it, and are thereby much confused. If the light be oblique, an object in the center of the field will appear to sway from side to side when the fine adjustment is turned back and forth.

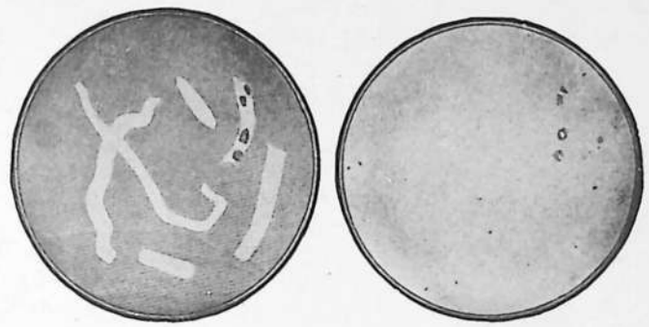
The **amount of light** admitted is also important. It is regulated by the diaphragm.

The bulk of routine work is done with central illumination, and, therefore, every examination should begin with it. Each of the forms of illumination, however—central and oblique, subdued and strong—has its special uses and demands some consideration here. The well-known rule, "Use the least light which will show the object well," is good, but it does not go far enough.

In studying any microscopic structure one considers: (1) Its color (2) its outline, and (3) its surface contour. No one form of illumination shows all of these to the best advantage. It may, therefore, be necessary to change the illumination many times during a microscopic examination.

To see color best, use central illumination with strong light. The principle is that by which a stained glass window shows the purest color when the light is streaming through it. Strong central light is, therefore, to be used for structures such as stained bacteria, whose recognition depends chiefly upon their color, and, alternating with other forms, for stained structures in general.

To study the outline of an object use very subdued central illumination. The diaphragm is closed to the point which trial shows to be best in each case. This illumination is required by delicate colorless objects, such as hyaline tube casts and cholesterol crystals, which



A B

Fig. 6.—A, Hyaline casts, one containing renal cells; properly subdued illumination; B, same as A; strong illumination. The casts are lost in the glare, and only the renal cells are seen. (From Greene's "Medical Diagnosis.")

are recognized chiefly by their outline. The usual mistake of beginners is to work with the diaphragm too wide open. Strong light will often render semitransparent structures entirely invisible (Fig. 6).

To study surface contour use oblique light of a strength suited to the color or opacity of the object. In routine work oblique illumination is resorted to only to study more fully some object which has been found with central illumination, as, for instance, to demonstrate the cylindric shape of a hyaline tube cast.

Dark-field illumination consists in blocking out the central rays of light and directing the peripheral rays against the microscopic object from the side. Only those rays which strike the object and are reflected upward pass into the objective. The object thus appears bright upon a black background. By means of this form of illumination very minute structures can be seen, just as particles of dust in

the atmosphere become visible when a ray of sunlight enters a darkened room.

Dark-field illumination for low-power work can be obtained by means of the ring stops with central disks which accompany most microscopes when purchased. The stop is placed in a special ring beneath the condenser. When the regular stop is not at hand, one can use the glass disk which is generally supplied with the microscope or an extra large round cover glass, in the center of which is pasted a circular disk of black paper. The size of the black disk depends upon the aperture of the objective with which it is to be used, and can be ascertained by trial. For best results the condenser should be oiled to the under surface of the slide and should be focused on the object under examination.

For oil-immersion work a special condenser is necessary. This is sold under the name of reflecting condenser or dark-field illuminator. In its most desirable form this is interchangeable with the regular substage condenser. One type has a lamp built in or attached beneath it (Fig. 7). Some makers now offer a condenser which allows one to change almost instantly from bright-field to dark-field, and vice versa, with, however, some loss of quality in both. Objectives used for dark-field work must be of relatively low numeric aperture (usually less than 0.9); hence, when the ordinary oil-immersion objective is used, its aperture must be reduced by placing in it a "funnel stop" obtainable from the maker of the objective. Oil-immersion objectives of low N. A., especially designed for this work, but also useful for bright-field work, have recently been placed on the market. If such examinations are made frequently, it is an advantage to use a specially designed dark-field microscope with the dark-field condenser and illuminant in positive alignment and a rheostat control on the substage lamp (Fig. 8).

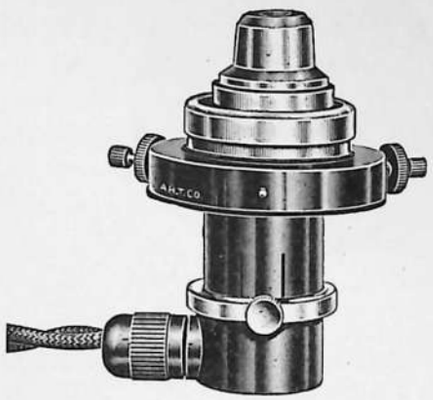


Fig. 7.—Dark-field condenser with lamp attached.

The chief use of dark-field illumination in clinical work is for demonstration of *Treponema pallidum* in fresh material (Fig. 345).

Method of Using Dark-field Condenser.—A small drop of the fluid to be examined is placed on a clean slide of the correct thickness and covered

with a clean cover glass. The thickness of the slide is important owing to the need of accurately focusing the condenser, and the proper thickness to be used with a particular condenser is generally engraved upon its mounting. This is usually between 1 and 1.55 mm. The layer of fluid must be thin. The slide and cover must be free from scratches; air-bubbles must be avoided, and also any excess of objects (blood corpuscles, pus cells, and so forth) other than those which are sought, since all of these tend to brighten the background and thus reduce contrast. Because of the short working distance of oil-immersion objectives, number 1 cover glasses are essential.

The source of light, other than the built-in lamp, may be direct sunlight, or, preferably, a strong artificial light with bull's-eye or water-bottle con-

Fig. 8.—Dark-field microscope.

denser to deliver parallel rays to the mirror. When the arc light is used, it is desirable to interpose a piece of lightly oiled ground glass between the light and the mirror, but this is not to be inserted until after the condenser has been focused.

The regular substage condenser is removed, the dark-field condenser is inserted in its place, and accurately centered in the optic axis. To facilitate centering, a series of concentric circles is generally ruled on the top of the condenser. These circles are brought to the center of the field of a low-power objective by means of centering screws provided for the purpose. A drop of immersion oil is then placed on the apex of the condenser, the slide is placed in position on the stage, and the condenser is raised until the oil is in contact with the under surface of the slide. The low-power objective

is now focused on the slide. If the light be sufficiently intense and the mirror properly adjusted, a circle or a spot of light should be seen in the center of the field. A circle indicates that the condenser is decidedly above or below its correct position (Fig. 9). The condenser is then focused by raising or lowering it until the circle becomes a spot of light and this spot becomes as small and as bright as it is possible to make it. This spot is also utilized for centering such condensers as are provided with the concentric centering circles mentioned above. The low-power objective is finally replaced by the higher power with which the examination is to be made and this is brought to focus and used in the ordinary way.

For dark-field work the various adjustments must be much more exact than for bright field. The most frequent causes of failure to secure a satisfactory dark field with brilliantly lighted objects which appear to be self-luminous are:

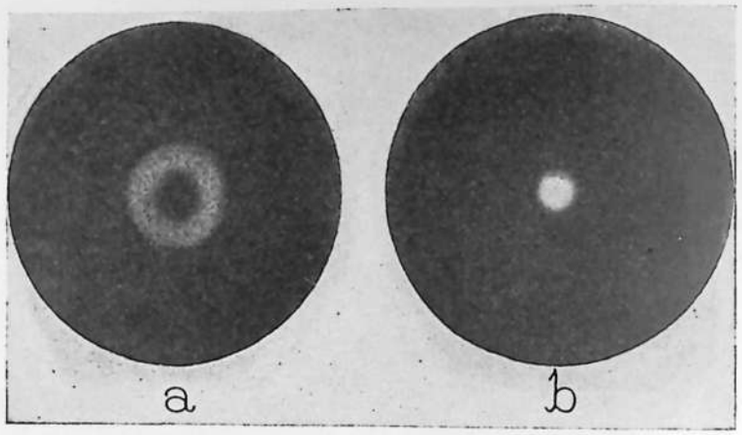


Fig. 9.—Dark-field illumination: a, Circle of light seen with a low-power objective when the condenser is above or below the correct focus. b, The bright spot of light seen with a low-power objective when the condenser is correctly focused.

1. Use of an objective of too high aperture. When the regular oil-immersion objective is used, its aperture must be reduced by means of the stop provided by the makers.

2. Failure accurately to focus and center the condenser. Very slight readjustments of condenser or mirror after the examination is begun may remedy matters, provided the slide is not too thick to permit accurate focusing.

3. Inclusion of air-bubbles in the preparation or in the oil above or below the slide. It is generally necessary to remove the oil and apply again.

4. Inclusion of too many microscopic objects in the field. This may be remedied by diluting the fluid to be examined, or by reducing the thickness of the preparation by means of slight pressure on the cover glass.

The Condenser.—For the work of the clinical laboratory a substage condenser is a necessity. Its purpose is to condense the light upon the object to be examined. For critical work the light must be focused on the object by raising or lowering the condenser by means of the screw provided for the purpose. The image of the light source will then appear in the plane of the object. This is best seen by using a low-power objective and ocular. Should the image of the window-frame or other nearby object appear in the field and prove annoying, the condenser may be raised or lowered a little. It is often advised to remove the condenser for certain kinds of work, but this is not necessary and is seldom desirable in the clinical laboratory.

The condenser is constructed for parallel rays of light. With daylight, therefore, the plane mirror should be used, while for the divergent rays of ordinary artificial light the concave mirror, which tends to bring the rays together, is best.

It is very important that the condenser be accurately centered in the optical axis of the instrument, and most high-grade instruments have centering screws by which it can be adjusted at any time. The simplest way to recognize whether the condenser is centered is to close the diaphragm beneath it to as small an opening as possible, then remove the eyepiece and look down the tube. If the diaphragm opening does not appear in the center of the field, the condenser is out of center.

The use of the condenser is further discussed in the following sections.

Objectives and Eyepieces.—Unfortunately, different makers use different systems of designating their lenses. The system used in this country is to designate objectives by their focal lengths in millimeters or by their "initial magnification," and eyepieces by their magnifying power, indicated by an "×." Most foreign makers use this system for their high-grade lenses, but still cling to arbitrary letters or numbers for the others.

Objectives are of two classes—achromatic and apochromatic. Those in general use are of the achromatic type, and they fulfil all requirements for ordinary work. Apochromatic objectives are more highly corrected for chromatic and spheric aberration, and represent the highest type of microscope lenses produced. They give crisp images with little or no trace of the color fringes which with achromatic objectives can readily be seen about the edges of black or colorless objects lying in a bright field, and are hence very desirable for photomicrography and research. A compensating ocular must be used with an apochromatic objective. A third type of objective, which is made of fluorite, requires no special eyepiece, and offers color correction midway between an apochromatic and an achromatic objective. This is sometimes designated as a "semi-apochromat."

The simple eyepieces which have long been used with achromatic objectives are known as Huygenian oculars. With apochromatic objectives it is necessary to use special "compensating eyepieces" which are corrected to overcome certain defects in this type of objective. The same compensating eyepieces may be used with oil-immersion and high dry objectives of the achromatic series. A third type of ocular has recently been introduced under the trade names "hyperplane," "periplan," and so forth. They have a compensation midway between the compensating and Huygenian eyepieces and may be used with either achromatic or apochromatic objectives. Their chief advantage is that they overcome to a very marked degree the curvature of field of any objective with which they may be used.

Objectives are "corrected" for use under certain fixed conditions, and *they will give the best results only when used under the conditions for which corrected.* The most **important corrections** are: (1) For tube length, (2) for thickness of cover glass, and (3) for the medium between objective and cover glass.

1. The tube length with which an objective is to be used is usually engraved upon it—in most cases it is 160 mm. The draw tube of the microscope should be pulled out until the proper length (measured with ocular and objective removed) is obtained. The length is indicated by the graduations upon the side of the draw tube, but in some cases this scale is made for use without a nosepiece. When a nosepiece is attached it adds about 15 mm. to the tube length.

2. The average No. 2 cover glass is about the thickness for which most objectives are corrected—usually 0.17 or 0.18 mm. One can get about the right thickness by buying No. 2 covers and discarding the thick ones, or by buying No. 1 covers and discarding the thinner ones. Slight differences in cover-glass thickness can be compensated by increasing the length of tube when the cover is too thin, and decreasing it when the cover is too thick. This should be done with a spiral motion while supporting the body tube with the other hand. The amount of correction necessary will depend upon the focal length and numeric aperture of the objective. With a 4-mm. objective of 0.85 numeric aperture a difference of 0.03 mm. in cover-glass thickness requires a change of 30 mm. in the tube length. Many high-grade objectives are supplied with a "correction collar," which accomplishes the same end. While for critical work, especially with apochromatics, cover-glass thickness is very important, one pays little attention to it in the clinical laboratory. A high-power dry lens always requires a cover, but its exact thickness is unimportant

in routine work. Very low-power and oil-immersion objectives may be used without any cover glass.

3. The correction for the medium between objective and cover glass is very important. This medium may be either air or some fluid, and the objective is hence either a "dry" or an "immersion" objective. The immersion fluid generally used is an especially prepared cedar oil, which gives great optical advantages because its index of refraction is the same as that of crown glass. It is obvious that only objectives with very short working distance, as the 2 mm., can be used with an immersion fluid.

To use an oil-immersion objective a suitable field for study should first be found with the low power. A drop of immersion oil is placed on the slide and the objective lowered until it is in contact with the oil and almost touches the slide. This is observed with the eye on a level with the stage. Then, with the eye looking into the microscope, the objective is very slowly raised until the objects on the slide are in focus. In order to avoid air bubbles the oil must be placed on the slide carefully and without stirring it. Bubbles are a frequent source of trouble, and should always be looked for when an immersion objective does poor work. They are readily seen by removing the eyepiece and looking down the tube. If they are present, the oil must be removed and a new drop applied. Immediately after use both objectives and slide should be wiped clean with lens paper or a soft linen handkerchief. In an emergency glycerin may be used instead of cedar oil, but, of course, with inferior results.

Curvature of field, through which it is impossible to focus both center and periphery sharply at the same time, is a very noticeable defect; but it is less serious than appears at first sight, particularly for visual work. It is easily compensated by frequent use of the fine focusing adjustment. Complete flatness of field cannot be attained without sacrifice of other and more desirable properties. Some of the finest objectives made, notably the apochromatics, show decided curvature.

The **working distance** of an objective should not be confused with its focal distance. The former term refers to the distance between the front lens of the objective, when it is in focus, and the cover glass. It is always less than the focal distance, since the "focal point" lies somewhere within the objective; and it varies considerably with different makes. Long working distance is a very desirable feature. Some oil-immersion objectives have such short working distance that only very thin cover glasses can be used.

The **formation of the microscopic image** demands brief consid-

eration (Fig. 10). The rays of light which are reflected upward from the mirror and which pass through the object are brought to a focus in a magnified, inverted real image. This can be focused to appear at different levels, but when the microscope is used in the ordinary way

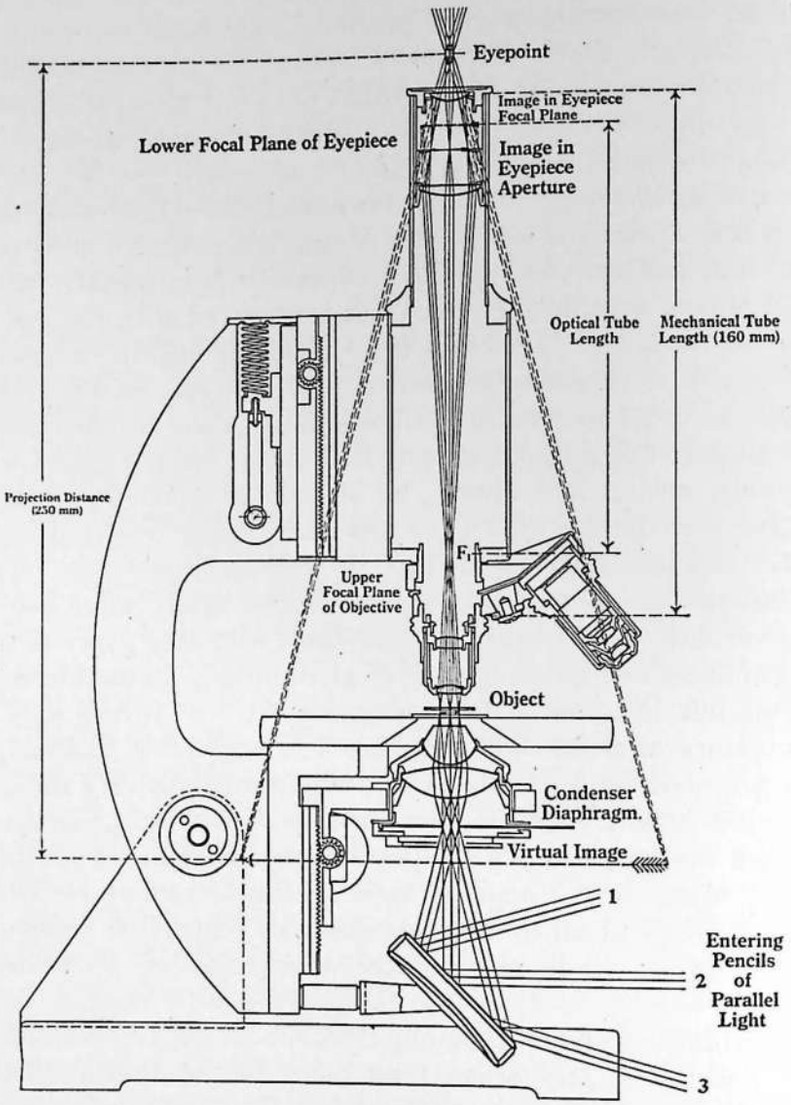


Fig. 10.—Path of light through the microscope.

it is formed at about the level of the diaphragm in the ocular. It can be seen by removing the ocular, placing a piece of ground glass on the top of the tube, and focusing upon it. When viewing this image a roll of paper or a cylindric mailing tube should be used to exclude extraneous light. This image, in turn, is magnified by the eye lens of

the ocular, producing a second real image, which is again inverted, and therefore, shows the object right side up. This can be seen upon a ground glass held a few inches above the ocular, provided strong artificial light be used and the room darkened. The eye, when it looks into the microscope, sees, not this real image, but rather an inverted *virtual image* which appears about 250 mm. (10 inches) in front of the eye.

Numeric Aperture.—This expression, usually written N. A., indicates the amount of light which enters an objective from a point in the microscopic field. In optical language N. A. is the sine of one-half the angle of aperture multiplied by the index of refraction of the medium between the cover and the front lens. Numeric aperture is extremely important, because upon it depends *resolving power*, which is the most important property of an objective.[1]

Resolving power is the ability to separate minute details of structure. For example, the dark portions of a good half-tone picture appear gray or black to the unaided eye, but a lens easily resolves this apparently uniform surface into a series of separate dots. Resolving power does not depend upon magnification. The fine lines and dots upon certain diatoms may be brought out clearly and crisply (that is, they are resolved) by an objective of high numeric aperture, whereas with an objective of lower numeric aperture, but greater magnifying power, the same diatom may appear to have a smooth surface, with no markings at all, no matter how greatly it is magnified. Knowing the N. A., it is possible to calculate how closely lines and dots may lie and still be resolved by a given objective. To state the numeric aperture, therefore, is to tell what the objective can accomplish, provided, of course, that spheric and chromatic aberrations are satisfactorily corrected. An objective's N. A. is usually engraved upon the mounting.

It is an important fact, and one almost universally overlooked by practical microscopists, that the proportion of the numeric aperture of an objective which is *utilized* depends upon the aperture of the cone of light delivered by the condenser. In practice, the numeric aperture of an objective is reduced nearly to that of the condenser (which is indicated by lower case letters, n. a.).[2] The condenser should, therefore, have a numeric aperture at least equal to that of the objective with which it is to be used. Lowering the condenser below

[1] Resolving power really depends on two factors, the N. A. and the wavelength of light, but the latter can be ignored in practice.

[2] The N. A. of the objective is not reduced wholly to that of the condenser, because, owing to diffraction phenomena, a part of the unilluminated portion of the back lens is utilized.

its focal distance and closing the diaphragm beneath it have the effect of reducing its working aperture. A condenser, whatever its numeric aperture, cannot deliver through the air a cone of light of greater N. A. than 1. From these considerations it follows that the proper adjustment of the substage condenser is a matter of great importance when using objectives of high N. A., and that, to gain the full benefit of the resolving power of such objectives, the condenser must be focused on the object under examination, it must be oiled to the under surface of the slide in the same way as the immersion objective is oiled to the cover glass, and the substage diaphragm must be wide open. The last condition introduces a difficulty in that colorless structures will appear "fogged" in a glare of light, making a satisfactory image impossible when the diaphragm is more than three quarters open (Fig. 6). Wright suggests that the size of the light source be so regulated by a diaphragm that its image, thrown on the slide by the condenser, coincides with the real field of the objective, and maintains that in this way it is possible to reduce the glare of light and to dispel the fog without closing the diaphragm.

One can easily determine how much of the aperture of an objective is in use by removing the eyepiece, looking down the tube, and observing what proportion of the back lens of the objective is illuminated. The relation of the illuminated central portion to the unilluminated peripheral zone indicates the proportion of the numeric aperture in use. The effect of raising and lowering the condenser and of oiling it to the slide can thus be easily seen.

Another property of an objective which depends largely upon N. A. is **depth of focus,** the ability to render details in different planes clearly at the same time. The higher the N. A. and the greater the magnification, the less the depth of focus. Any two objectives of the same focal length and same N. A. will have exactly the same depth of focus. Depth of focus can be increased by closing down the diaphragm, and thus reducing the N. A. Great depth is desirable for certain low-power work, but for high powers it does not offer advantages to balance the loss of N. A. by which it is attained. In some cases, indeed, it is a real disadvantage.

Magnification.—The degree of magnification should always be expressed in *diameters*, not *times*, which is a misleading term. The former refers to increase of *diameter;* the latter, to increase of *area.* The comparatively low magnification of 100 diameters is the same as the apparently enormous magnification of 10,000 times.

According to the system of rating magnification in use in this country, the magnifying power of an objective is ascertained by

dividing the *optical tube length* (Fig. 10) by the focal length of the objective. The optical tube length is usually somewhere near 165 mm., but it varies with the different objectives; and the makers' catalogs must be consulted for an accurate statement of magnifying power. Some makers follow the commendable plan of engraving both the focal length of the objective and its initial magnification upon its barrel.

This system of rating magnification measures the enlarged image at the level of the diaphragm in the ocular, and this image is, in turn, magnified by the ocular, so that when an objective and ocular are used together the total magnification is the product of the two. In the case, for example, of the 1.9 mm. oil-immersion objective, whose initial magnification is 95 diameters, the total magnification with the 5× ocular is 475 diameters.

It is easy to find the magnifying power of any combination of objective and ocular by actual trial. Place the counting slide of the hemacytometer upon the microscope and focus the ruled lines. Now adjust a sheet of paper upon the table close to the microscope in such a position that when the left eye is in its proper place at the ocular the paper will lie in front of the right eye at the normal visual distance, that is, 250 mm. (10 inches). The paper may be supported upon a book, if necessary. If both eyes are kept open, the ruled lines will appear to be projected on the paper. With a pencil mark on the paper the apparent location of the lines which bound the small squares used in counting red blood corpuscles and measure the distance between the marks. Divide this distance by 0.05 mm., which is the actual distance between the lines on the slide. The quotient gives the magnification. If, to take an example, the lines in the image on the paper are 5 mm. apart, the magnification is 100 diameters. The figures obtained in this way will vary somewhat as one is near- or far-sighted, unless the defect of vision is corrected with glasses.

In practice, magnification can be increased in one of three ways:

1. *Drawing Out the Tube.*—Since the increased tube length interferes with spheric correction, it should be used only with the knowledge that an imperfect image will result.

2. *Using a Higher Power Objective.*—As a rule, this is the best way, because resolving power is also increased; but it is often undesirable because of the shorter working distance, and because the higher objective often gives greater magnification than is desired, or cuts down the size of the real field to too great an extent.

3. *Using a Higher Power Eyepiece.*—This is the simplest method. It has, however, certain limitations. When too high an eyepiece is used, there results a hazy image in which no structural detail is seen

clearly. This is called "empty magnification," and depends upon the fact that the objective has not sufficient resolving power to support the high magnification. It has been aptly compared to the enlargement, by stretching in all directions, of a picture drawn upon a sheet of rubber. No new detail is added no matter how great the enlargement. The extent to which magnification can be satisfactorily increased by eyepieces depends wholly upon the resolving power of the objective, and consequently upon the N. A. The greatest total or combined magnification which will give an *absolutely* crisp picture is found by multiplying the N. A. of an objective by 400. The greatest magnification which can be used at all satisfactorily is 1000 times the N. A. For example: The ordinary 1.9-mm. objective has a N. A. of 1.30; the greatest magnification which will give an absolutely sharp picture is 520 diameters, which is obtained approximately by using a 5.5 × eyepiece. Higher eyepieces can be used, up to a total magnification of 1300 diameters (12.5 × eyepiece), beyond which the image becomes wholly unsatisfactory.

The Microscope in Use.—Optically, it is a matter of indifference whether the instrument be used in the vertical position or inclined. Examination of fluids requires the horizontal stage, and since much of the work of the clinical laboratory is of this nature it is well to accustom one's self to the use of the vertical microscope. While working one should sit as nearly upright as is possible compatible with comfort, and the height of the seat should be adjusted with this in view.

It is always best to "focus up," which saves annoyance and probable damage to slides and objectives. This is accomplished by bringing the objective nearer the slide than the proper focus, and then, with the eye at the eyepiece, turning the tube up until the object is clearly seen. *The fine adjustment should be used only to get an exact focus with the higher power objectives after the instrument is in approximate focus*. It should not be turned more than one revolution.

There will be less fatigue to the eyes if both are kept open while using the microscope, and if no effort is made to see objects which are out of distinct focus. Fine focusing should be done with the fine adjustment, not with the eye. An experienced microscopist keeps his fingers almost constantly upon one or other of the focusing adjustments.

Although the ability to use the eyes interchangeably is sometimes very desirable, greater skill in recognizing objects will be acquired if the same eye be always used. The left eye is the more convenient, because the right eye is thus left free to observe the

drawing one may wish to do with the right hand. After a little practice one can cause the microscopic image to appear as if projected upon a sheet of paper placed close to the microscope under the free eye. This gives the effect of a camera lucida, and it becomes very easy to trace outlines. When one is accustomed to spectacles they should not be removed.

It is very desirable that one train oneself to work with the low-power objective as much as possible, reserving the higher powers for detailed study of the objects which the low power has found. This makes both for speed and for accuracy. A search for tube casts, for example, with the 4-mm. objective is both time consuming and liable to failure. Even such minute structures as nucleated red corpuscles in a stained blood-film are more quickly found with an 8-mm. or even a 16-mm. objective combined with a high ocular than with the oil-immersion lens. It is difficult for one who has not measured it to realize how small is the "real field," that is, the actual area of the slide which is seen through the microscope (Fig. 11).

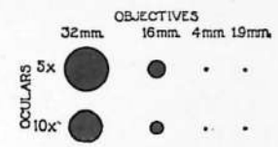


Fig. 11.—Showing the size of the "real fields" (actual areas seen through the microscope) with various objectives and oculars and tube length of 160 mm. The size differs slightly with different makes.

To be seen most clearly, an object should be brought to the center of the field. *Acuity of vision will be greatly enhanced and fatigue lessened if all light except that which enters through the microscope be excluded from both eyes*. Strong light should not be allowed to fall directly upon the surface of the slide, as this clouds the image, especially with low powers. To this end various eye shades have been devised, and some workers go so far as to work inside a small tent constructed of strips of wood covered with black cloth, the source of illumination being placed outside the tent.

A useful **pointer** can be made by placing a straight piece of a hair across the opening of the diaphragm of the eyepiece, cementing one end with a tiny drop of balsam, and cutting the hair in two in the middle with small scissors. When the eyepiece is in place, the hair appears as a black line extending from the periphery to the center of the microscopic field. If the pointer does not appear sharply defined it is out of focus, and the diaphragm must be raised or lowered a little within the ocular.

One often wishes to mark a particular field upon a permanent preparation so as to refer to it again. If the mechanical stage is permanently fastened to the stage of the microscope, the vernier can

be used for marking the position of an object on the slide. There are on the market several "object markers" by which a desired field can be marked with ink, or by a circle scratched on the cover glass by a minute diamond, while the slide is in place on the microscope. The circle is easily located with a low power. In the absence of these one can, while using the low power, place minute spots with a fine pen at the edge of the field on opposite sides.

A good marking material is a cement which Todd used for marking cells, ringing cover glasses, and so forth. To a few ounces of white shellac in wood alcohol add an equal volume of gasoline, shake thoroughly, and let stand for twenty-four hours, or until well separated into two layers. Pipet off the clear lower portion, add 5 to 10 drops of castor oil to each ounce, and color with any aniline dye dissolved in absolute alcohol. When too thick, thin with alcohol. This makes a beautiful, transparent, easy-flowing cement which does not crack and which is not readily attacked by xylol.

Many good workers advise against the use of spring clips to hold the slide against the stage of the microscope. Manipulation of the slide with the fingers alone certainly gives good training in delicacy of touch, and is desirable when examining infectious material which might contaminate the clips, or when one must detect slight pressure of the objective upon the cover glass, as in studying a hanging-drop preparation. For the majority of examinations, however, it is more satisfactory to use a clip at one end of the slide, with just sufficient pressure to hold the slide without interfering with its freedom of movement.

Occasionally when one wishes a very low-power objective for some special work it may be desirable to unscrew the front lens of the 16-mm. objective and use the back lens only. This procedure is not recommended for critical work, and it should not be tried with high-power objectives, *which must never be taken apart.*

To attach an objective it should be supported in position against the nosepiece by means of the index finger and middle finger, which grasp it as one would a cigar. It is then screwed into place with the fingers of the other hand.

Care of the Microscope.—The microscope is a delicate instrument and should be handled accordingly. Even slight disturbance of its adjustments may cause serious trouble. It is so heavy that one is apt to forget that parts of it are fragile. It seems unnecessary to say that when there is unusual resistance to any manipulation, force should never be used to overcome it until its cause has first been sought;

and yet it is no uncommon thing to see students, and even graduates, push a high-power objective against a microscopic preparation with such force as to break not only the cover glass, but even a heavy slide.

To bend the instrument at the joint the force should be applied to the pillar and never to the tube or the stage.

The microscope should be kept scrupulously clean, and dust must not be allowed to settle upon it. When not in use the instrument should be kept in its case or under a cover. An expensive glass bell jar is not needed, and, in fact, is undesirable, except for display. It is heavy and awkward to handle, and when lifted is almost certain (unless great care is exercised) to strike the microscope. It is particularly liable to strike the mechanical stage and disturb its adjustment. The simplest, cheapest, lightest, and probably the best cover for the microscope is a truncated cone or pyramid of pasteboard, covered with cretonne or similar material. This is easily made at home. In the absence of a special cover a square of lintless cloth may be draped over the microscope.

Lens surfaces which have been exposed to dust only should be cleaned with a camel's-hair brush. A small brush and a booklet of lens paper should always be at hand in the microscope case. Those surfaces which are exposed to finger marks should be cleaned with lens paper, or a soft linen handkerchief, moistened with water if necessary. The rubbing should be done very gently and with a circular motion. Particles of dirt which are seen in the field are upon the slide, the eyepiece, or the condenser. Their location can be determined by moving the slide, rotating the eyepiece, and lowering the condenser. Dirt on the objective cannot be seen as such; it causes a diffuse cloudiness. When the image is hazy, the objective probably needs cleaning; or in case of an oil-immersion lens, there may be bubbles in the oil.

Oil and balsam which have dried upon the lenses—an insult from which even dry objectives are not immune—may be removed with alcohol or xylol; but these solvents must be used sparingly and carefully, as there is danger of softening the cement between the components of the lens. Some manufacturers now claim to use a cement which resists xylol. Care must be taken not to get any alcohol upon the brass parts, as it will remove the lacquer. Balsam and dried oil are best removed from the brass parts with xylol.

When the vulcanite stage becomes brown and discolored the black color can be restored by rubbing well with petrolatum.

Measurement of Microscopic Objects.—The importance of size in identification of microscopic structures cannot be too strongly em-

phasized. Even very rough measurements will often prevent humiliating blunders. The principal microscopic objects which are measured clinically are animal parasites and their ova and abnormal blood corpuscles. The metric system is used almost exclusively. For very small objects, 0.001 mm. has been adopted as the unit of measurement, under the name *micron*. It is represented by the Greek letter μ. For larger objects, where exact measurement is not essential, the diameter of a red blood corpuscle (7 to 8 μ) is sometimes taken as a unit. Of the several methods of measurement, the most convenient and accurate is the use of a micrometer eyepiece. In its simplest form this is similar to an ordinary eyepiece, but it has within it a glass disk upon which is ruled a graduated scale. When this eyepiece is placed in the tube of the microscope, the ruled lines appear in the microscopic field, and the size of an object is readily determined in *terms of the divisions of this scale*. The value of these divisions in millimeters manifestly varies with different magnifications. Their value must, therefore, be determined separately for each objective. This is accomplished through use of a stage micrometer—a glass slide with carefully ruled scale divided into subdivisions, usually hundredths of a millimeter. The stage micrometer is placed upon the stage of the microscope and brought into focus. The tube of the microscope is then pushed in or pulled out until two lines of the one scale exactly coincide with two lines of the other. From the number of divisions of the eyepiece scale which then correspond to each division of the stage micrometer the value of the former in micra or in fractions of a millimeter is easily calculated. *This value, of course, holds good only for the objective and the tube length with which it was found*. The counting slide of the hemacytometer will answer in place of a stage micrometer, the lines which form the sides of the small squares used in counting red blood corpuscles being 50 micra apart. When using the counting chamber with an oil-immersion lens a cover must be used, otherwise the oil will fill the ruled lines and cause them to disappear. Any eyepiece can be converted into a micrometer eyepiece by placing a micrometer disk—a small circular glass plate with ruled scale—ruled side down upon its diaphragm. If the lines upon this are at all hazy, the disk has probably been inserted upside down, or else the diaphragm is out of its proper position.

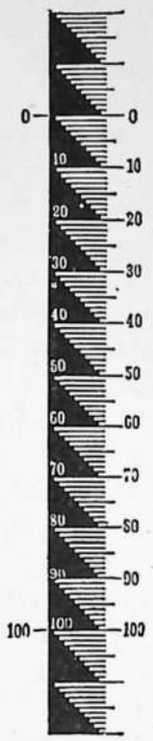


Fig. 12.—Scale of the step micrometer eyepiece.

Usually it can be pushed up or down as required. The "step" micrometer eyepiece is very satisfactory. The steplike arrangement of the scale (Fig. 12) makes it easy to read, and the divisions are such that they read in microns or easy multiples of microns with little or no change from the regular tube length.

The following method of micrometry is less accurate, but is fairly satisfactory for comparatively coarse objects, such as the ova of parasites. A ruled scale corresponding to the magnified image of the hemacytometer ruling is drawn upon cardboard in the manner described for ascertaining magnifications (p. 15), except that the card is placed upon the table beside the microscope and not necessarily at a distance of 10 inches from the eye. This card may then be used as a micrometer, and should be inscribed with the value of its gradua-

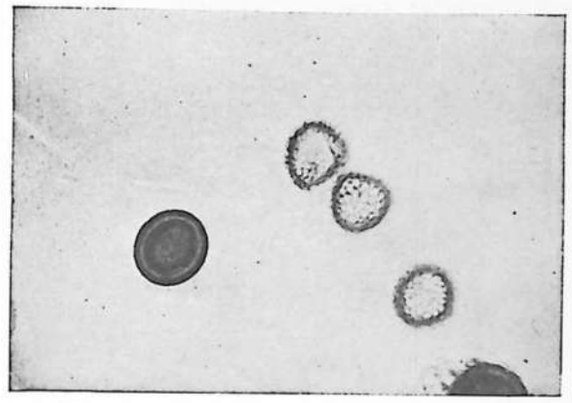


Fig. 13.—Egg of *Taenia saginata*. Lycopodium granules used as micrometer as described in the text. Since the granules average 30 μ in diameter, the egg is seen by comparison to be about 35 by 40 μ (photograph × 250).

tions, and the objective, ocular, and tube length with which it is to be used. In the example cited upon page 15 the lines on the card are 5 mm. apart, corresponding to an actual distance of 50 μ. To measure an object, the cardboard is placed in the position which it occupied when made (upon the table at the right of the microscope). The lines and the objects on the slide can then be seen together, and the space covered by any object indicates its size. The graduations made as above indicated are too coarse for most work, and they should be subdivided. If five subdivisions are made, each will have a value of 10 μ.

Tuttle has suggested that in fecal and other examinations a little lycopodium powder be mixed with the material. The granules are of fairly uniform size—30 μ in diameter—and are easily recognized (Fig. 13). They furnish a useful standard with which the size of

other structures can be compared. Care must be exercised not to use too much powder. The lycopodium is conveniently kept in a gelatin capsule, and a faint cloud can be dusted over the slide by gently scraping the edge of the lid upon the rim of the capsule.

Photomicrography.—Although high-grade photomicrography requires expensive apparatus and considerable skill in its use, fairly good pictures of microscopic structures can be made by any one with simple instruments.

Any camera which is equipped with a focusing screen may be used. It is best to remove the photographic lens. The camera is placed with the lens opening looking into the eyepiece of the microscope, which may be in either the vertical or the horizontal position. One can easily rig up a standard to which the camera can be attached in the proper position by means of a tripod screw. A light-tight connection can be made of a cylinder of paper or a cloth sleeve with drawstrings. The image will be thrown upon the ground-glass focusing screen, and is focused by means of the fine adjustment of the microscope. The degree of magnification is ascertained by placing the ruled slide of the blood-counting instrument upon the microscope and measuring the image on the screen. The desired magnification is obtained by changing objectives or eyepieces or lengthening the camera draw.

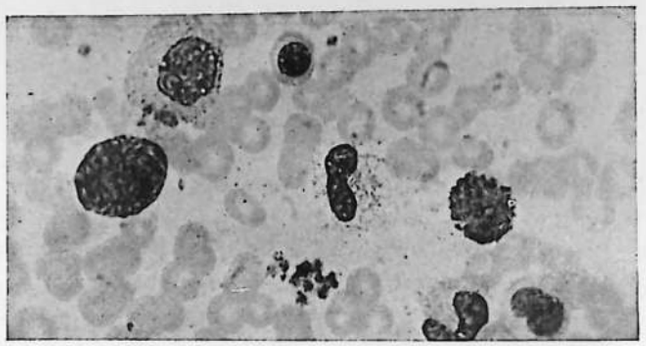


Fig. 14.—Leukemic blood (about × 650). Photograph taken with a Kodak, as described in the text.

Focusing is comparatively easy with low powers, but when using an oil-immersion objective it is a difficult problem unless the source of light be very brilliant. If one always uses the same length of camera and microscope tube, a good plan is as follows: Ascertain by trial with a strong light how far the fine adjustment screw must be turned from the correct eye focus to bring the image into sharp focus upon the ground-glass screen. At any future time one has

only to focus accurately with the eye, bring the camera into position, and turn the fine adjustment the required distance to right or left. When the camera draw is 10 inches little or no change in the focusing adjustment will be necessary.

The light should be as intense as possible in order to shorten exposure, but any light that is satisfactory for ordinary microscopic work will answer. The light must be carefully centered. It is nearly always necessary to insert a colored filter between the light and the microscope. The filter should have a color complementary to that which it is desired to bring out strongly in the photograph: For blue structures, a yellow filter; for red structures, a green filter. For the average stained preparation, a picric-acid yellow or a yellowish green will be found satisfactory. For deeply stained objects whose strength it is desired to reduce, and for all objects within which it is desired to bring out as much of the internal detail as possible, a filter of the same color should be used.

Very fair pictures can be made on Kodak film, but orthochromatic plates (of which Cramer's "Iso" and Eastman's D. C. "Ortho" are examples) give much better results. Panchromatic plates, like the Wratten "M," or Eastman's Process Panchromatic, are still better, but are more difficult to handle because more sensitive to red light. In order to avoid halation all plates should if possible be "backed." The length of exposure depends upon so many factors that it can be determined only by trial. It will probably vary from a few seconds to fifteen minutes. Plates are developed in the usual way. Either the tray or tank method may be used, but in order to secure good contrast it is often desirable to overdevelop somewhat. Metol-hydroquinone is an excellent developer, as it gives good contrast with full detail.

Choice of a Microscope.—It is poor economy to buy a cheap instrument.

For work in a clinical laboratory the microscope should preferably be of the handle-arm type, and should have a large stage. It should be provided with a substage condenser (preferably of 1.40 n. a.), three or more objectives on a revolving nosepiece, and two or more eyepieces. After one has learned to use them, the new single objective binocular microscopes (Fig. 15) are extremely satisfactory, giving an impression of stereoscopic vision, also enabling the worker to keep both eyes open with no feeling of strain. The distance between the two eyepieces is adjustable to allow for differences in pupillary distances of different individuals; the left eyepiece may also be focused by turning it slightly and thus adjusting it for any slight refractive

differences of the two eyes. If there is a bad refractive error it may be necessary to have special lenses ground for the eyepieces to obtain stereoscopic effects. Microscopists generally prefer binocular to monocular instruments when they become familiar with their use.

The most generally useful objectives are: 16 mm., 4 mm., and an oil-immersion, which usually is 1.9 or 1.8 mm. The 4-mm. objective may be obtained with N. A. of 0.65 to 0.85. If it is to be used for blood counting, the former is preferable, since its working distance is sufficient to take the thick cover of the blood counting instrument. For coarse objects a 32-mm. objective is very desirable. The eyepieces

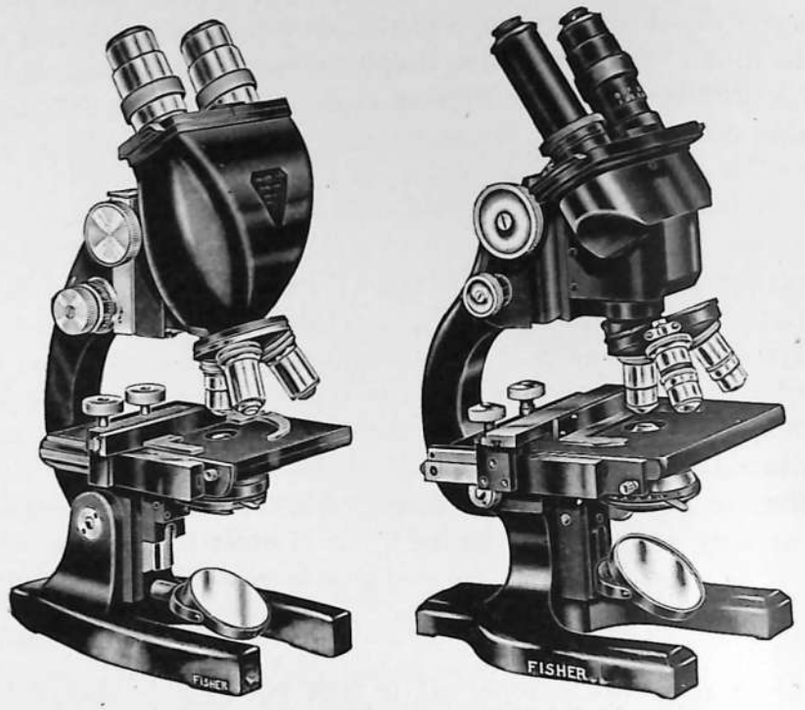


Fig. 15.—Two American made binocular microscopes.

most frequently used are 5× and 10×. A very low power (2×) and a very high (15×) will sometimes be found useful. The micrometer eyepiece is almost a necessity. A mechanical stage is almost indispensable for differential counting of leukocytes and certain other work of the clinical laboratory. An attachable mechanical stage is illustrated in Figure 16.

Practical Exercises.—The following is a brief outline of certain exercises which have been found useful in teaching microscopy. The student must learn as early as possible what can be expected of his microscope with proper manipulation. When he sits down to work

his first glance should tell him whether the instrument is giving its best results. If the microscopic picture falls short of the best, he must locate the difficulty and correct it before proceeding.

1. Clean the microscope and study its parts, familiarizing yourself with the names, purposes, and movements of each (Fig. 1).

2. Practice the manipulations necessary to locate particles of dust or dirt which appear in the microscopic field (p. 19).

3. Place the microscope before a window, focus upon a dusty slide, and adjust condenser and mirror so that the image of the window-frame, or better, of trees just outside the window appear in the microscopic field. Try the effect of raising and lowering the condenser, and of changing from plane to concave mirror, upon these images. Note that they cause an unevenly illuminated or mottled field when a little out of focus.

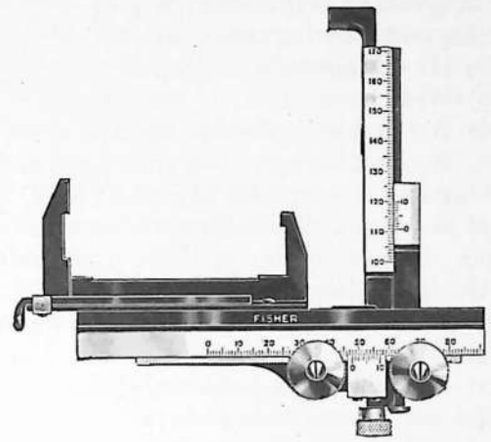


Fig. 16.—An attachable mechanical stage with graduated scales.

4. Insert a "pointer" in one of the oculars (p. 17).

5. Study illumination. Use a slide of some colorless structures, such as cholesterol crystals, and two preparations of blood, one a dried film stained with eosin or any blood stain (p. 252) and mounted in balsam, the other an unstained wet preparation made as described for the malarial parasite (p. 531). Study only the areas in which the corpuscles are well separated.

(1) Place one of these on the microscope, bring to a focus, and practice the manipulations necessary to secure (p. 4)—

(*a*) Central illumination.
(*b*) Oblique illumination.
(*c*) Strong and subdued illumination.

The field in each case must be evenly lighted throughout without mottling. Continue until you can adjust any desired form of illumination quickly and surely, and can recognize each by a glance into the microscope.

(2) Using the three slides mentioned above, ascertain the best form of illumination to study (p. 5)—

(*a*) Color.

(*b*) Outlines.

(*c*) Surface contour. The unstained normal and crenated red corpuscles are excellent objects for study of surface contour (Figs. 89 and 90).

(3) Try dark-field illumination by means of the substage disk (p. 6). Study the unstained blood smear and draw a few corpuscles. Also examine the cholesterol crystals, a drop of diluted milk, and a bit of lens paper, using a 16-mm. objective for this.

6. With central illumination, focus upon a slide and observe how much of the numeric aperture is in use (p. 13). Try the effect upon numeric aperture of—

(1) Opening and closing the diaphragm.

(2) Raising and lowering the condenser.

(3) Using the oil-immersion objective—

(*a*) Without oil.

(*b*) With oil between objective and cover glass.

(*c*) With oil between slide and condenser.

7. Practice using the oil-immersion objective (p. 11) upon an unstained film preparation of blood or a slide strewn with diatoms. These are nearly colorless and hence difficult to see. If there is difficulty in finding the specimen, move the slide about while lowering the objective to a focus. Moving objects will catch the eye as the objective approaches the correct focus. If a cover glass is used, its edge can be easily found, but it must be borne in mind that when the upper surface of the cover is in focus, objects beneath it are so far out of focus as to be invisible.

Produce some bubbles in the oil by stirring it about on the slide; observe their effect on the image of the blood cells or diatoms, and learn to detect their presence (p. 11).

8. Image formation (p. 11). Mount a bit of paper printed with very small type, using oil or balsam to render it transparent. Focus upon this with a low-power objective. Remove the ocular and lay a piece of ground glass across the top of the tube. This forms a screen upon which an image can be focused by means of the coarse adjustment. If the ground glass be placed over only half of the top of the tube, half of the image can be seen on the screen, half in the air. Note whether it is right side up or reversed. Repeat this with the ocular in place, holding the ground glass some inches above the ocular. These exercises, especially the last, are best done in a darkened room with strong artificial illumination, but extraneous light can usually be sufficiently excluded by viewing the image through a pasteboard mailing cylinder.

9. Find by trial the magnification produced by your 16-mm. objective

with the 5× or 10× oculars (p. 15). Compare your result with that listed by the maker of the microscope.

10. Micrometry.

(1) Evaluate the scale of your micrometer eyepiece with a high-power objective, and measure accurately 10 red blood corpuscles and 10 leukocytes (p. 20).

(2) Prepare a cardboard micrometer and measure 10 lycopodium granules (p. 21).

11. Focus upon a stage micrometer or hemacytometer slide and measure the diameter of the real field of each of your objectives with each of the oculars (p. 17). Note the effect of increasing the tube length.

12. Study the following structures, chiefly with a view to best illumination. Examine separately the color, outline, and surface contour of each. Many of these are met as accidental contaminations in microscopic preparations and one must learn to recognize them. Make drawings of each.

Fluids are examined by placing a drop in the center of a clean slide and applying a cover glass. The drop should be large enough to fill the space between the slide and cover, but not large enough to float the cover about. Fibers or insoluble powder may be placed in a drop of water and covered.

(1) With 16-mm. and 4-mm. objectives examine the upper surface of a new cover glass without cleaning. Usually it will show dirt and often crystals; if not, make finger-prints upon it and produce faint scratches by rubbing two covers together.

(2) Air-bubbles produced by shaking a little diluted mucilage.

(3) Fresh milk diluted with three or four volumes of water. Prepare three slides.

(*a*) Examine one untreated.

(*b*) Treat one with solution of sudan III. (For method see p. 127.) Note color assumed by the fat globules. This is one of the most useful tests for microscopic fat.

(*c*) Treat one with dilute acetic acid. Note clumping of globules similar to that of typhoid bacilli in the Widal test.

(4) A drop of diluted India ink. Note the dancing motion of the smaller particles ("brownian motion").

(5) Starch granules. Gently scrape the freshly cut surface of a potato with a knife, place a drop of the cloudy fluid upon a slide with a drop of water, and apply a cover glass. Make two preparations.

(*a*) Examine one untreated. Note the variously sized starch granules, oval, colorless, concentrically striated. Make sure that you find the best form of illumination to bring out the striations clearly. The starch granules themselves are easy to see because of their broad

dark outlines. This means that they are "highly refractive"—a term much used in describing microscopic structures—or, more correctly, that their index of refraction differs greatly from that of the medium in which they are mounted.

(*b*) Treat one with dilute Lugol's or Gram's iodine solution. Note the change in color of the granules. This is the standard test for starch.

(6) Yeast which has been growing in a dextrose solution. Make two preparations.

(*a*) Examine one unstained. Note "budding."

(*b*) Treat one with iodine solution. Compare color of yeast with that taken by starch.

(7) Mold from moldy food. Note hyphae and spores. Try the effect of iodine.

(8) Various fibers and other structures mounted in a drop of water:

(*a*) Cotton.
(*b*) Wool.
(*c*) Linen.
(*d*) Silk.
(*e*) Feather tip.
(*f*) Some dust from a carpeted room.
(*g*) A hair.
(*h*) Pollen from as many species of flowers as possible.

(9) Some of the scum from the bottom of a stagnant pool. Note the abundance of microscopic life. Look especially for diatoms, amebae, and ciliated organisms.

(10) Test your proficiency in using the microscope by trying to resolve diatoms. For the 4-mm. objective use *Pleurosigma angulatum*. The dots should be clearly seen. For the oil-immersion lens use *Surirella gemma*. The fine lines between the ribs should be seen as rows of dots. As a most critical test, both of the oil-immersion lens and of your skill in manipulation, use *Amphipleura pellucida*. Select a diatom of large size. Use oblique illumination and endeavor to bring out the cross striations. Try the same with central light, although you are not likely to succeed. These striations consist of rows of extremely minute dots, which can be seen only under the most favorable conditions such as are rarely attained in clinical work.

A NOTE ON THE MAGNIFICATIONS USED FOR THE ILLUSTRATIONS

The appearance of microscopic structures can generally be well shown by photomicrographs or good drawings, but as to their size,

which is equally important for their identification, the picture itself gives no hint unless some object which is familiar to us has been included. Circular pictures, indeed, are often actually misleading. They generally represent only a small area from the center of the microscopic field of view; but to many persons they appear to represent the entire field, and objects which occupy a large portion of the picture are accordingly visualized as occupying a correspondingly large part of the microscopic field and therefore as being much larger than they really are.

In order to derive the greatest possible benefit from study of the illustrations of microscopic structures, it is necessary that the student train himself to interpret the size of the pictured objects in terms of the magnifications afforded by his own microscope. To this end, the magnifications at which these objects are depicted are indicated beneath the illustrations. For example, Fig. 8 of Plate XII shows a tapeworm egg photographed at 250 diameters, and represents it about as it appeared with the 4-mm. objective and 6× ocular, and about twice as large as it appeared with a 16-mm. objective and 12.5-mm. ocular. The actual size of the object is found by measuring its picture and dividing by the stated magnification. In this case the pictured egg is about 9 mm. long, and the egg itself was approximately 36 μ long. In order to make up for unavoidable loss of detail in reproduction, the illustrations have in most cases been made at somewhat higher magnifications than are generally used for study of the objects in question. In many cases the same object is shown at both high and low magnifications.

The magnifications afforded by the different combinations of one's objectives and oculars can easily be found by the method given on page 15, or by consulting the maker's catalog.

CHAPTER I

THE SPUTUM

Preliminary Considerations.—Before beginning the study of the sputum the student will do well to familiarize himself with the structures which may be present in the normal mouth, and which frequently appear in the sputum as contaminations. Nasal mucus and material obtained by scraping the tongue and about the teeth should be studied as described for unstained sputum. A drop of Lugol's solution should then be placed at the edge of the cover, and, as it runs under, the effect upon different structures noted. Another portion should be spread upon slides or covers and stained by some simple stain and by Gram's method. The structures likely to be encountered are epithelial cells of columnar and squamous types; leukocytes, chiefly mononuclear, the so-called "salivary corpuscles"; food particles; *Leptotrichia buccalis*; great numbers of saprophytic bacteria; and frequently "spirochetes" and endamebae. These structures are described later. The so-called "normal morning sputum" is described on page 60.

When **collecting the sample** for examination the morning sputum, or the whole amount for twenty-four hours, should be saved. In beginning tuberculosis tubercle bacilli can often be found in that first coughed up in the morning, when they cannot be detected at any other time of day. Sometimes, in these early cases, there are only a few mucopurulent flakes which contain the bacilli, or only a small purulent mass every few days, and these may easily be overlooked by the patient.

Patients should be instructed to rinse the mouth well, in order to avoid contamination with food particles which may prove confusing in the examination, and to make sure that the sputum comes from the lungs or bronchi and not from the nose and nasopharynx. Many persons find it difficult to distinguish between the two sources. It is desirable that the material be raised with a distinct expulsive cough, but this is not always possible. In some cases of chronic tuberculosis there may be no cough at all, the small masses of sputum rising by action of bronchial and tracheal cilia, and the patient becoming conscious of them only when they reach the larynx, and often swallowing them without realizing their significance. Material

from the upper air passages can usually be identified by the large proportion of mucus and the character of the epithelial cells.

The sputum of infants and young children is usually swallowed and therefore cannot be collected. In such cases examination of the feces for tubercle bacilli will sometimes establish a diagnosis of tuberculosis.

As a receptacle for the sputum a clean, wide-mouthed bottle with tightly fitting cork may be used. The patient must be particularly cautioned against smearing any of it upon the outside of the bottle. This is probably the chief source of danger to those who examine sputum. Disinfectants should not be added. Although some of them (phenol, for example) do not interfere with detection of tubercle bacilli, they generally so alter the character of the sputum as to render it unfit for other examinations.

The following outline is suggested for the **routine examination:**

1. Spread the material in a thin layer in a large Petri dish or between two plates of glass. The use of glass plates is messy, but is to be recommended for careful work. The top plate should be much smaller than the lower one, or have some sort of handle.
2. Examine all parts carefully with the naked eye or with a hand lens. This is best done over a black background which is placed some distance below. The ordinary paper picnic plate, one-half of which is painted black, makes a convenient tray, but the black background is too close for greatest efficiency. The portions most suitable for further examination may thus be easily selected. *This macroscopic examination should never be omitted.*
3. Transfer various portions, including all suspicious particles, to clean slides, cover, and examine unstained with the microscope (p. 35). A wooden applicator may be used to transfer selected portions of sputum to the glass slide. The applicator is then used as a spreader, and can be burned in the Bunsen flame.
4. Slip the covers from some or all of the above unstained preparations, leaving a thin smear on both slide and cover.
5. Dry and fix the smears and stain one or more by each of the following methods:
   (*a*) For tubercle bacilli (p. 45).
   (*b*) Gram's method (p. 780).
6. When indicated, make special examinations for—
   (*a*) Capsules of bacteria (p. 53).
   (*b*) Eosinophilic cells (p. 57).
   (*c*) Presence of albumin (p. 59).

After the examination the sputum must be destroyed by heat or chemicals, and everything which has come in contact with it must

be sterilized. The utmost care must be taken not to allow any of it to dry and become disseminated through the air. If flies are about, it must be kept covered. It is a good plan to conduct the examination upon a large newspaper, which can then be burned. Contamination of the work table is thus avoided. If this is not feasible, the table should be washed off with 10 per cent lysol or other disinfectant solution, and allowed to dry slowly, as soon as the sputum work is finished.

Examination of the sputum is most conveniently considered under four heads: I. Physical examination. II. Microscopic examination. III. Chemical examination. IV. Characteristics of the sputum in various diseases.

I. PHYSICAL EXAMINATION

1. Quantity.—The quantity expectorated in twenty-four hours varies greatly. It may be so slight as to be overlooked entirely in beginning tuberculosis. It is usually small in acute bronchitis and lobar pneumonia. It may be very large—sometimes as much as 1000 c.c.—in advanced tuberculosis with large cavities, edema of the lung, bronchiectasis, and following rupture of an abscess or empyema. It is desirable to obtain a general idea of the quantity, but accurate measurement is unnecessary.

2. Color.—Since the sputum ordinarily consists of varying proportions of mucus and pus, it may vary from a colorless, translucent mucus to an opaque, whitish or yellow, purulent mass. A yellowish green is frequently seen in advanced phthisis and chronic bronchitis. In jaundice, in caseous pneumonia, and in slowly resolving lobar pneumonia it may assume a bright green color, due to bile or altered blood pigment.

A red or reddish-brown color usually indicates the presence of blood or of a pigment derived from it. Bright red blood, most commonly in streaks, is strongly suggestive of phthisis. It may be noted early in the disease and generally denotes an extension of the tuberculous process. One must, however, be on his guard against blood-streaked mucus or mucopus originating in nasopharyngeal catarrh. Tuberculous patients not infrequently mistake this for true sputum and become much worried because of it. Blood-stained sputum is also sometimes seen in bronchiectasis. A rusty red sputum is the rule in croupous pneumonia, and was at one time considered pathognomonic of the disease. Exactly similar material may be raised in pulmonary infarction. "Prune-juice" sputum is said to be characteristic of "drunkard's pneumonia." It at least indicates a dangerous

type of the disease, as it is apparently referable to coincident edema of the lung. A brown color, due to altered blood pigment, follows hemorrhages from the lungs, and is present, to greater or less degree, in chronic passive congestion of the lungs, which is most frequently due to a heart lesion.

Gray or black sputum is observed among those who work much in coal dust, and is occasionally seen in smokers who are accustomed to "inhale."

3. Consistence.—According to their consistence sputa are usually classified as serous, mucoid, purulent, seropurulent, or mucopurulent, which names explain themselves. As a rule, the more mucus and the less pus and serum a sputum contains, the more tenacious it is.

The rusty sputum of croupous pneumonia is extremely tenacious, so that the vessel in which it is contained may be inverted without spilling it. The same is true of the almost purely mucoid sputum ("sputum crudum") of beginning acute bronchitis, and of that which follows an attack of asthma. A purely serous sputum, usually slightly blood tinged, is fairly characteristic of edema of the lungs.

Formerly much attention was paid to the so-called "nummular sputum." This consists of definite mucopurulent masses which flatten out into coinlike disks and sink in water. It is fairly characteristic of tuberculous and bronchiectatic cavities.

4. Layer Formation.—Some sputa show a striking tendency to separate into three sharply defined layers when a large volume is allowed to stand in a tall vessel. This is notably true in bronchiectasis, gangrene, and abscess of the lung.

5. Dittrich's Plugs.—While these bodies sometimes appear in the sputum, they are more frequently expectorated alone. They are yellowish or gray caseous masses, usually about the size of a pin-head, but sometimes reaching that of a bean. When crushed, they emit a foul odor. Microscopically, they consist of granular débris, fat globules, fatty acid crystals, and bacteria. They are formed in the bronchi, and are sometimes expectorated by healthy persons, but are more frequent in putrid bronchitis and bronchiectasis. The laity commonly regard them as evidence of tuberculosis. The similar caseous masses which are formed in the crypts of the tonsils are sometimes also included under this name.

6. Lung Stones.—At times during the course of chronic tuberculosis small calcified nodules of tuberculous tissue may be expectorated, and these constitute the great majority of the so-called "pneumoliths." Small foreign bodies, bits of clothing, and so forth, carried into the lung by gunshot and other injuries, may sometimes

remain for years and finally ulcerate into a bronchus and be expectorated, usually with hemorrhage.

7. Bronchial Casts.—These are branching, treelike casts of the bronchi, frequently, but not always, composed of fibrin (Figs. 17 and 18). In color they are usually white or grayish, but may be reddish or brown from the presence of blood pigment. Their size varies with that of the bronchi in which they are formed. Casts 15 or more centimeters in length have been observed, but they are usually very much smaller. Ordinarily they are rolled into a ball or tangled mass, and can be recognized only by floating out in water—best over a black background—when their treelike structure becomes evident. The naked-eye examination will usually suffice; occasionally a hand lens may be required.

Bronchial casts appear in the sputum in croupous pneumonia,

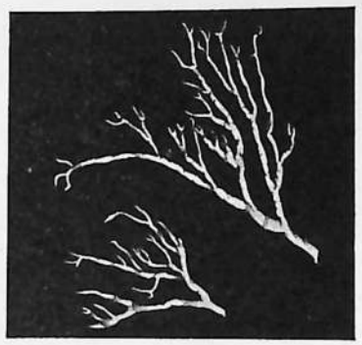


Fig. 17.—Bronchial casts as seen when carefully spread out and viewed over a black background. Natural size. More frequently only broken pieces are found.

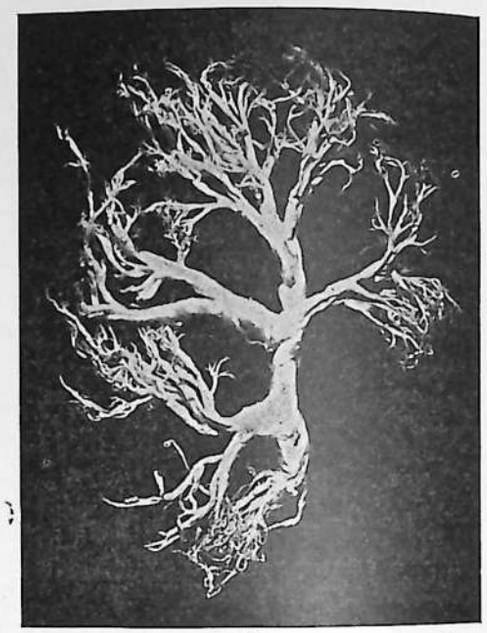


Fig. 18.—Unusually large and perfect bronchial cast. One half natural size (Spencer).

in fibrinous bronchitis, and in diphtheria when the process extends into the bronchi. In diphtheria they are usually large. In fibrinous or chronic plastic bronchitis they are of medium size and usually of characteristic structure. Their demonstration is essential for the diagnosis of this disease. In some cases they may be found every day for considerable periods; in others, only occasionally. In almost every case of croupous pneumonia the casts are present in the sputum in variable numbers during the stage of hepatization and beginning resolution. Here they are usually small (0.5 to 1 cm. in length) and are often not branched.

II. MICROSCOPIC EXAMINATION

The portions most likely to contain structures of interest should be very carefully selected, as already described. *The few minutes spent in this preliminary examination will sometimes save hours of work later.* Opaque, white or yellow particles are most frequently bits of food, but may be cheesy masses from the tonsils; particles, sometimes caseous, derived from tuberculous cavities and containing many tubercle bacilli and elastic fibers; Curschmann's spirals, or small fibrinous casts, coiled into little balls; or shreds of mucus with great numbers of entangled pus corpuscles. The food particles most apt to cause confusion are bits of bread, which can be recognized by the blue color which they assume when touched with iodine solution.

Some structures are best identified without staining; others require that the sputum be stained.

A. Unstained Sputum

A careful study of the unstained sputum should be included in every routine examination. Unfortunately, it is almost universally neglected. It best reveals certain structures which are seen imperfectly or not at all in stained preparations. It gives a general idea of the other structures which are present, such as pus corpuscles, eosinophils, epithelial cells, and blood, and thus suggests appropriate stains to be used later. It enables one to select more intelligently the portions to be examined for tubercle bacilli.

The particle selected for examination should be transferred to a clean slide, covered with a clean cover glass, and examined with the 16-mm. objective, followed by the 4-mm. The oil-immersion lens should not be used for this purpose. It is convenient to handle the bits of sputum with a wooden toothpick or with a wooden cotton-applicator, which may be burned when done with. The platinum wire used in bacteriologic work is unsatisfactory because not usually stiff enough. A little practice is necessary before one can handle particles of sputum readily. The bit desired should be separated from the bulk of the sputum by cutting it free with the toothpick and drawing it out upon the dry portion of the glass dish. It can then be picked up by rotating the end of a fresh toothpick against it. *The slide must never be dipped into the sputum, nor must any of the sputum be allowed to run over its edges in spreading.*

The more important structures to be seen in unstained sputum are: Elastic fibers, Curschmann's spirals, Charcot-Leyden crystals, pigmented cells, myelin globules, the ray fungus of actinomycosis,

and molds. Forming the background for these are usually pus corpuscles, granular detritus, and mucus in the form of translucent, finely fibrillar, or jelly-like masses. The pus cells appear as finely granular grayish or yellowish balls, about 10 to 12 μ in diameter, and generally without visible nuclei (Figs. 25 and 26). They are best studied in stained preparations.

1. Elastic Fibers.—These are the elastic fibers of the pulmonary substance, where they are distributed in the walls of the alveoli, the bronchioles, and the blood vessels. When found in the sputum they always indicate destructive disease of the lung, provided they do not come from the food, which is a not infrequent source. They are found in abscess and gangrene of the lung, but in the vast majority of cases their presence indicates tuberculosis. Advanced cases of tuberculosis often show great numbers, and, rarely, they may be found in early

Fig. 19.—Elastic fibers in tuberculous sputum, unstained, as seen with a low-power objective (× 100).

tuberculosis, when the bacilli cannot be detected. After the diagnosis is established they furnish a valuable clue as to the existence and rate of lung destruction. In gangrene of the lung, contrary to the older teaching, elastic tissue is probably always present in the sputum, usually in large fragments.

The portion of sputum to be searched for elastic tissue should be selected by careful inspection. Small bits of necrotic tissue, or yellowish, greenish, or rusty particles, which are often minute, are most favorable; when these are absent the most purulent portion of the sputum should be taken. The selected bit is taken on a slide, and a cover glass is applied and pressed down so as to give a moderately thin preparation, which is examined before it dries. Careful selection of the portion examined is more efficient than is the concentration method—boiling with 10 per cent caustic soda and centrifugalizing—which is widely recommended.

The search should be conducted with the 16-mm. objective, although a higher power is often needed to identify the fibers with certainty. They are slender, highly refractive, wavy fibrils with double contour and curled, often split, ends. Very characteristic are their graceful curves without sharp bends, their uniform diameter, and their smoothness, although in old sputum they may become much roughened. The fibers may lie singly or in bundles. Frequently they are found in alveolar arrangement, preserving the original outline of the alveoli of the lung (Figs. 19 and 20). This arrangement is positive proof of their origin in the lung.

Leptotrichia buccalis, which is a normal inhabitant of the mouth, may easily be mistaken for elastic tissue with the low power. It can

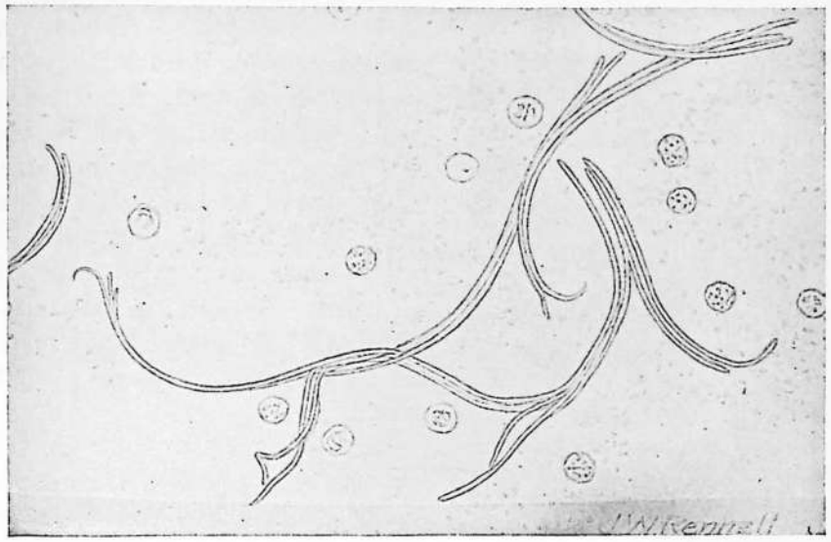


Fig. 20.—Elastic fibers in tuberculous sputum, unstained, as seen with a high-power objective (× 400).

usually easily be distinguished when studied with the 4-mm. objective. In case of doubt the iodine reaction (p. 613) may be tried, although not all forms of leptothrix react characteristically. Fatty acid crystals, which are often present in Dittrich's plugs and in sputum which has lain in the body for some time, also simulate elastic tissue when very long, but they are more like stiff, straight or curved needles than wavy threads. They show varicosities when the cover glass is pressed upon and melt into droplets when the slide is heated. The structures which most frequently confuse the student are the cotton fibrils, which are often present as a contamination from the air. These are usually coarser than elastic fibers and flat, with one or

two twists, and often have longitudinal striations and frayed-out ends. The color, too, is somewhat different. Cotton fibers lack the faint yellowish tinge of elastic tissue. Very important also is the relative degree of refractility: If the diaphragm be opened slowly, elastic fibers can be seen long after the slightly refractile mucous strands have disappeared, but finally they also are practically lost in the glare, while cotton fibers still remain visible. In stained preparations students frequently report the fibrils of precipitated mucus as elastic tissue.

Elastic fibers from the food are coarser, generally shorter, less frequently wavy, and not arranged in alveolar order.

2. Curschmann's Spirals.—These peculiar structures are found most frequently in bronchial asthma, of which they are fairly characteristic. Although not present in every attack, they probably occur at some time in every case. Sometimes they can be found only near the end of the attack. They may occasionally be met with in chronic bronchitis and other conditions, but in these there is nearly always an underlying asthmatic tendency. Their nature has not been definitely determined.

Fig. 21.—Curschmann's spirals in asthmatic sputum as seen when pressed out between two plates of glass and viewed over a black background. Each is embedded in a mass of grayish mucus. (Natural size.)

Macroscopically, they are whitish or yellow wavy threads, frequently coiled into little balls (Fig. 20). Their length is rarely over 1.5 cm., though it sometimes exceeds 5 cm. They can sometimes be definitely recognized with the naked eye. Under a 16-mm. objective they appear as mucous threads with a bright, colorless, central line—the so-called "central fiber"—about which are wound many fine fibrils (Figs. 22 and 23). The bright central line is best seen when the objective is raised a little above the true focus; and it has been interpreted as an optical phenomenon due to tight coiling of the spiral. In some cases one or more definite dark colored threadlike fibers can be seen at the true focus. The spiral fibrils are sometimes loosely, sometimes tightly wound. Eosinophils are usually present within them, and sometimes Charcot-Leyden crystals also. Not infrequently the spirals are imperfectly formed, consisting merely of twisted strands of mucus enclosing leukocytes. The central fiber is absent from these.

3. Charcot-Leyden Crystals.—Of the crystals which may be found in the sputum, the most interesting are the Charcot-Leyden crystals. They may be absent when the sputum is expectorated, and appear in large numbers after it has stood for some time. They are rarely found except in cases of bronchial asthma, and were at

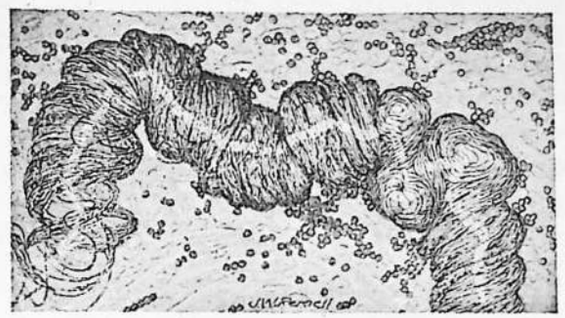


Fig. 22.—End of a large, tightly wound Curschmann's spiral in sputum from a case of bronchial asthma. Unstained (× 70).

one time thought to be the cause of the disease. They frequently adhere to Curschmann's spirals. Their exact nature is unknown. Their formation seems to be in some way connected with the presence of eosinophilic cells. Outside of the sputum they are found in

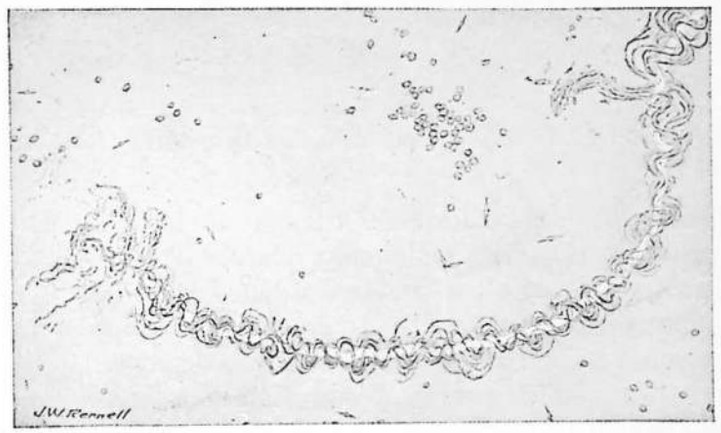


Fig. 23.—Slender, loosely wound Curschmann's spiral in sputum from a case of bronchia, asthma. A few Charcot-Leyden crystals are also shown. Unstained (× 70).

the feces in association with animal parasites, and in the coagulated blood in leukemia.

They are colorless, pointed, often needle-like crystals (Fig. 24). Formerly they were described as octahedral, but are now known to be hexagonal in cross section. Their size varies greatly, the average

length being about three or four times the diameter of a red blood corpuscle.

Other crystals—hematoidin, cholesterol, and, most frequently, fatty acid needles (Fig. 45)—are common in sputum which has remained in the body for a considerable time, as in abscess of the lung and bronchiectasis. The fatty acid crystals are regularly found in Dittrich's plugs. They might be mistaken for elastic fibers (p. 36). Sometimes they form rounded masses with the individual crystals radially arranged, and they then bear considerable resemblance to the clumps of *Actinomyces hominis*.

4. Pigmented Cells.—Granules of pigment are sometimes seen in ordinary pus corpuscles, but the more common and important pigment-containing cells are large mononuclear cells whose origin is in

Fig. 24.—Charcot-Leyden crystals and eosinophilic leukocytes in sputum from a case of bronchial asthma. Unstained (× 475). The magnification is greater than is usually used in studying these structures.

some doubt. They were formerly thought to be the flattened epithelial cells which line the pulmonary alveoli. The present tendency is to identify them with the endothelial leukocytes, which are known to take up pigment granules readily; but in view of recent studies the question must be left open. Two kinds of pigmented cells deserve mention: Those which contain blood pigment, chiefly hemosiderin; and those which contain carbon. As a rule, these cells also have a coarsely granular appearance from the presence of many small colorless myelin globules.

To those which contain blood pigment the name **heart-failure cells** has been given, because they are most frequently found in long-continued passive congestion of the lungs resulting from poorly compensated heart disease. The presence of these cells in considerable numbers, by directing one's attention to the heart, will some-

times clear up the etiology of a chronic bronchitis. They are sometimes so numerous as to give the sputum a brownish tinge. Such cells are also found in the sputum in pulmonary infarction and for some time after a pulmonary hemorrhage. In fresh unstained sputum heart-failure cells appear as round, grayish, or colorless bodies filled with variously sized rounded granules of yellow to brown pigment (Plate II, Fig. 1). Sometimes the pigmentation takes the form of a diffuse staining. The nucleus is usually obscured by the pigment. The cells are large, averaging about 35 μ in diameter.

To demonstrate the nature of the brown pigment apply a 10 per cent solution of potassium ferrocyanide for a few minutes and follow with weak hydrochloric acid. Iron-containing pigment assumes a blue color. Many of the granules will, however, fail to respond. The test may be applied either to wet preparations or to dried smears.

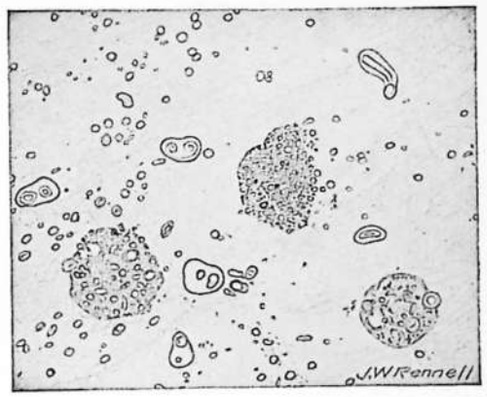


Fig. 25.—Myelin globules, free and contained within cells. From a "normal morning sputum" (× 350).

Carbon-laden cells (Plate II, Fig. 1) are less important. They are especially abundant in the sputum of anthracosis where angular black granules, both intracellular and extracellular, may be so numerous as to color the sputum. Similar cells with smaller carbon particles are often abundant in the morning sputum of those who inhale tobacco smoke to excess, or those who live in a smoky atmosphere.

5. Myelin Globules.—These have little or no clinical significance, but require mention because of the danger of confusing them with more important structures, notably blastomyces. They are colorless, round, oval, or pear-shaped globules of various sizes, often resembling fat droplets, but the larger ones more frequently showing peculiar concentric or irregularly spiral markings (Figs. 25 and 34). Such globules are abundant in the scanty morning sputum of apparently

healthy persons, but may be found in any mucoid sputum. They lie both free in the sputum and contained within the large cells which have long been known as alveolar cells, but which are possibly endothelial leukocytes. The intracellular globules are generally small, and when closely packed give the cells a yellowish tinge which may mislead the unwary into calling them heart-failure cells.

6. Actinomyces Hominis (Ray-fungus).—In the sputum of pulmonary actinomycosis and in the pus from actinomycotic lesions elsewhere, small gray or yellowish, "sulfur" granules can be detected with the unaided eye. Without a careful macroscopic examination they are almost certain to be overlooked. The fungus can be seen by crushing one of these granules between slide and cover, and examining with a low power. *Actinomyces hominis* is very similar to, and perhaps identical with, *Actinomyces bovis*, the cause of "lumpy-jaw"

Fig. 26.—A "sulfur granule" crushed beneath the cover glass. From the pus of a case of actinomycosis of submaxillary lymph nodes. Unstained (× 60).

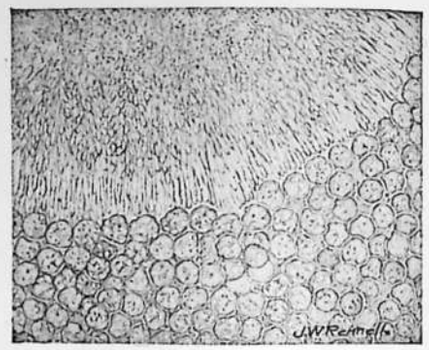


Fig. 27.—A portion of Fig. 25, more highly magnified (× 300).

in cattle. It consists of a network of threads having a more or less radial arrangement (Figs. 26 and 27). In cattle, and to a less extent in man, the filaments at the periphery of the nodule present club-shaped extremities. It can be brought out more clearly by running a little solution of eosin in alcohol and glycerin under the cover. This organism apparently stands midway between the bacteria and the molds and may be classed with the Trichomycetes or higher bacteria (p. 56). It stains by Gram's method.

Actinomycosis of the lung is rare. The clinical picture is that of tuberculosis.

7. Molds and Yeasts.—The hyphae and spores of various molds are occasionally met with in the sputum. The hyphae are rods, usually jointed or branched (Fig. 82), and often arranged in a mesh-

work (mycelium); the spores are highly refractive spheres and ovoids. Both stain well with the ordinary stains. Molds in the sputum are usually the result of contamination and have little significance. Occasionally they grow in the pus of lung cavities whether of tuberculous or other origin. Very rarely there may be true infection of the lung—a pneumomycosis—usually resembling tuberculosis and most often due to *Aspergillus fumigatus*. Bronchopulmonary mycosis is said to be common in the Orient.

In the extremely rare condition of pulmonary blastomycosis specific yeasts have been found in the sputum in large numbers. In the tissues they multiply by budding and the presence of budding forms in the sputum is sufficient for their identification as blastomyces. In cultures they form hyphae. The similar organism, *Coccidioides immitis*, the cause of coccidioidal granuloma of the Pacific Coast, does not form buds, but multiplies by endosporulation. For both these organisms it is advisable to add a little 10 per cent caustic soda solution and examine unstained. Both may also be studied in stained smears, but cultural methods are essential for their complete identification.

As a contamination one sometimes finds forms of the fungus *Mycoderma*. These have a striking appearance, resembling gigantic sausage-shaped bacilli.

8. Animal Parasites.—These are extremely rare in the sputum in this country. A trichomonad, perhaps identical with *Trichomonas hominis*, has been seen in the sputum of putrid bronchitis and gangrene of the lung, but its causal relationship is doubtful. In Japan infection with the lung flukeworm, *Paragonimus westermanii*, is common, and the ova are found in the sputum. The lung is not an uncommon seat for echinococcus cysts, and hooklets and scolices may appear. Larvae of *Strongyloides stercoralis* and of the hookworm have been reported. *Endamoeba histolytica* has been found after rupture of an hepatic abscess into the lung. Ciliated body cells, with cilia in active motion, are not infrequently seen, and may easily be mistaken for infusoria. All the above-mentioned parasites are described in Chapter VI.

B. Stained Sputum

The principal structures which are best seen in stained sputum are bacteria and cells.

A number of smears should be made upon slides or covers. These films must, of course, be thin, but it is easily possible to get them too thin. This is a common error of students who have just finished

a course in bacteriology and who have there been accustomed to work with scarcely perceptible films of bacteria. It is a good plan to slide off the cover glass from the preparation used for the unstained microscopic examination. If this is properly done, satisfactory smears will be left on both slide and cover. They are then dried in the air and fixed in the flame, as described on page 779, or better, by immersion for one or two minutes in pure wood alcohol or 1 per cent solution of corrosive sublimate. Fixation will ordinarily kill the bacteria and the smears may be kept indefinitely; but smears on slides when fixed by heat are often not sterile, and should be handled accordingly. As a matter of routine one of the smears should be stained for the tubercle bacillus and one by Gram's method with a good counterstain (p. 780). These preparations will give a good idea of the various cells and bacteria present and may suggest further procedure.

1. Micro-organisms.—Saprophytic bacteria from mouth contamination are frequently present in large numbers and will prove confusing to the inexperienced. The presence of squamous cells in their neighborhood will suggest their source. Among the pathogenic organisms are: Tubercle bacilli; staphylococci and streptococci; pneumococci; bacilli of Friedländer; influenza bacilli, and *Micrococcus catarrhalis*. Of these, the tubercle bacillus is the only one whose recognition has great clinical value and the only one which is easily identified in stained smears. Their cultural characteristics are described in Chapter X. When cultures are to be made the teeth and tongue should be well cleaned with a sterile brush, the mouth should be well rinsed, and the sputum, preferably only one expectoration, should be expectorated directly into a dry, sterile, wide-mouthed bottle. This should be kept on ice. As soon as possible the most purulent portions should be picked out, washed in several changes of sterile salt solution, and planted upon appropriate media.

(1) **Tubercle Bacillus** (*Mycobacterium tuberculosis*).—The presence of tubercle bacilli may be taken as positive evidence of the existence of tuberculosis somewhere along the respiratory tract, most likely in the lung, but when only one or two are found on a slide the result should be confirmed by a second examination. There is always the rare possibility that bacilli from a previous examination may have clung to an imperfectly cleaned slide, that bacilli may have reached the sputum from the dust of the air, or that the suspected bacillus may be some other acid-fast organism.

The importance of carefully selecting the portion for examination cannot be too strongly emphasized. It is always best to select the more purulent portions of the sputum, keeping away from the mucoid

parts. If bits of necrotic tissue are present, they may show immense numbers of tubercle bacilli when other portions of the specimen contain very few. One must, however, be on his guard against bits of food which resemble these "caseous particles." The specimen should be examined while fresh. It will usually liquefy upon standing, and this, by preventing the selection of particles favorable for examination, will greatly reduce one's chances of finding bacilli.

Recognition of the tubercle bacillus depends upon the fact that it stains with difficulty; but that when once stained, it retains the stain tenaciously even when treated with a mineral acid, which quickly removes the stain from other bacteria. This "acid-fast" property is due to the presence of a waxy or lipoid substance. A number of the best staining methods are included here. Since Gabbet's method is convenient, inexpensive, and widely used in office work, it is given in greater detail than the others. If it is carried out as here recommended, there is no danger of decolorizing the tubercle bacillus. In large laboratories the Ziehl-Neelsen method is to be preferred.

Tubercle bacilli can often be found in very poorly prepared slides, but for dependable results when bacilli are scarce, properly spread, fixed, and stained preparations free from precipitated stain are absolutely essential. *The person who is content with an imperfect preparation because it is "good enough for diagnosis" will succeed only in the most obvious cases.*

Gabbet's Method.—1. Spread suspicious particles thinly and evenly upon a slide or a cover glass held in the grasp of cover glass forceps. In general, slides are more satisfactory, but cover glasses are easier to handle while staining. Do not grasp a cover too near the edge or the stain will not stay on it well. Tenacious sputum will spread better if gently warmed while spreading.

2. Dry the film in the air.

3. Fix the film by immersing in 1 per cent aqueous solution of corrosive sublimate or in methyl alcohol for two or three minutes and then rinse well in water. This is much to be preferred, particularly for beginners, to the usual practice of fixing in the flame (p. 779). Should the film be washed off during future manipulations, fixation has been insufficient.

4. Apply as much carbolfuchsin (p. 831) as will stay on, and hold over a flame so that it will steam for three minutes or longer, replacing the stain with a dropper as it evaporates. If the stain be allowed to evaporate completely, the preparation is ruined. If the bacilli be well stained in this step, there will be little danger of decolorizing them later. Too great heat will interfere with the staining of some of the bacilli, probably by destroying the waxy substance upon which the acid-fast property depends. A number of slides may be stained at the same time by placing them upon a rack

consisting of two parallel rods about 2 inches apart placed across a sink or on a tripod.

It has been shown that twenty to thirty minutes' staining at room temperature will suffice, and this may be recommended on the score of avoiding precipitates, the slides being immersed in the fluid in a staining jar. With some batches of carbolfuchsin even five minutes' staining is sufficient.

5. Wash the film in water.

6. Apply Gabbet's stain (p. 832) to the under side of the cover glass to remove excess of carbolfuchsin, and then to the film side. Allow this to act for one-quarter to one-half minute.

7. Wash in water.

8. If, now, the thinner portions of the film are blue, proceed to the next step; if they are still red, repeat steps 6 and 7 until the red has disappeared. Too long application of Gabbet's stain will decolorize the tubercle bacilli.

9. Place the preparation between layers of filter paper and dry by rubbing with the fingers, as one would in blotting ink. Warm over the flame until thoroughly dry.

10. Put a drop of Canada balsam upon a clean slide, place the cover glass film side down upon it, and examine with an immersion objective. Cedar oil or water may be used in place of balsam for temporary preparations. Smears on slides may be examined directly with an oil-immersion lens, no cover being necessary.

Ziehl-Neelsen Method.—The objection is often made to the above method that decolorization is masked by the blue in Gabbet's stain. Although this will not make trouble if step 8 is carefully carried out, most experienced workers prefer the Ziehl-Neelsen method. This resembles Gabbet's method, with the following exceptions: After the staining with carbolfuchsin the smear is washed in 5 per cent nitric acid (or, better, a mixture of 3 c.c. concentrated hydrochloric acid and 97 c.c. 70 per cent alcohol) until only a faint pink remains in the thinner portions, washed in water, stained lightly with Löffler's methylene blue, again washed, and finally dried and mounted. In place of methylene blue, some prefer a counterstain consisting of equal parts of alcohol and saturated aqueous solution of picric acid. With this, tubercle bacilli stand out clearly against a faint yellow background, but nonacid-fast bacteria and cells are not recognizable.

Pappenheim's Method.—This is the same as Gabbet's method, except that Pappenheim's methylene blue solution (p. 832) is substituted for Gabbet's stain and is allowed to act several times as long. To avoid evaporation the blue stain must be kept in a tightly corked bottle.

The method is very satisfactory for routine work. Decolorization of the tubercle bacillus is practically impossible: it retains its red color even when soaked overnight in Pappenheim's solution. The stain was originally recommended as a means of differentiating the smegma bacillus, which is decolorized by it, but it is not to be absolutely relied upon for this purpose.

In films stained by these methods tubercle bacilli, if present, will be seen as slender red rods upon a blue background of mucus, which appear as delicate threads and strands, granular detritus, and cells (Plate II, Fig. 3). They vary considerably in size, averaging 3 to 4 μ in length—about one half the diameter of a red blood corpuscle. Beginners must be warned against mistaking the edges of cells, or particles which have retained the red stain, for bacilli. The appearance of the bacilli is almost always typical, and if there seems room for doubt, the structure in question is probably not a tubercle bacillus. They may lie singly or in groups. They are very frequently bent and often have a beaded appearance. It is possible that the larger beaded bacilli indicate a less active tuberculous process than do the smaller uniformly stained ones. Sometimes they are present in great numbers—thousands in a field of the 2-mm. objective. Sometimes, even in advanced cases, several slides must be examined to find a single bacillus. At times they are so few that none are found in stained smears, and special methods are required to detect them. The number may bear some relation to the severity of the disease but this relation is by no means constant. The mucoid sputum from an incipient case sometimes contains great numbers, while sputum from large tuberculous cavities at times contains very few. Failure to find them is not conclusive, though *their absence is much more significant when the sputum is purulent than when it is mucoid.*

The approximate number of bacilli present should always be indicated in the record of the examination. This may be done by recording the average number seen in a field. Since the sputum raised at various times in the day, and even different parts of the same sample, may vary greatly in bacillary content, such a record is not an accurate index of the comparative number of bacilli thrown off, even when the twenty-four-hour sputum is collected and uniformly mixed before preparing the slides. It is, however, a useful clinical guide.

Fluorescent Dye Method.—Using a principle described by Hagemann,[1] Richards and Miller[2] have presented an efficient method of using a simple fluorescence microscope for demonstrating *Mycobacterium tuberculosis* which may in time supersede the older methods

[1] Hagemann, P. K. H.: Fluoreszenzmikroskopische Untersuchungen über Virus und andere Mikroben. Zentralbl. f. Bakt., Abt. I, *140:*184–187, 1937. Fluoreszenzfärbung von Tuberkelbakterieren mit Auramin. München. med. Wchnschr., *85:*1066–1068 (July 15) 1938.

[2] Richards, O. W. and Miller, D. K.: An efficient method for the identification of M. tuberculosis with a simple fluorescence microscope. Amer. Jour. Clin. Path. Tech. Suppl,. no. 5, *11:*1–8 (Jan.) 1941.

described above. It can be entrusted as a valuable aid in finding this organism, although it necessitates the use of a darkened room in which to examine the slides stained with the fluorescent dye, Auramine O. Tubercle bacilli stained with this dye in ultra-violet light are visible as bright, shining rods.

The required accessories for an ordinary monocular microscope are illustrated in Figure 28.

The staining solution is composed of Auramine O (National Aniline Company) 0.1 gm. dissolved in distilled water, 97 ml. and liquefied

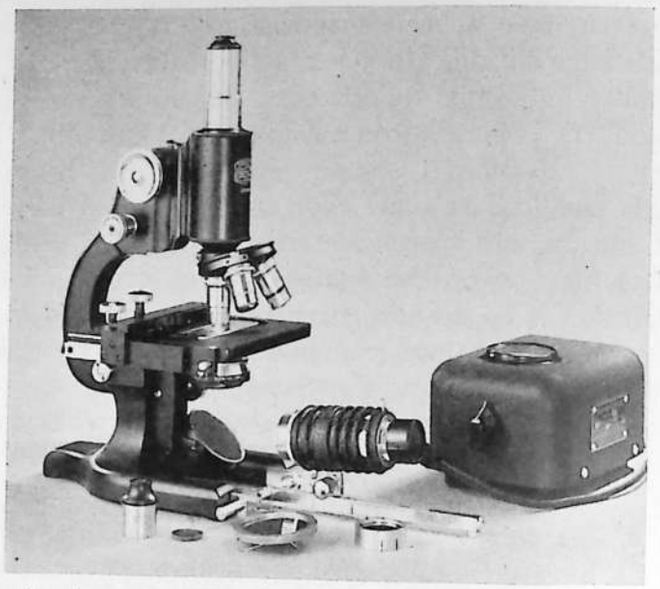


Fig. 28.—Attachments used for fluorescent demonstration of acid-fast organisms; *a*, yellow filter to fit into ocular; *b*, front surfaces, aluminized mirror to fit over microscope mirror; *c*, blue, ultraviolet transmitting filter for lamp. (Richards and Miller, Am. J. of Clin. Path., Vol. 11.)

phenol, 3 ml. Thompson[1] has found that the dye is more easily dissolved by the following method:

Solution A:

Distilled water	87 c.c.
Liquefied phenol	3 c.c.
Mix completely.	

Solution B:

Alcohol (95 per cent)	10 c.c.
Auramine O	0.1 gm.

[1] Thompson, Luther: Comparison of carbolfuchsin with the fluorescent dye auramine for the demonstration of acid-fast bacteria. Proc. Staff. Meet. Mayo Clinic. *16*:673–675 (Oct. 22) 1941.

When solution A is well mixed and the dye is completely dissolved in solution B, solution B is added to solution A. The stain will stay clear and will be free from precipitate. The decolorizing solution recommended by Richards and Miller is made as follows:

Alcohol (70 per cent)	100 c.c.
Hydrochloric acid (concentrated)	0.05 c.c.
Sodium chloride	0.5 gm.

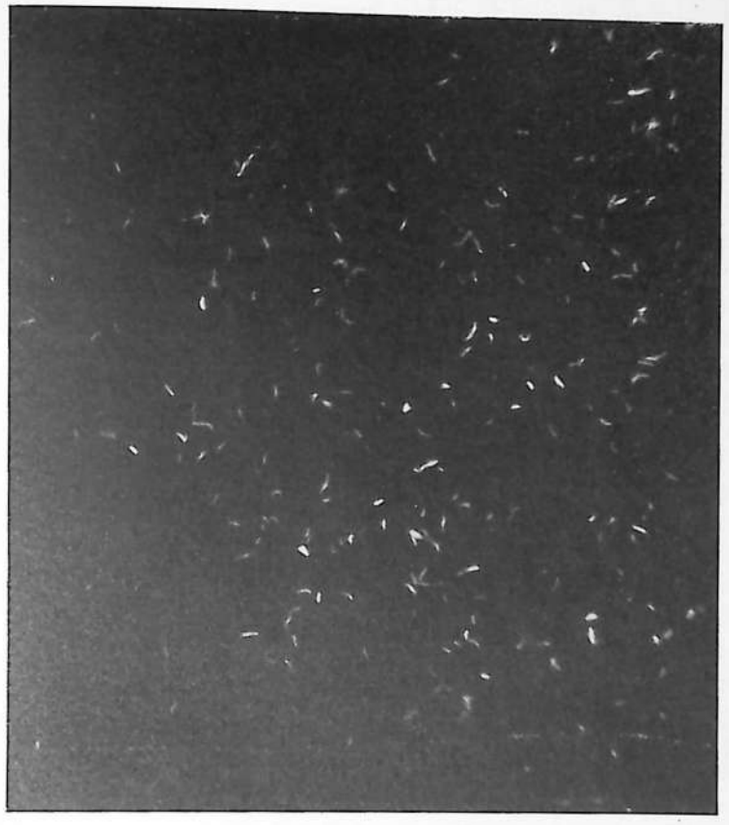


Fig. 29.—Fluorescent photomicrograph showing tubercle bacilli in sputum. (Richards, Kline and Leach, Am. Review of Tub., Vol. 44.)

Make the smear in the usual manner on a microscope slide and stain for two to three minutes. Wash in tepid water, decolorize for two minutes and then bleach still further in a fresh solution for another two to five minutes. Wash and dry the slides. Thompson also suggested that, for material that is spread thickly on slides, it is better to double the amount of hydrochloric acid in the decolorizing solution and to bleach for ten minutes.

Set up the microscope with the yellow filter resting on the diaphragm inside the ocular. Place the aluminized mirror over the microscope mirror and the blue, ultraviolet-transmitting filter over the microscope lamp. The room should be darkened as the tubercle bacilli will appear as bright, luminous rods against the black background (Fig. 29). The lens combination that is best used is an 8-mm. objective with a 20× ocular.

When bacilli are not found in suspected cases, one of the following methods should be tried:

1. **Antiformin Method.**—This has largely superseded the older methods of concentration. The chief difficulty with the older methods, such as boiling with caustic soda, is that the bacilli are so injured in the process that they do not stain characteristically. Except in special cases, when the bacilli are both scarce and uniformly scattered through the sputum, it is doubtful whether any method of concentration offers any advantage over the usual direct smear made from carefully selected particles.

Antiformin is a trade name for a preparation consisting essentially of equal parts of a 15 per cent solution of caustic soda and a 20 per cent solution of sodium hypochlorite. Substitutes appear to be less satisfactory than the original preparation. The solution slowly loses strength upon standing.

Löffler's method is probably the best for clinical work. It kills the bacilli, so that there is no danger in handling the material. Upon this account, however, it is not applicable to isolation of tubercle bacilli for cultures.

Place 10 to 20 c.c. of the sputum in a small flask, with an equal amount of 50 per cent antiformin, and heat to the boiling point. The sputum will be thoroughly liquefied, usually within a few seconds. For each 10 c.c. of the resulting fluid add 1.5 c.c. of a mixture of 1 volume of chloroform and 9 volumes of alcohol. Insert a rubber stopper and shake vigorously for several minutes or until emulsification has taken place. The object is to impregnate the lipoid capsules of the bacilli with chloroform, thus increasing their specific gravity. Pour off the emulsion into centrifuge tubes and centrifugalize at high speed for about fifteen minutes. The chloroform will go to the bottom, and the sediment which collects on its surface in a thin firm layer will contain the tubercle bacilli. Pour off the supernatant liquid and transfer the sediment to glass slides, removing the excess of fluid with filter paper. To facilitate removal of the disk of sediment *in toto* Williamson recommends the use of a centrifuge tube, the lower $\frac{1}{2}$ inch of which is of uniform caliber and the bottom of which is open and plugged with a rubber stopper. Add a little of the original sputum to cause the film to adhere to the slide, mix well, spread into a uniform layer, and finally dry, fix, and stain by the Ziehl-Neelsen method. Löffler recommends 0.1 per cent solution of malachite green for counterstain.

2. **Culture of Tubercle Bacilli.**—Corper and Uyei[1] developed a method for the isolation of tubercle bacilli from sputum, urine (p. 158), and tissues in the presence of other organisms. Their method as now perfected has proved very satisfactory in the hands of many laboratory workers.

Thoroughly mix the sputum. Place 1 c.c. of the homogeneous mixture in a 15-c.c. centrifuge tube, and add an equal amount of 5 per cent oxalic acid. Mix thoroughly with a sterile loop. Stopper with a sterile cork, and incubate at 37° C. for thirty minutes, shaking occasionally during this time. Add 10 c.c. of sterile 0.9 per cent salt solution, mix thoroughly and centrifuge. Decant the supernatant fluid and seed the residue on the surface of the special glycerol water crystal violet potato cylinder medium described on page 766. Cap the tube with tin foil, or impregnate the cotton plug lightly with hot paraffin. Incubate at 37° C. for several weeks. Growth may be evident in two weeks, but cultures should not be discarded as negative for several months.

3. **Animal Inoculation.**—Inoculation of guinea-pigs is the court of last appeal in detection of tubercle bacilli, but even this is not infallible, for it has been shown that the injected material must contain 10 to 150 bacilli in order to produce tuberculosis in the guinea-pig, the number required depending upon the virulence. The method is described on page 607.

There are a number of bacilli which stain in the same way as the tubercle bacillus and, therefore, belong to the group known as **acid-fast bacilli.** They stain with difficulty, and when once stained give up the color only very slowly when treated with a mineral acid, but, unlike the tubercle bacillus, most of them can be decolorized with alcohol or with Pappenheim's solution. Among them are the leprosy, smegma, butter and grass bacilli, and a bacillus which has been found in old distilled water. For this reason old distilled water should never be used for rinsing films or making staining solutions. Of the acid-fast bacilli, the smegma bacillus is the only one likely to cause confusion. It occurs normally about the genitals and other parts of the body, as the axillae, where secretions are prone to collect, and is often present in the urine and in the wax of the ear. It, or a similar bacillus, is sometimes found in the sputum of gangrene of the lung. The method of distinguishing it from the tubercle bacillus is given later (p. 157). A streptothrix (p. 57), which resists Gabbet's solution but is readily decolorized by Pappenheim's, has also been found in the sputum.

Other bacteria than the acid-fast group are stained blue by Gabbet's and the Ziehl-Neelsen method. Those most commonly found are staphylococci, streptococci, and pneumococci. Their presence in com-

[1] For references to this method see footnote on page 766.

pany with the tubercle bacillus constitutes *mixed infection*, although it is doubtless true that some of them in many cases exist as saprophytes. It is to be remembered that a few of the bacteria may reach the sputum from the upper air passages, and that great numbers are usually present in decomposing sputum.

There are many varieties of tubercle bacilli pathogenic for different animals, for example, the human, bovine, avian, and reptilian bacilli. Both human and bovine types are pathogenic for man, the latter being most frequently found in intestinal and lymphatic lesions of infancy and childhood. In clinical work it is not practicable to distinguish between the two.

Much's granules which are illustrated in Plate II, Fig. 4, were formerly thought to be significant. They are not acid-fast, and were demonstrated by a technic which in many respects constituted a Gram stain. Kretschmer[1] has shown that the tubercle bacillus in its acid-fast, pathogenic form does not possess true Gram-staining properties because of the acid-fast material. It cannot be stained differentially by Gram's method.

(2) **Staphylococcus and Streptococcus.**—One or both of these organisms is commonly present in company with the tubercle bacillus in the sputum of advanced phthisis (Plate II, Figs. 2 and 3). They are often found in bronchitis, catarrhal pneumonia, and many other conditions. The streptococcus is much the more important. It is a common cause of severe sore throat and tonsillitis. Staphylococci and streptococci are sometimes found in sputum and normal mouths as saprophytes. These cocci are discussed more fully on page 588, and their cultural characteristics are described on pages 790–792.

(3) **Pneumococcus** (*Diplococcus pneumoniae*).—The pneumococcus is the causative agent in nearly all cases of croupous pneumonia, and is commonly found in large numbers in the rusty sputum of this disease. It is frequently met with in the sputum of catarrhal pneumonia, bronchitis, and tuberculosis, although here it is not infrequently a harmless saprophyte. It is also an important factor in the causation of pleurisy, meningitis, otitis media, and other inflammations. It is frequently present in the saliva in health.

Pneumococci are about the size of streptococci. They are ovoid in shape, and lie in pairs, end to end, often forming short chains. Each pair is surrounded by a gelatinous capsule, which is its distinctive feature (Fig. 30).

The pneumococcus is closely related to the streptococcus, and it is sometimes extremely difficult to differentiate them even by cul-

[1] Kretschmer, O. S.: The Gram Property of the Acid-fast Form of the Tubercle Bacillus. Jour. Lab. and Clin. Med., *19*:350–358 (Jan.), 1934.

PLATE II

Fig. 1.—Heart-failure cells and carbon-laden cells in unstained sputum. Two small squamous epithelial cells and four red blood-corpuscles are included for comparison of size. (× 200.)

Fig. 2.—Eosinophilic leukocytes and staphylococci in asthmatic sputum. Eosin and methylene-blue. (× 1000.)

Fig. 3.—Tubercle bacilli, streptococci, pus corpuscles, and mucous threads in tuberculous sputum. Ziehl-Neelsen method. (× 1000.)

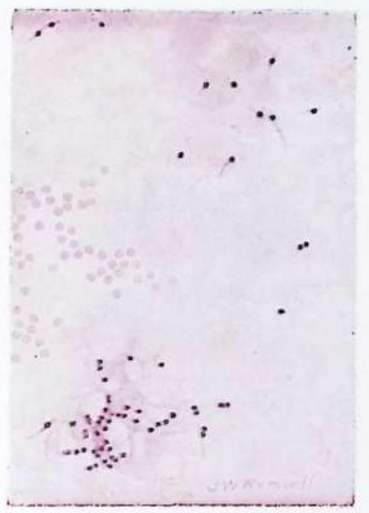


Fig. 4.—Much's granules; group of half-digested staphylococci is also shown. (× 1500.)

tural methods (p. 792). The morphology of the pneumococcus, the fact that it is gram-positive, and the presence of a capsule are, however, generally sufficient for its recognition in smears from sputum or pus. The capsule is often seen as a halo around pairs of cocci in smears stained by the ordinary methods, particularly Gram's method, but to show it well special methods are required. There are numerous special methods of staining capsules, which are applicable to other encapsulated bacteria, as well as to the pneumococcus. Smith's and Rosenow's methods, described below, and Hiss' method, described on page 588, can be recommended; but any of the standard methods will give beautiful results if capsules are well developed. The chief

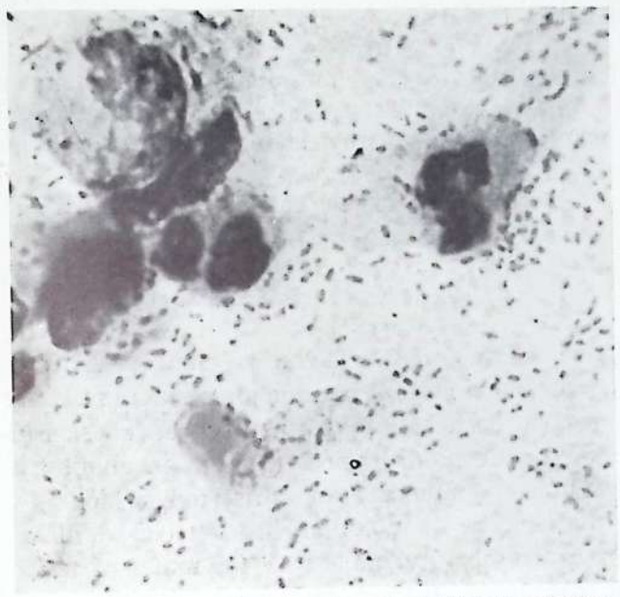


Fig. 30.—Pneumococci in sputum of pneumonia patient. (Frisch and Price, Detroit Med. News, Vol. 32.)

requisite is that the sputum or pus be very fresh—not more than two or three hours old.

W. H. Smith's Method for Capsules.—1. Make thin smears of the sputum or other material, which should be as fresh as possible.

2. Fix in the flame in the usual manner.

3. Apply a 10 per cent aqueous solution of phosphomolybdic acid (Merck) for four to five seconds.

4. Rinse in water.

5. Appy anilin-gentian violet (p. 831), steaming gently for fifteen to thirty seconds.

6. Rinse in water.
7. Apply Gram's iodine solution, steaming gently for fifteen to thirty seconds.
8. Wash in 95 per cent alcohol until the purple color ceases to come off.
9. Rinse in water.
10. Apply a 6 per cent aqueous solution of eosin and gently warm for one-half to one minute.
11. Rinse in water.
12. Wash in absolute alcohol.
13. Clear in xylol.
14. Mount in balsam.

This is essentially Gram's method (p. 780), preceded by treatment with phosphomolybdic acid and followed by eosin. Gram-positive bacteria like the pneumococcus are deep purple; capsules are pink and stand out clearly.

When the method is applied to gram-negative bacteria steps 5 to 9 inclusive are omitted; and between steps 11 and 12 the preparation is counterstained with Löffler's methylene blue, gently warming for fifteen to thirty seconds.

Rosenow's Method.—This is the same as Smith's, with the exception that a 10 per cent solution of tannic acid, applied while the film is still moist and allowed to act for ten to twenty seconds, takes the place of the heat and phosphomolybdic acid in steps 2 and 3.

The cultural characteristics of the pneumococcus are described on page 792. By means of certain immunologic reactions it can be shown that there are at least thirty distinct types. These differ in their virulence. For organisms of Type I (present in about a third of the cases of croupous pneumonia) an antiserum, which is valuable in treatment of this type of infection, has been used for a number of years. Antisera are also available now for many of the other types. Methods of determining pneumococcus types, either by agglutinins or precipitins, are described on page 793.

(4) **Bacillus of Friedländer** (*Klebsiella pneumoniae*).—In a small percentage of cases of pneumonia this organism is found alone or in company with the pneumococcus. It is commonly called "Bacillus mucosus capsulatus." Its pathologic significance is uncertain. It is often present in the respiratory tract under normal conditions. Friedländer's bacilli are nonmotile encapsulated rods, sometimes arranged in short chains (Fig. 31). Very short individuals in pairs closely resemble pneumococci, from which they are distinguished by the fact that they are gram-decolorizing.

(5) **Bacillus of Influenza** (*Hemophilus influenzae*).—For many years this organism was regarded as the cause of true influenza,

although it was recognized that clinically similar or identical conditions could be produced by the pneumococcus and streptococcus. It is present, sometimes in great numbers, in the nasal and bronchial secretions of most cases of influenza and in the local lesions which follow as complications. Chronic bronchitis, clinically suggesting tuberculosis, but apparently of influenzal origin, with great numbers of the bacilli in the sputum, has been described.

The many studies during the great pandemic of 1918–19 have, however, failed to confirm the specific importance of the influenza bacillus. It now seems probable that influenza is a virus disease.

Recognition of influenza bacilli in smears depends on the facts that they are extremely small bacilli; that most of them lie within

Fig. 31.—Bacillus mucosus capsulatus. *Klebsiella pneumoniae*. (Ford, "Textbook of Bacteriology.")

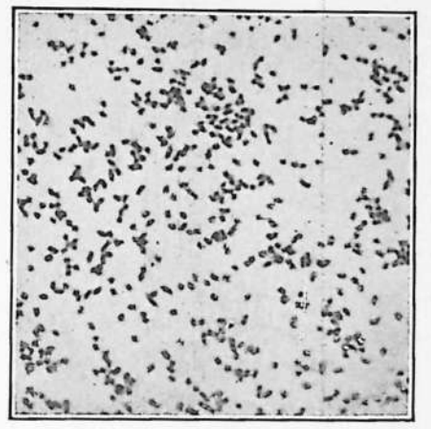


Fig. 32.—*Hemophilus influenzae*. (Ford, "Textbook of Bacteriology.")

the pus cells; that their ends often stain more deeply than their centers, sometimes giving the appearance of minute diplococci; and that they are decolorized by Gram's method of staining (Figs. 32 and 306).

They are well stained by dilute fuchsin or by Pappenheim's pyronin-methyl green, but are more certainly recognized by Gram's method with the pyronin-methyl green for counterstain.

(6) **Bacillus Pertussis** (*Hemophilus pertussis*).—The whooping-cough bacillus, which is found in the sputum in large numbers early in the disease, is a minute, ovoid, gram-negative bacillus which stains feebly with the ordinary dyes, and sometimes, though not usually, lies within pus cells. It can be demonstrated by the method given for the influenza bacillus which it resembles morphologically.

(7) **Micrococcus Catarrhalis** (*Neisseria catarrhalis*).—This organism is frequently present in the sputum in inflammatory conditions of the respiratory tract resembling influenza. It is sometimes present in the nasal secretions in health and is probably of very little pathogenic significance. It is a gram-negative diplococcus, frequently intracellular, and can be distinguished from the meningococcus and gonococcus only by means of cultures (Fig. 33). The staining method recommended for the influenza bacillus is best. It grows readily on ordinary media.

(8) **Higher Bacteria.**—Standing between the bacteria and the molds is a group of higher bacteria at one time called Trichomycetes

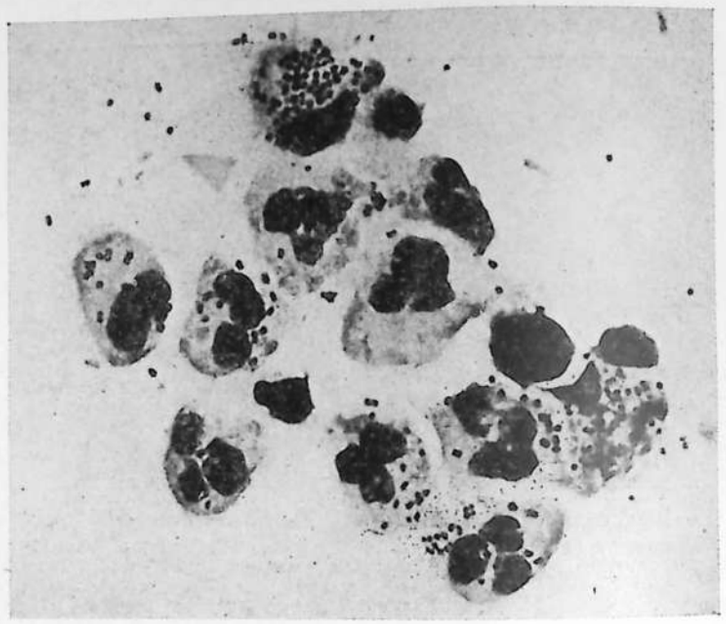


Fig. 33.—*Micrococcus catarrhalis* in smear from sputum. (F. T. Lord; photo, by L. S. Brown.)

or "hair fungi." They all form filaments, which are more slender than the hyphae of molds. Their classification is somewhat unsettled. Buchanan, Bergey, and the Society of American Bacteriologists now include them in the order of Actinomycetales, and in the family of Actinomycetaceae. The genera Leptotrichia and Actinomyces, as well as two others not found in sputum, are in the family. *Leptotrichia buccalis* forms long, unbranched filaments, with partition walls. Its normal habitat is the oral cavity. There are numerous species of the genus Actinomyces. They may be aerobic, or anaerobic, acid-fast, or nonacid-fast. The most common forms found in sputum are *Actinomyces hominis* (p. 42) and *Actinomyces farcinicus* (also known as

Nocardia, and also as Streptothrix). This last named species forms filaments, but no "clubs," is aerobic, and produces yellowish-white pigment on agar or potato medium. It is moderately acid-fast, but may be partially decolorized by alcohol and readily by Pappenheim's solution. In the sputum it appears in the form of short, very slender, branching filaments which usually lie in small tangled clusters, that might be mistaken for clumps of tubercle bacilli by the inexperienced.

2. Cells.—These include various types of leukocytes, epithelial cells, and red blood corpuscles. In general, a stain of the nature of Wright's blood stain is most satisfactory.

(1) **Leukocytes.**—(*a*) *Polymorphonuclear neutrophils* are present as pus corpuscles in every sputum, and at times the sputum may consist of little else. They appear as granular, rounded cells 10 to 12 μ in diameter, with several nuclei or one very irregular nucleus which when unstained is obscured by the granules. In preparations stained by any of the usual methods the nuclei stand out clearly and their polymorphous character makes identification of these cells easy (Plate II, Fig. 3). In old sputa the cells may be much disintegrated and hence difficult to recognize even when stained. When these cells predominate in the sputum a pyogenic infection may be assumed.

(*b*) *Lymphocytes* are generally present in small numbers along with the ordinary pus corpuscles, from which they are distinguished by the possession of a single round nucleus. In early or mild cases of pure tuberculous infection they are usually the predominating cell, and may be of much help in distinguishing such cases from those due to pyogenic organisms. If, in a case of known tuberculosis, the "cell formula" changes from lymphocytic to polynuclear, the occurrence of a secondary infection is strongly suggested.

(*c*) *Eosinophilic leukocytes* are quite constantly found in large numbers in the sputum of bronchial asthma near the time of the paroxysm, and constitute one of the most distinctive features of the sputum of this disease. However, while of much diagnostic importance, they are by no means pathognomonic of asthma. They resemble ordinary pus corpuscles, except that their cytoplasm is filled with coarse granules having a marked affinity for eosin. It is worthy of note that sometimes many of them are mononuclear. These are involution forms and not myelocytes. The eosinophils are very fragile; and large numbers of free granules, derived from disintegrated cells, are also found (Plate II, Fig. 2). Eosinophils can often be recognized in unstained sputum by the coarseness of their granules (Fig. 24), but for positive identification some method which includes eosin must be

used. A simple method is to stain the dried and fixed film two or three minutes with saturated solution of eosin, and then with Löffler's methylene blue for one-half minute or until the thinner portions of the film become blue; nuclei and bacteria will be blue, eosinophilic granules, bright red. Either Wright's or Jenner's stain (pp. 253 and 256) will also be found satisfactory.

(*d*) *Endothelial leukocytes* are best studied in unstained sputum, and have been described in the sections upon Pigmented Cells and Myelin Globules (p. 41).

(2) **Epithelial cells** may come from any part of the respiratory tract. A few are always present, since desquamation of cells goes on

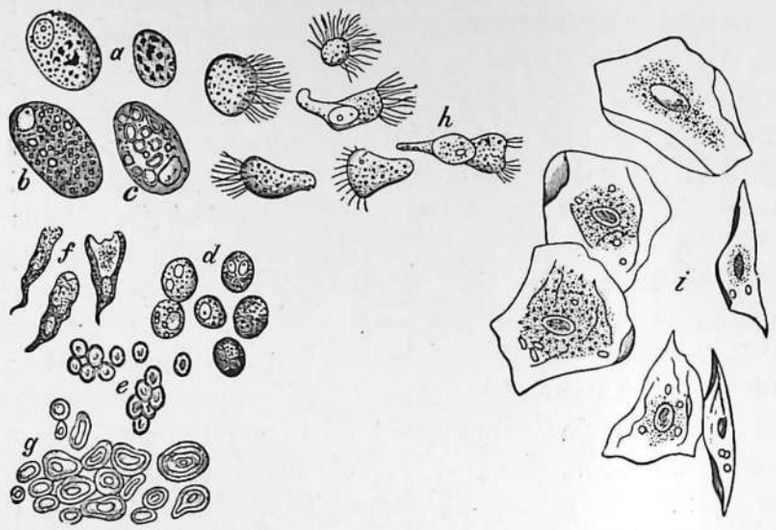


Fig. 34.—Different morphologic elements of the sputum (unstained): *a*, *b*, *c*, Pulmonary or alveolar epithelium—*a*, with normal lung pigment (carbon); *b*, with fat droplets; *c*, with myelin globules; *d*, pus corpuscles: *e*, red blood corpuscles; *f*, cylindric beaker-shaped bronchial epithelial cells; *g*, free myelin globules; *h*, ciliated epithelium of different kinds from the nose, altered by coryza; *i*, squamous cells from the pharynx. (After Bizzozero.)

constantly. Their recognition is important, chiefly as an aid in deciding upon the source of the portion of the sputum in which they are found. For this reason they are sometimes spoken of as "guide cells." In suspected lung conditions it is manifestly useless to study material from the nose only, yet this is not infrequently done. They have little diagnostic value, although a considerable excess would indicate a pathologic condition at the site of their origin. Any of the stains mentioned above will show them, and they can usually be identified without difficulty in unstained sputum. In general, three forms are found:

(*a*) *Squamous Cells.*—Large, flat, polygonal cells with a comparatively small nucleus (Fig. 34, *i*). They come from the upper air passages, and are especially numerous in laryngitis and pharyngitis. They are frequently studded with bacteria—most commonly diplococci.

(*b*) *Cylindric Cells from the Nose, Trachea, and Bronchi* (Fig. 34, *f*, *h*).—These are not usually abundant, and, as a rule, they are not identified because much altered from their original form, being usually round and swollen. Cylindric cells with cilia intact are rare, but are sometimes seen in bronchial asthma and very acute bronchitis. When very fresh the cilia may still be in active motion, suggesting infusoria.

(*c*) *Alveolar Cells.*—Rather large, round, or oval cells, three to six times the diameter of a red corpuscle, with one or two round nuclei (Fig. 34, *a*, *b*, and *c*). Their source is presumably the pulmonary alveoli. It is probable that many of the cells which have been included in this group are really endothelial leukocytes.

(3) **Red blood corpuscles** may be present in small numbers in almost any sputum. When fairly constantly present in considerable numbers they are suggestive of phthisis. The corpuscles, when fresh, can easily be recognized in unstained sputum, or may be shown by any of the staining methods which include eosin. They are, however, commonly so much degenerated as to be unrecognizable, and often only altered blood pigment is left. Ordinarily, blood in the sputum is sufficiently recognized with the naked eye.

III. CHEMICAL EXAMINATION

There is little to be learned from a chemical examination, and it is rarely undertaken. The presence or absence of albumin may have clinical significance. Albumin is almost constantly present in the sputum in pneumonia, pulmonary edema, and tuberculosis. It is usually absent in bronchitis. A test for albumin may, therefore, be of some value in distinguishing between bronchitis and tuberculosis, but it is not much relied upon. It is carried out as follows:

Method for Albumin in Sputum.—1. To 10 c.c. of the sputum add 30 c.c. of 1 per cent acetic acid and shake until thoroughly mixed. This may be done in a stoppered bottle. Dilution and addition of acetic acid precipitate the mucus.

2. Filter through filter paper.

3. Test the filtrate for albumin qualitatively and quantitatively, as described in Chapter II.

Active cases of phthisis, whether early or far advanced, generally show 0.2 per cent or more albumin; slightly active cases, less than 0.2

per cent. The sputum must be fresh, otherwise a negative reaction may have changed to positive owing to disintegration of cells.

IV. THE SPUTUM IN DISEASE

Strictly speaking, any appreciable amount of sputum is abnormal. A great many healthy persons, however, raise a small quantity each morning, owing chiefly to the irritation of inhaled dust and smoke. Although not normal, this can hardly be spoken of as pathologic. Ordinarily it reaches the larynx without cough. It is particularly frequent in city dwellers and in those who smoke cigarettes to excess. In the latter the amount is sometimes so great as to arouse suspicion of tuberculosis. Such "normal morning sputum" or "sputum of irritation" generally consists of small, rather dense, mucoid masses, translucent, white, or, when due to inhaled smoke, gray in color. Microscopically, there are a few pus corpuscles, and, usually, many endothelial leukocytes, both of which may contain carbon particles. The endothelial leukocytes commonly show myelin degeneration, and free myelin globules may be present in large numbers. Saprophytic bacteria may be present, but are not abundant.

1. Acute Bronchitis.—There is at first a small amount of tenacious, almost purely mucoid sputum, frequently blood streaked. This gradually becomes more abundant, mucopurulent in character, and yellowish or gray in color. At first the microscope shows a few leukocytes and bronchial cells; later the leukocytes become more numerous. Bacteria are not usually abundant.

2. Chronic Bronchitis.—The sputum is usually abundant, mucopurulent, and yellowish or yellowish-green in color. Nummular masses like those of tuberculosis are sometimes seen. Microscopically, there are great numbers of pus corpuscles, often much disintegrated. Epithelium is not abundant. Bacteria of various kinds, especially staphylococci, are usually numerous.

In fibrinous bronchitis there are found, in addition, fibrinous casts, usually of medium size.

In the chronic bronchitis accompanying long-continued passive congestion of the lungs, as in poorly compensated heart disease, the sputum may assume a rusty brown color, owing to presence of large numbers of the "heart-failure cells" previously mentioned.

3. Bronchiectasis.—The characteristic sputum is greenish or grayish, purulent, very abundant—sometimes as much as a liter in twenty-four hours—and has an offensive odor. It is thinner than that of chronic bronchitis, and upon standing separates into three layers of pus, serum, and frothy mucus. It contains great numbers of miscellaneous bacteria. Small hemorrhages are common. A feature of cases with a single large cavity is the periodic emptying of the cavity, usually upon rising in the morning; in other cases no periodicity is evident.

4. Gangrene of the Lung.—The sputum is abundant, fluid, very offensive, and brownish in color. It separates sharply into three layers upon standing—a thick brownish deposit of pus, débris, and blood pigment, a clear fluid, and a frothy layer. Microscopically, few cells of any kind are found. Bacteria are extremely numerous; among them may sometimes be found an acid-fast bacillus probably identical with the smegma bacillus. As before stated, elastic fibers are usually present in large fragments.

5. Pulmonary Edema.—Here there is an abundant, watery, frothy sputum, varying from faintly yellow or pink to dark brown in color; a few leukocytes and epithelial cells and varying numbers of red blood corpuscles are found with the microscope.

6. Bronchial Asthma.—The sputum during and following an attack is scanty, mucoid, and very tenacious. Most characteristic is the presence of Curschmann's spirals, Charcot-Leyden crystals, and eosinophilic leukocytes.

7. Croupous Pneumonia.—Characteristic of this disease is a scanty, rusty red, very tenacious sputum, containing red corpuscles or altered blood pigment, leukocytes, epithelial cells, usually many pneumococci, and often very small fibrinous casts. This sputum is seen during the stage of red hepatization. During resolution the sputum assumes the appearance of that of chronic bronchitis. When pneumonia occurs during the course of a chronic bronchitis, the characteristic rusty red sputum may not appear.

8. Pulmonary Tuberculosis.—The sputum is variable. In the earliest stages it may appear only in the morning, and is then scanty and almost purely mucoid, with an occasional yellow flake; or there may be only one small mucopurulent mass no larger than a match head. When the quantity is small there may be no cough, the sputum reaching the larynx by action of the bronchial cilia. This is not well enough recognized by practitioners. A careful inspection of all the sputum brought up by the patient on several successive days, and a microscopic examination of all yellow portions, will not infrequently establish a diagnosis of tuberculosis when physical signs are negative. Intelligent cooperation of the patient is essential in such cases. Tubercle bacilli will sometimes be found in large numbers at this stage. Blood-streaked sputum is strongly suggestive of tuberculosis, and is more common in the early stages than later. It usually indicates an advancing process.

The sputum of more advanced cases resembles that of chronic bronchitis, with the addition of tubercle bacilli and elastic fibers. Nummular masses—circular, "coinlike" disks, which sink in water—may be seen. Caseous particles containing immense numbers of the bacilli are common. Far-advanced cases with old cavities often show rather firm, spheric or ovoid grayish masses in thin fluid—the so-called "globular sputum." These globular masses usually contain many tubercle bacilli. Considerable hemorrhages are not infrequent, and for some time thereafter the sputum may contain clots of blood or be colored brown.

CHAPTER II

THE URINE

Preliminary Considerations.—The urine is an extremely complex aqueous solution of various organic and inorganic substances. Most of the substances are either waste products from the body metabolism or products derived directly from the foods eaten. Normally, the total amount of solid constituents carried off in twenty-four hours is about 60 Gm., of which the organic substances make up about 35 Gm. and the inorganic about 25 Gm.

The most important organic constituents are urea, uric acid, and creatinine. Urea constitutes about one half of all the solids, or about 30 Gm., in twenty-four hours.

The chief inorganic constituents are the chlorides, phosphates, sulfates, and ammonia. The chlorides, practically all in the form of sodium chloride, make up about one half of the inorganic substances, or about 13 Gm., in twenty-four hours.

Certain substances appear in the urine only in pathologic conditions. The most important of these are proteins, sugars, acetone and related substances, bile, and hemoglobin. Hormones may at times be excreted in the urine. On page 647 is a description of new pregnancy tests dependent on the excretion of an excess of the hormone from the anterior lobe of the pituitary gland.

In addition to the substances in solution all urines contain various microscopic structures.

While, under ordinary conditions, the composition of urine does not vary much from day to day, it varies greatly at different hours of the same day. It is evident, therefore, that the **collection of the specimen** is important, and that *no quantitative test can be of value unless a sample of the mixed twenty-four-hour urine be used.* The patient should be instructed to void all the urine during the twenty-four hours into a clean vessel kept in a cool place, to mix it well, to measure the whole quantity, and to bring 8 or more ounces for examination. In order to avoid annoying misunderstandings it is well to make these directions specific, telling him to empty the bladder at a specified time, say 8 A.M., and to discard this urine, to save all the urine voided up to 8 A.M. of the next day, and at that time to empty the bladder whether he feels the need for it or not, and to add this final amount to

the quantity collected. When it is desired to make only qualitative tests, as for albumin or sugar, a "sample" voided at random will answer. It should be remembered, however, that urine passed about three hours after a meal is most likely to contain pathologic substances. That voided first in the morning is least likely to contain them. To diagnose cyclic albuminuria samples obtained at various periods during the twenty-four hours must be examined.

The urine must be examined while fresh. **Decomposition** sets in rapidly, especially in warm weather, and greatly interferes with all the examinations. Decomposition may be delayed by adding 5 grains of boric acid (as much of the powder as can be heaped upon a 10-cent piece) for each 4 ounces of urine, but this causes precipitation of rhombic crystals of uric acid and does not prevent the growth of yeast. Formalin, in proportion of 1 drop to 1 ounce, is a still more efficient preservative, and is especially useful for microscopic structures; but it will interfere with Obermayer's test for indican, and if larger amounts be used, it may give reactions for sugar and albumin, and is likely to cause a precipitate which greatly interferes with the microscopic examination. Thymol, toluol, and chloroform are likewise much used. The use of thymol is very convenient. A small lump, floating on the surface, will preserve a bottle of urine for several days, but enough may dissolve to simulate the albumin reaction. The chief objection to toluol is the fact that it floats upon the surface, and the urine must be pipeted from beneath it. It is, however, the best preservative for the chemical constituents, particularly acetone and diacetic acid. Chloroform is probably the least satisfactory. It reduces Fehling's solution; and it settles to the bottom in the form of globules which it is impossible to avoid when removing the sediment for microscopic examination. One of these preservatives may be placed in the vessel when collection of the twenty-four-hour sample is begun. Whenever possible the urine should be kept on ice.

Normal and **abnormal pigments,** which interfere with certain of the tests, can be removed by filtering the urine through animal charcoal, or precipitating with a solution of normal acetate of lead (sugar of lead), or with powdered lead acetate in substance, and filtering.

Certain cloudy urines cannot be **clarified** by ordinary filtration through paper, particularly when the cloudiness is due to bacteria. Such urines can usually be rendered comparatively clear by adding a small amount of purified talc or infusorial earth, shaking well, and filtering.

A suspected fluid can be **identified as urine** by detecting any considerable quantity of urea in it (p. 78). Traces of urea may, however, be met with in ovarian cyst fluid, while urine from very old cases of hydronephrosis may contain little or none.

The **frequency of micturition** is often suggestive in diagnosis. Whether it is unduly frequent can best be ascertained by asking the patient whether he has to get up at night to urinate. Increased frequency may be due to restlessness; to increased quantity of urine; to irritability of the bladder, usually an evidence of cystitis; to obstruction ("retention with overflow"); or to paralysis of the sphincter.

The points to be covered in a **routine examination** will vary with circumstances. The following are suggested for office work and for the small hospital: Quantity, if twenty-four-hour amount is available; color and transparency; reaction; specific gravity; qualitative tests for albumin, sugar, indican, and acetone bodies; and a careful microscopic examination of the sediment.

Clinical examination of the urine may conveniently be considered under five heads: I. General characteristics. II. Chemical examination. III. Microscopic examination. IV. Functional tests. V. The urine in disease.

I. GENERAL CHARACTERISTICS

1. Quantity.—The quantity passed in twenty-four hours varies greatly with the amount of liquids ingested, perspiration, etc. The normal average may be taken as 1200 to 1500 c.c., or 40 to 50 ounces for an adult in this country. German writers give higher figures. For children the amount is somewhat greater in proportion to body weight.

The quantity is increased (polyuria) during absorption of large serous effusions and in many nervous conditions. It is usually much increased in chronic interstitial nephritis, diabetes insipidus, and diabetes mellitus. In these conditions a permanent increase in amount of urine is fairly constant—a fact of much value in diagnosis. In diabetes mellitus the urine is usually 2 to 5 liters, and may, though rarely, reach the enormous amount of 28 liters.

The quantity is decreased (oliguria) in severe diarrhea; in fevers; in all conditions which interfere with circulation in the kidney, as poorly compensated heart disease; in the parenchymatous forms of nephritis; and during accumulation of fluid in the serous cavities. In uremia the urine is usually very greatly decreased and may be entirely suppressed (anuria).

Ordinarily, more urine is voided during the day than during the night, the normal ratio being about 4 to 1 or 3 to 1. *Nocturnal polyuria*, in which the night urine (8 P.M. to 8 A.M.) is increased and may equal or exceed that passed during the day, is of value as a sign

of early functional derangement of the kidneys provided no water is taken after the evening meal (Mosenthal's Test, p. 169).

2. Color.—This varies considerably in health, and depends largely upon the quantity of urine voided, dilute urines being pale and concentrated urines highly colored. The usual color is yellow or reddish yellow, due to the presence of several pigments, chiefly urochrome, which is yellow. Traces of hematoporphyrin, uro-erythrin, and urobilin are frequent. Uro-erythrin is chiefly responsible for the deep reddish tinge of urine in acute fevers. Urobilin and porphyrins have clinical significance and are discussed later (pp. 112 and 116). Acid urine is generally darker than is alkaline.

Color is sometimes greatly changed by abnormal pigments. Blood pigment gives a red or brown, smoky color. Urine containing bile is yellowish or brown, with a yellow foam when shaken. It may assume a greenish hue after standing, owing to oxidation of bilirubin into biliverdin. Ingestion of small amounts of methylene blue gives a pale green; large amounts give a marked greenish blue. Santonin produces a yellow; rhubarb, senna, cascara, and some other cathartics, a brown color; these change to red upon addition of an alkali, and if the urine be alkaline when voided may cause suspicion of hematuria. A bright pink or red color appearing when the urine is alkalinized may be due to phenolphthalein taken as a laxative. Thymol gives a yellowish green. Following poisoning from phenol and related drugs the urine may have a normal color when voided, but becomes olive green to brownish black upon standing. Urine which contains melanin, as sometimes in melanotic tumors, and very rarely in wasting diseases, also becomes brown or black upon long standing. A similar darkening upon exposure to the air occurs in alkaptonuria (p. 118). A milky color may be due to presence of chyle, or milk may have been added by a malingering patient.

A pale greenish urine with high specific gravity strongly suggests diabetes mellitus.

3. Transparency.—Freshly passed normal urine is clear. Upon standing, a faint cloud of mucus, leukocytes, and epithelial cells settles to the bottom—the so-called "nubecula." This is more abundant in women, owing to vaginal cells and mucus. In urines of high specific gravity it may float near the middle of the fluid.

Abnormal cloudiness is usually due to presence of phosphates, urates, pus, blood, or bacteria. Epithelial cells and tube casts are rarely present in sufficient number to produce more than a slight cloudiness, although they may add to turbidity due to other causes. There are on record a very few cases in which cloudiness was caused

by spontaneous precipitation of albumin, but, in general, albumin does not affect the transparency of the urine. The presence of albumin does, however, cause marked foaming when the urine is shaken.

Amorphous phosphates are precipitated in neutral or alkaline urine. They form a white cloud and sediment, which disappear upon addition of an acid.

Amorphous urates are precipitated only in acid urine. They form a white or pink cloud and sediment ("brick-dust deposit"), which disappear upon heating.

Pus resembles amorphous phosphates to the naked eye. Its nature is easily recognized with the microscope, or by adding a strong solution of caustic soda to the sediment, which is thereby transformed into a gelatinous mass (Donné's test).

Blood gives a reddish or brown, smoky color, and may be recognized with the microscope or by tests for hemoglobin.

Bacteria, when present in great numbers, give a uniform cloud, which cannot be removed by ordinary filtration. They are detected with the microscope.

The cloudiness of decomposing urine is due mainly to precipitation of phosphates and multiplication of bacteria.

4. Odor.—The characteristic aromatic odor has generally been attributed to volatile acids. A substance, called "urinod," has also been held responsible. The odor is most marked in concentrated urines. During decomposition the odor becomes ammoniacal. A fruity odor is sometimes noted in diabetes, due probably to acetone. Urine which contains cystine may develop an odor of sulfurated hydrogen during decomposition.

Various articles of diet and drugs impart peculiar odors. Notable among these are asparagus, which gives a characteristic offensive odor, and turpentine, which imparts an odor somewhat suggesting that of violets.

5. Reaction.—Normally, the mixed twenty-four-hour urine is slightly acid in reaction. The acidity sometimes increases for a time after the urine is voided, the so-called "acid fermentation." The acidity was formerly held to be due wholly to acid phosphates, but Folin has shown that the acidity of a clear urine is ordinarily greater than the acidity of all the phosphates, the excess being due to free organic acids. Individual samples may be slightly alkaline, especially after a full meal; or they may be amphoteric, turning red litmus paper blue and blue paper red, owing to presence of both alkaline and acid phosphates. The reaction is ordinarily determined by means of litmus paper, which must be of good quality.

Determination of Hydrogen-ion Concentration.—The hydrogen-ion concentration or true reaction of the urine is ordinarily about *p*H 6, with the normal range of 4.8 to 7.5. Simple outfits for the determination of hydrogen-ion concentration colorimetrically can be purchased from the chemical supply houses. Dilute the sample of urine with distilled water if too highly colored. The phosphates in the urine act as a natural buffer. Place 10 c.c. of the sample in a pyrex test tube, 16 mm. internal diameter. Add 0.5 c.c. of 0.04 per cent solution of bromthymol-blue, which has a range in its color changes from 6.1 to 7.7. If the urine is acid, less than 7, use brom-cresol-purple, 5.4. to 7, and if alkaline use phenol red, 6.6 to 8.2. If the urine is very alkaline use thymol-blue, 8.2 to 9.8, and if very acid use chlor-phenol-red, 5.1 to 6.7. The colors are readily matched by referring to a color chart now generally used for this purpose, or by using a comparator as described on page 778.

A very satisfactory method of testing the reaction of urine and roughly determining the *p*H is with the use of nitrazine paper.[1]

Acidity is increased after administration of certain drugs, by excess of protein in the diet, in acidosis, and whenever the urine is concentrated from any cause, as in fevers. A strongly acid urine may cause frequent micturition because of its irritation. This is often an important factor in the troublesome enuresis of children.

Quantitative estimation of acidity of urine is not of much clinical value. When, however, it is desired to make it, the method of Folin will be found satisfactory. In every case the sample must be from the mixed twenty-four-hour urine and as fresh as possible.

Folin's Method.—Into a small flask measure 25 c.c. of the urine, and add 1 or 2 drops 0.5 per cent alcoholic solution of phenolphthalein and 15 or 20 Gm. of neutral potassium oxalate. Shake for a minute, and immediately titrate with decinormal sodium hydroxide, shaking meanwhile, until the first permanent pink appears. Read off from the buret the amount of decinormal sodium hydroxide solution added, and calculate the number of cubic centimeters which would be required for the entire twenty-four hours' urine. Most estimations run between 25 and 40 c.c. of decinormal solution for 100 c.c. of urine. Folin places the normal acidity for the twenty-four-hour specimen at 554 to 669 c.c. of decinormal solution, but most other authors give lower figures. Much depends on the diet.

The urine always becomes alkaline upon long standing, owing to decomposition of urea with formation of ammonia. Marked alkalinity of the freshly voided urine usually indicates such "ammoniacal decomposition" in the bladder, which is the rule in neglected chronic

[1] Nitrazine (sodium dinitrophenyl-azo-naphthol disulfonate) is manufactured by E. R. Squibb & Sons, New York, N. Y.

cystitis, especially that due to paralysis or obstruction. This form of alkalinity is known as *volatile alkalinity*, and can be recognized by the odor or by the fact that litmus paper turned blue by the urine again becomes red upon gentle heating, or that the paper will turn blue when held in the steam over the boiling urine. Such accumulation of free ammonia, derived from breaking down of urea after the urine is secreted and leading to volatile alkalinity of the urine, is frequently confused in the student's mind with increased elimination of ammonium salts in acidosis, which is associated with increased acidity. A second form of alkalinity, *fixed alkalinity*, is due to alkaline salts, and is often observed during frequent vomiting, after the crisis of pneumonia, in various forms of anemia, during digestion of full meals ("alkaline tide"), after abundant eating of fruits, and after administration of certain drugs, especially salts of vegetable acids. In some cases, as a result of the alkalinity, there is regularly a white deposit of amorphous phosphates, and the condition has been called "phosphatic diabetes."

With normal individuals, administration of 3 to 5 Gm. of sodium bicarbonate by mouth will cause the urine to become alkaline. In conditions of acidosis, upon the other hand, very much larger amounts of bicarbonate may be given without bringing about this change. It appears that a large proportion of the carbonate is retained in the body to fortify the depleted alkali reserve of the blood and tissues. Only after the reserve is restored does the carbonate pass into the urine and change its reaction. This fact forms the basis for the "bicarbonate tolerance test" of Sellards,[1] which is a simple and useful test for acidosis. Unlike the determination of ammonium salts in urine or detection of acetone, it is applicable in all forms of acidosis. Moreover, it furnishes a reasonably accurate index of the degree of acidosis, although Palmer and Van Slyke have shown that, judged by the CO_2-combining power of blood plasma, it generally indicates a somewhat greater degree of acidosis than really exists.

The test consists in giving the patient 5 Gm. of sodium bicarbonate, dissolved in a little water, by mouth, every two or three hours until the urine, voided before each dose, becomes neutral or faintly alkaline to litmus paper. The urine is thoroughly boiled before testing.

Tolerance of 20 to 30 Gm. of bicarbonate indicates a moderate grade of acidosis which usually produces no clinical symptoms. Tolerance of 40 to 50 Gm. is noted in more marked grades which still do not lead to symptoms beyond dyspnea upon exertion. When the tolerance reaches 75 to 100 Gm. there may be very definite and serious clinical symptoms. In extreme cases the tolerance may reach 150 Gm.

[1] For further details and directions for sterilization and intravenous use of the bicarbonate, see Sellards, A. W.: The Principles of Acidosis and Clinical Methods for Its Study, Harvard University Press, 1919.

Since, however, large amounts of bicarbonate are not well borne by the stomach it is well, in severe acidosis, not to push the administration until the urine becomes alkaline, but to discontinue as soon as the existence of marked acidosis is established.

6. Specific Gravity.—In a general way this varies inversely with the quantity of urine. The normal average is about 1.017 to 1.020. Samples of urine taken at random may go far above or below these figures. That first voided in the morning is generally most concentrated. Normal kidneys should dilute the urine to a specific gravity of 1.003 or less following ingestion of 1500 c.c. of water upon an empty stomach in the morning, and should be able to concentrate the urine to about 1.030 when the patient is upon a diet of solid food without liquids for a day. Inability to dilute or concentrate to this degree is evidence of defective renal function (Concentration and Dilution Test of Volhard and Fahr, p. 170). Variations in specific gravity of specimens, taken at stated intervals during the day when the patient is upon a standard diet with no intake between meals, are also of value in detecting functional incapacity of the kidneys and are discussed on page 169.

Pathologically, the specific gravity of the mixed twenty-four-hour urine may vary from 1.001 to 1.060. It is low in chronic interstitial nephritis, diabetes insipidus, and many functional nervous disorders. It is high in fevers and in parenchymatous disease of the kidney. In any form of nephritis a sudden fall without a corresponding increase in quantity of urine may foretell approaching uremia. It is highest in diabetes mellitus. A high specific gravity when the urine is not highly colored, or when the quantity is above the normal, should lead one to suspect this disease. A normal or low specific gravity, even below 1.005, does not, however, exclude it.

The specific gravity is most conveniently estimated by means of the urinometer (Fig. 35). Squibb's urinometer is adjusted to give accurate readings at 22.5° C.; the Taylor instrument, at 25° C. If the urine be brought to about the right temperature, a correction for temperature will seldom be necessary in clinical work. For accuracy, however, it is necessary to add 0.001 to the urinometer reading for each 3° C. above the temperature for which the urinometer is standardized, and to subtract 0.001 for each 3° C. below that point. Care should be taken that the urinometer does not touch the side of the tube, and that air bubbles are removed from the surface of the urine. Bubbles are easily removed with a strip of filter paper. With most instruments the reading is taken from the bottom of the meniscus.

A long scale on the stem is desirable because of the greater ease of accurate reading. Urinometers which show slight slipping of the scale may be checked by students as a special project, by comparing them carefully with an accurate instrument. Instead of being discarded entirely, they thus are made useful by using a correction factor.

The specific gravity of extremely small quantities of fluid can be ascertained by the following method which has been much used for the specific gravity of the blood: Two fluids, such as benzol and chloroform, which differ widely in specific gravity and which readily mix with each other, but do not mix with the fluid to be tested, are mixed in a cylinder. A drop of the fluid to be tested is then placed in the mixture, and the specific gravity of the mixture is adjusted by adding the lighter or the heavier of the fluids until

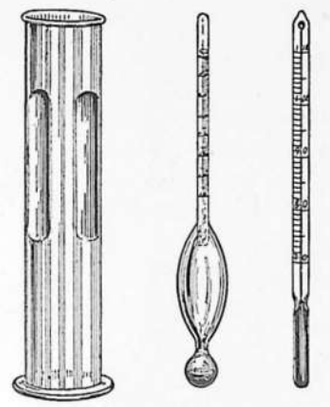


Fig. 35.—Squibb's urinometer with thermometer and cylinder.

the drop remains suspended near the middle. The mixture then has the same specific gravity as the fluid which is being tested, and this is readily ascertained with an accurate hydrometer. The following precautions must be observed: (*a*) The two fluids must be well mixed. (*b*) The drop of fluid to be tested must be of medium size, it must not contain an air-bubble, it must not cling to the side of the cylinder, and it must not remain long in the fluid. (*c*) After the specific gravity has been adjusted as accurately as possible with the first drop, it should be verified with a fresh drop. (*d*) The hydrometer must be standardized for the chloroform-benzol mixture or other fluid used in the test. Make a mixture such that a drop of distilled water will remain suspended in it (that is, with specific gravity of 1.000) and correct the hydrometer by this.

Exton's "immiscible balance" for the determination by the above method of the specific gravity of small quantities of urine, blood, or most aqueous solutions is especially satisfactory (Fig. 36). He uses a mixture

of carbon tetrachloride and a petroleum product sold as "varnolene," "stanisol," and possibly under other trade names.

7. Total Solids.—The total amount of solids which pass through the kidneys in twenty-four hours is about 60 Gm., or 950 grains for a man of 150 pounds. The principal factors which influence this amount are body weight (except with excessive fat), diet, exercise, age, the activity of metabolism, and the ability of the kidneys to excrete. Simple estimations of the total solids, therefore, may under uniform conditions of diet and exercise furnish an important clue to the functional efficiency of the kidneys. After about the forty-fifth

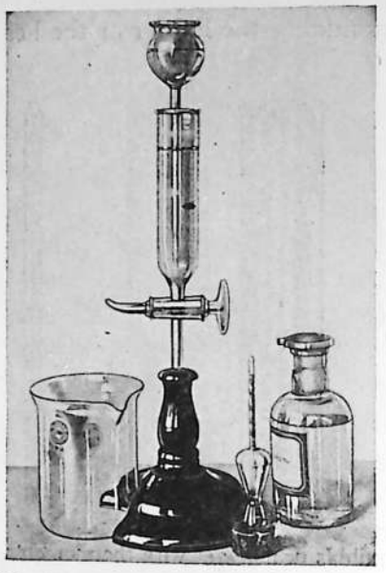


Fig. 36.—Exton's immiscible balance.

year the solids become gradually less; after the seventy-fifth the normal amount is about one half that given above.

The total solids can be estimated roughly, but accurately enough for most clinical purposes, by multiplying the last two figures of the specific gravity of the mixed twenty-four-hour urine by the number of ounces voided and to the product adding one tenth of itself. This gives the amount in grains. If, for example, the twenty-four-hour quantity is 3 pints or 48 ounces, and the specific gravity is 1.018, the total solids would approximate 950 grains, as follows:

$$48 \times 18 = 864; 864 + 86.4 = 950.4.$$

This method is especially convenient for the practitioner, because patients nearly always report the amount of urine in pints and ounces,

and it avoids the necessity of converting into the metric system. Häser's method, which uses the metric system, is more widely used, but is less convenient. The last two figures of the specific gravity are multiplied by 2.33. The product is then multiplied by the number of cubic centimeters voided in twenty-four hours and divided by 1000. This gives the total solids in grams.

II. CHEMICAL EXAMINATION

A. Normal Constituents

Of the large number of organic and inorganic substances normally present in the urine, only a few demand any consideration from the clinician. The table given below therefore outlines the average composition from the clinical, rather than from the chemical, standpoint. Only the twenty-four-hour quantities are given, since they alone furnish an accurate basis for comparison. *The student cannot too soon learn that percentages mean little or nothing except as they furnish a means of calculating the twenty-four-hour elimination.*

Although the conjugate sulfates are organic compounds, they are, for the sake of convenience, included with the inorganic sulfates in the table.

COMPOSITION OF NORMAL URINE

	Grams in twenty-four hours.	Grams, approximate average.
Water	1000–1500	1200
Total substances in solution	55–70	60
Inorganic substances	20–30	25
Chlorides (chiefly sodium chloride)	10–15	12.5
Phosphates (estimated as phosphoric acid), total	2.5–3.5	3
Earthy, one third of total		1
Alkaline, two thirds of total		2
Sulfates (estimated as sulfuric acid), total	1.5–3	2.5
Mineral, nine tenths of total		2.25
Conjugate, one tenth of total		0.25
Includes indican		Trace
Ammonia	0.5–1	0.7
Organic substances	30–40	35
Urea	25–35	30
Uric acid	0.4–1	0.7

Among constituents which are of little clinical importance, or are present only in traces, are:

Inorganic.—Iron, carbonates, nitrates, silicates, and fluorides.

Organic.—Creatinine, hippuric acid, purine bases, oxalic acid, volatile fatty acids, pigments, and acetone.

Variations in body weight, diet, and exercise cause marked fluctuations in the total solids and in individual substances.

1. Chlorides.—These are derived from the food, and are mainly in the form of sodium chloride. The amount excreted normally is 10 to 15 Gm. in twenty-four hours. It is much affected by the diet, and is reduced to a minimum in starvation.

Excretion of chlorides is diminished in some forms of nephritis and in fevers, especially in pneumonia and inflammations, leading to the formation of large exudates. In nephritis, particularly the form designated chronic parenchymatous nephritis, the kidneys are less permeable to the chlorides; and it is possible that the edema is due largely to an effort of the body to dilute the chlorides which have been retained. Certainly an excess of chlorides in the food will in many cases increase both the albuminuria and the edema of nephritis. In fevers the diminution is due largely to decrease of food, though probably in some measure to impaired renal function. In pneumonia chlorides are constantly very low, and in some cases are absent entirely. Following the crisis they are increased. In inflammations leading to formation of large exudates—for example, pleurisy with effusion—chlorides are diminished because a considerable amount becomes "locked up" in the exudate. During absorption chlorides are liberated and appear in the urine in excessive amounts. In extensive burns, there is a diminution of urinary chlorides. Saline injections form an important part of the treatment. Diminution of chlorides is also sometimes observed in severe diarrhea, anemic conditions, and carcinoma of the stomach.

Detection of Chlorides.—The following simple test will show the presence of chlorides, and at the same time roughly indicate any pronounced alteration in amount. It is especially useful in following the excretion of chlorides in cases of pneumonia:

To a few cubic centimeters of albumin-free urine in a test tube add a few drops of nitric acid to prevent precipitation of phosphates and then a few drops of silver nitrate solution of about 12 per cent strength. A white, curdy precipitate of silver chloride forms. If the urine merely becomes milky or opalescent, chlorides are markedly diminished.

Quantitative Estimation.—The well-known and reliable Volhard method has been simplified by Strauss, and this modification has been still further simplified by Bayne-Jones and by McLean and Selling, so that the method is now available for ordinary clinical work. The only difficulty is the preparation of solutions, and these can be purchased ready prepared. Other methods for the determination of chlorides, which can be applied to urine, are described in the chapter on Clinical Chemistry.

Simplified Volhard Method (McLean and Selling[1]).—As a rule, albumin need not be removed. In an accurately graduated 50-c.c. cylinder place 5 c.c. of the urine and 10 c.c. of Solution No. 1. Mix by inverting several times. If a reddish color appears, add 3 drops of 10 per cent potassium permanganate. After five minutes add Solution No. 2, a very little at a time, mixing after each addition until a permanent reddish-brown color (best seen against a white background) appears. This is the end-point.

The solutions are so balanced that if the urine be chloride free the volume of fluid when the end-point is reached will be 35 c.c., and that for each gram per liter of chlorides in the urine the volume will be 1 c.c. less. Therefore, the difference between 35 c.c. and the height of the fluid at the end of the test gives directly the number of grams of chlorides per liter of urine, expressed as sodium chloride. If, for example, the fluid reaches the 28-c.c. mark, 35 − 28 = 7 Gm. of sodium chloride per liter of urine.

A certified 50-c.c. graduated cylinder, with glass stopper, is required. The ordinary 50-c.c. graduate is not sufficiently accurate.

The **solutions** are as follows:

No. 1.—*Standard silver nitrate solution:*

Silver nitrate (C. P., anhydrous, crystallized)	29.055 Gm.
Nitric acid (25 per cent)	900 c.c.
Ammonioferric alum (cold saturated solution)	50 "
Distilled water to	1000 "

No. 2.—*Ammonium sulfocyanate solution:*

Ammonium sulfocyanate	7 Gm.
Distilled water	1000 c.c.

This solution is intentionally made too strong, and it must be standardized by diluting with distilled water until exactly 20 c.c. (and no less) will produce a red color when mixed with exactly 10 c.c. of Solution No. 1.

2. Phosphates are derived largely from the food, only a small proportion resulting from metabolism. The normal daily output of phosphoric acid is about 2.5 to 3.5 Gm.

The urinary phosphates are of two kinds: *Alkaline*, which make up two thirds of the whole, and include the phosphates of sodium and potassium, and *earthy*, which constitute one third, and include the phosphates of calcium and magnesium. Earthy phosphates are frequently thrown out of solution in neutral and alkaline urines, and as "amorphous phosphates" form a very common sediment. This sediment seldom indicates an excessive excretion of phosphoric acid.

[1] McLean, F. C., and Selling, L.: Further Simplification of Quantitative Determination of Chlorids in the Urine, Jour. Am. Med. Assn., *62*:1081–1082 (April 4), 1914

It is usually merely an evidence of diminished acidity of the urine, or of an increase in the proportion of phosphoric acid eliminated as earthy phosphates. This form of "phosphaturia" is most frequent in neurasthenia and hysteria. When the urine undergoes ammoniacal decomposition some of the ammonia set free combines with magnesium phosphate to form ammoniomagnesium phosphate ("triple phosphate"), which is only slightly soluble in alkaline urine and is deposited in typical crystalline form (p. 136).

From the clinical point of view variations in the amount of phosphates in the urine are unimportant and no method for their determination need be given here.

3. Sulfates.—The urinary sulfates are derived partly from the food, especially meats, and partly from body metabolism. The normal output of sulfuric acid is about 1.5 to 3 Gm. daily. It is increased in conditions associated with active metabolism, and in general may be taken as a rough index of protein metabolism.

About nine tenths of the sulfuric acid is in combination with various mineral substances, chiefly sodium, potassium, calcium, and magnesium (*mineral* or *preformed sulfates*). One tenth is in combination with certain aromatic substances, which are mostly products of protein putrefaction in the intestine, but are derived in part from destructive metabolism (*conjugate* or *ethereal sulfates*). Among these aromatic substances are indol, phenol, and skatol. By far the most important of the conjugate sulfates and representative of the group is potassium indoxyl sulfate.

Potassium indoxyl sulfate, or **indican,** is derived from indol. Indol is absorbed and oxidized into indoxyl, which combines with sulfuric acid and potassium and is thus excreted. Under normal conditions the amount in the urine is small. It is increased by a diet rich in protein.

Unlike the other ethereal sulfates, which are derived in part from metabolism, indican originates practically wholly from putrefactive processes. It alone, therefore, and not the total ethereal sulfates, can be taken as an index of such putrefaction. A marked increase is called *indicanuria* and is often associated with lassitude and headache. Not infrequently it is merely evidence of too much meat in the diet. Pathologically indicanuria is noted in:

(*a*) *Diseases of the Small Intestine.*—This is by far the most common source. Intestinal obstruction gives the largest amounts of indican. It is also much increased in intestinal indigestion—so-called "biliousness"; in inflammations, especially in cholera and typhoid fever; and in paralysis of peristalsis, such as occurs in peritonitis.

Simple constipation and diseases of the *large* intestine alone rarely cause indicanuria.

(*b*) *Diseases of the stomach* associated with deficient hydrochloric acid, as chronic gastritis and gastric cancer. Diminished hydrochloric acid favors intestinal putrefaction. Simon has called attention to the rather frequent occurrence of indicanuria in cases of gastric ulcer in which hyperchlorhydria is the rule, a fact which is as yet unexplained.

(*c*) *Diminished Flow of Bile.*—Since the bile serves as a stimulant to peristalsis and in several ways retards putrefaction, a diminished flow from any cause favors occurrence of indicanuria.

(*d*) *Decomposition of exudates* anywhere in the body, as in empyema, bronchiectasis, and large tuberculous cavities.

Detection of indican depends upon its decomposition and subsequent oxidation of the indoxyl set free into indigo-blue. This change sometimes takes place spontaneously in decomposing urine, causing a dirty blue color. Crystals of indigo (Fig. 45) may then be found both in the sediment and the scum.

Obermayer's Method.—Take a test tube, about one third full of the urine and add an equal volume of Obermayer's reagent and a few cubic centimeters of chloroform. For best results the proportion of urine and reagent must be closely adhered to and the mixture should be warmed before adding the chloroform. Mix by inverting a few times. Avoid shaking violently as this may emulsify the chloroform. If indican be present in excess, the chloroform, which sinks to the bottom, will assume an indigo-blue color. The indican in normal urine may give a faint blue by this method. The depth of color indicates the comparative amount of indican if the same proportions of urine and reagents are always used, but one should bear in mind the total amount of urine voided. It is well to keep on hand one or two test tubes with marks indicating the amounts of urine and reagents to be used. An accurate quantitative method is unnecessary in clinical work.

Urine of patients taking iodides gives a reddish-violet color with Obermayer's reagent, and this may obscure even a fairly strong indican reaction. However, upon addition of a few drops of strong sodium hyposulfite solution and shaking, the violet color will disappear, leaving the blue if indican be present. Occasionally, owing to slow oxidation, indigo-red will form instead of indigo-blue. This gives a color much like that due to iodides, but it does not disappear when treated with sodium hyposulfite. A color somewhat similar to that of indigo-blue may be produced by guaiacol, but this soon changes to yellowish green. Hexamethylenamine prevents the reaction even when a large amount of indican is present, as does formaldehyde when added to the urine as a preservative. Bile pigments, which interfere with the test, must be removed if present (p. 64).

Obermayer's reagent consists of strong hydrochloric acid (sp. gr. 1.19), 1000 c.c., and ferric chloride, 2 Gm. This makes a yellow, fuming liquid which keeps well.

4. Urea.—From the standpoint of physiology urea is the most important constituent of the urine. It is the principal waste product of metabolism, and constitutes about one half of all the solids excreted—about 20 to 35 Gm. in twenty-four hours. It represents 85 to 90 per cent of the total nitrogen of the urine, and its quantitative estimation is a simple, though not very accurate, method of ascertaining the state of nitrogenous excretion. This is true, however, only in normal individuals upon average mixed diet. On a low protein diet it may fall to 60 per cent of the total nitrogen. Under pathologic conditions the proportion of nitrogen distributed among the various nitrogen-containing substances undergoes great variation. The only accurate index of protein metabolism is, therefore, the total output of nitrogen, which can be estimated by the Kjeldahl method or one of its modifications. The whole subject of "nitrogen partition" (distribution of nitrogen among the nitrogen-containing bodies) and "nitrogen equilibrium" (relation of excretion to intake) is an important one, but is out of the province of this book, since as yet it concerns the biochemist more than it does the clinician.

It may be helpful to state here, however, that upon a mixed diet the nitrogen of the urine is distributed about as follows: Urea nitrogen, 86.9 per cent; ammonia nitrogen, 4.4 per cent; creatinine nitrogen, 3.6 per cent; uric acid nitrogen, 0.75 per cent; "undetermined nitrogen," chiefly in amino-acids, 4.3 per cent.

Normally, the amount is greatly influenced by exercise and diet. It is increased by copious drinking of water and administration of ammonium salts of organic acids.

Pathologically, urea is increased in fevers, in diabetes when acidosis is not marked, and especially during resolution of pneumonia and absorption of large exudates. As above indicated, when other factors are equal, the amount of urea indicates the activity of metabolism. In deciding whether in a given case an increase of urea is due to increased metabolism the relation between the amounts of urea and of the chlorides is a helpful consideration. On a mixed diet the amount of urea is normally about twice that of the chlorides. If the proportion is much increased above this, increased tissue destruction may be inferred, since other conditions which increase urea also increase chlorides.

In general, a pathologic decrease in amount of urea is due either

to lessened formation within the body or to diminished excretion. *Decreased formation* of urea occurs in diseases of the liver with destruction of liver substance, such as marked cirrhosis, carcinoma, and acute yellow atrophy. The state of acidosis likewise decreases formation of urea, because nitrogen which would otherwise be built into urea is eliminated in the form of ammonia (p. 83). *Retention* of urea occurs in most cases of nephritis. In acute nephritis the amount of urea in the urine is markedly decreased, and a return to normal denotes improvement. In the early stages of chronic nephritis, when diagnosis is difficult, it is usually normal. In the late stages, when diagnosis is comparatively easy, it is decreased. Hence estimation of urea is of little help in the diagnosis of this disease, and is of no value whatever when, as is so frequently the case, a small quantity of

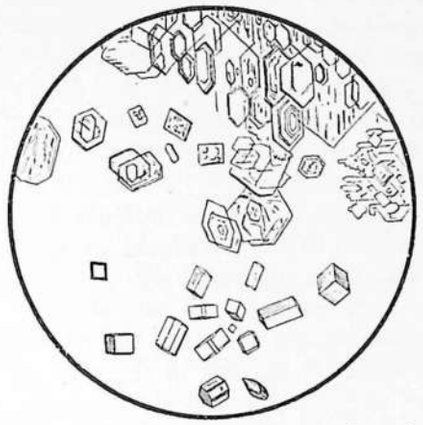


Fig. 37.—Crystals of nitrate of urea (upper half) and oxalate of urea (lower half) (after Funke).

urine taken at random is used. When, however, the diagnosis is established, estimations made at frequent intervals under the same conditions of diet and exercise are of much value, *provided a sample of the mixed twenty-four-hour urine be used*. A steady decline is a very bad prognostic sign, and a sudden marked diminution is usually a forerunner of uremia. Much more helpful in the study of nephritis is the estimation of the extent of retention of urea in the blood, which is discussed on pages 171 and 182. Estimations of urea in urine are, in fact, made much less frequently than formerly.

The presence of urea can be shown by allowing a few drops of the fluid partially to evaporate upon a slide, and adding a small drop of pure, colorless nitric acid or saturated solution of oxalic acid. Crystals of urea nitrate or oxalate (Fig. 37) will soon appear and can be recognized with the microscope.

Modern quantitative methods are based upon the conversion of urea into ammonium carbonate by urease, a ferment first extracted by Takeuchi from the soy bean in 1909. The urea is estimated from the amount of ammonium carbonate produced by the fermentation. There are several clinical methods, three of which are here given in detail. In the first, the urine after fermentation is titrated with decinormal hydrochloric acid in the presence of the indicator, methyl orange. It is not entirely accurate, but is much superior to the hypobromite method. In the second the ammonia is determined by nesslerization. This method is sufficiently accurate for the most exacting work, but is too complicated for use in a physician's office laboratory. The third method, which employs the Van Slyke and Cullen aeration technic, is particularly useful if a number of samples of urine or blood are to be tested at one time.

Neither albumin nor sugar nor any other substance likely to be present in body fluids interferes with the action of urease.

Marshall's Urease Method.—1. Into each of two 200-c.c. flasks measure 5 c.c. of the urine and about 100 c.c. of water, and to one add 1 c.c. of 5 per cent solution of urease.[1]

2. Overlay the fluid in each flask with about 1 c.c. of toluol, insert corks, and let stand over night at room temperature (or for three hours in the incubator at 37° C.).

3. At the end of this time titrate the contents of each flask to a distinct pink color with decinormal hydrochloric acid, using a few drops of 0.5 per cent methyl orange solution as indicator.

4. Find the difference between the number of cubic centimeters of decinormal acid used in the two titrations and multiply this by the factor 0.06 to obtain the percentage of urea in the urine. From the percentage calculate the twenty-four-hour elimination.

Urease Method of Folin and Youngburg.—*Reagents Required.*—(*a*) Permutit powder.

(*b*) Urease solution, pyrophosphate solution, Nessler's reagent, and standard ammonium sulfate solution as described for blood urea on page 364.

(*c*) Ammonia-free distilled water must be used throughout. Water may be freed of its ammonia by shaking with permutite powder and decanting.

Method.—1. Remove ammonia from the urine as follows: Carefully dilute 5 c.c. of the mixed twenty-four-hour urine to 50 c.c., or 10 c.c. to 50 c.c. if the specific gravity be very low. Mix well and filter. Place about 25 c.c. of the diluted urine in a 200-c.c. flask with about 4 Gm. of dry permutit powder and agitate gently for five minutes. Allow to settle for fifteen to thirty seconds or centrifugalize and decant the clear fluid.

[1] Urease powder with the necessary phosphate added to increase its activity is prepared by the Arlington Chemical Co. and E. R. Squibb & Sons. The physician will find it more convenient to use the 0.025-Gm. tablets sold by Hynson, Westcott & Dunning, Baltimore. Two of these are crushed and dissolved in 5 c.c. of water, and the whole of this solution is used for the test.

2. Place exactly 1 c.c. of the diluted ammonia-free urine in a test tube and add 1 c.c. of urease solution and 1 drop of the pyrophosphate solution.

3. Place in a large beaker of water at 40° to 55° C. for five minutes, or let stand at room temperature for fifteen minutes.

4. Transfer the fluid to a 200-c.c. volumetric flask, rinsing the tube with distilled water and adding the rinsings to the flask, and dilute to about 150 c.c.

5. At this stage prepare the standard by placing in another 200-c.c. volumetric flask 10 c.c. of the standard ammonium sulfate solution (representing 1 mg. of nitrogen), 1 c.c. of urease solution, and about 140 c.c. of water.

6. Add 20 c.c. of Nessler's reagent to each flask as nearly simultaneously as possible, dilute to the 200-c.c. marks with distilled water, and mix well. In adding the Nessler's reagent give the flask a whirl and run in the reagent from a pipet while the fluid is still whirling.

7. Compare the unknown with the standard in a colorimeter. The calculation is based upon the fact that the unknown represents 0.1 or 0.2 c.c. of urine, depending upon the dilution employed, while the standard contains 1 mg. of nitrogen. With the Duboscq and Denison Laboratory colorimeters the following formula may be used, D representing the dilution of the urine, that is, the number of cubic centimeters to which 1 c.c. of the urine was diluted in Step 1:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{D} \times 100 = \text{mg. urea-nitrogen in 100 c.c. of urine.}$$

8. It is generally desirable to record results in terms of urea instead of urea-nitrogen. To this end multiply the figure for urea-nitrogen by 2.14.

Van Slyke and Cullen Aeration Method.—The principle of this method is similar to that for the determination of blood urea, which is described on page 368.

Reagents Required.—(*a*) Urease solution, 10 per cent aqueous solution.

(*b*) 1. "Nonbubbler," rosin 20 Gm., turpentine 80 c.c. 2. Amyl alcohol 30 c.c., kerosene 70 c.c.

(*c*) Potassium carbonate, 50 per cent solution.

(*d*) Buffer solution, 0.6 per cent monobasic potassium sulfate.

(*e*) Volumetric solutions, fiftieth normal sulfuric acid, and fiftieth normal sodium hydroxide.

(*f*) Indicator, 1 per cent sodium alizarin sulfonate in distilled water.

Method.—1. Dilute the urine 10 times with distilled water.

2. Place 5 c.c. of diluted urine (represents 0.5 c.c. of original urine) in a urease tube.

3. Add 2 c.c. of urease solution (*a*).

4. Add 1 c.c. nonbubbler (*b*, 1).

5. Add 10 c.c. potassium carbonate solution (*c*).

6. Add 5 c.c. buffer solution (*d*).

7. Place 25 c.c. of fiftieth normal sulfuric acid (*e*) in the acid tube.
8. Add 1 c.c. nonbubbler (*b*, 2).
9. Add 1 drop indicator (*f*).
10. Connect the apparatus with a suction pump and aerate for forty-five minutes.
11. Titrate the acid tube with fiftieth normal sodium hydroxide (*e*).
12. Also titrate a blank tube, which has been set up and aerated without urine, to determine the amount of ammonia in the reagents.

Calculation.—(*A*) Urea-nitrogen + ammonia-nitrogen (mg. per 100 c.c.) = (blank titration − titration of unknown) × 56.

(*B*) Ammonia-nitrogen = (10 − titration of unknown) × factor (14, 28, or 56 according to the amount of urine used; see p. 86).

Urea-nitrogen per 100 c.c. = (*A*) − (*B*).

Urea = Urea-nitrogen × 2.14.

5. Uric acid is the most important of a group of substances, called *purine bodies*, which are derived chiefly from the nucleins of the food, *exogenous uric acid*, and from metabolic destruction of the nuclei of the body, *endogenous uric acid*. The daily output of uric acid is about 0.4 to 1 Gm. The amount of the other purine bodies together is about one tenth that of uric acid. Excretion of these substances is greatly increased by a diet rich in nucleins, as sweetbreads and liver.

Uric acid exists in the urine in the form of urates, chiefly of sodium and potassium, which in concentrated urines are readily thrown out of solution and constitute the familiar sediment of "amorphous urates." This, together with the fact that uric acid is frequently deposited as crystals, constitutes its chief interest to the practitioner. It is a very common error to consider these deposits as evidence of excessive excretion.

Pathologically, the greatest increase of uric acid occurs in leukemia, where there is extensive destruction of leukocytes, in diseases with active destruction of the liver and other organs rich in nuclei, and during absorption of a pneumonic exudate. There is generally an increase during *x*-ray treatment. Uric acid is decreased before an attack of gout and increased for several days after it, but its etiologic relation is still uncertain. An increase is also noted in acute fevers.

For exact determinations the method of Benedict and Franke,[1] given below in detail, or that of Folin and Wu,[2] is recommended. The Benedict and Franke method is a direct colorimetric method depending on the blue color that develops when uric acid is treated with arsenophosphotungstic acid and sodium cyanide. In the Folin and Wu method the uric acid is first precipitated as silver urate, and redissolved in sodium cyanide; when treated with Folin's uric acid reagent a blue color develops, the depth of which is proportional to the amount of uric acid.

[1] Benedict, S. R., and Franke, Elizabeth: A Method for the Direct Determination of Uric Acid in Urine, Jour. Biol. Chem., *52*:387–391 (June), 1922.

[2] Folin, O., and Wu, H.: A Revised Colorimetric Method for Determination of Uric Acid in Urine, Jour. Biol. Chem., *38*:459–460 (July), 1919.

Method of Benedict and Franke.—*Reagents Required.*—(*a*) Standard uric acid solution. This is prepared from Benedict and Hitchcock's solution (p. 360) as follows:

Place 50 c.c. of the stock solution, containing 10 mg. uric acid, in a 500-c.c. volumetric flask, add about 350 c.c. water and 25 c.c. of diluted hydrochloric acid (concentrated hydrochloric acid 1 part, water 9 parts). Make up to 500 c.c. with water and mix well. This solution contains 0.2 mg. uric acid in 10 c.c., and remains good for at least two weeks.

(*b*) Sodium cyanide solution, 5 per cent: This should be freshly prepared about once in two months.

(*c*) Benedict's uric acid reagent, described on page 371.

Method.—Albumin if present must be removed by adding a drop of acetic acid, boiling and filtering. Other substances do not interfere.

1. Dilute the filtered urine with water so that 10 c.c. will contain between 0.15 and 0.30 mg. of uric acid. The usual dilution is 1 in 20.
2. Place 10 c.c. of the diluted urine in a 50-c.c. volumetric flask and add 5 c.c. of the 5 per cent sodium cyanide from a buret. Mark this flask U.
3. In another 50-c.c. volumetric flask place 10 c.c. of the standard uric acid solution, containing 0.2 mg. uric acid, and add 5 c.c. of the 5 per cent sodium cyanide solution.
4. To each flask add 1 c.c. of the uric acid reagent and mix.
5. Let stand five minutes, dilute to the 50-c.c. mark, and mix.
6. Compare the unknown with the standard in a colorimeter. With the Duboscq or Denison Laboratory colorimeter the following formula may be used, D representing the dilution of the urine, that is, the number of cubic centimeters to which 1 c.c. of the urine was diluted:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 2 \times \text{D} = \text{mg. uric acid in 100 c.c. of urine.}$$

6. Ammonia.—A small amount of ammonia, combined with hydrochloric, phosphoric, and sulfuric acids, is always present. Estimated as NH_3, the normal average is about 0.7 Gm. in twenty-four hours. This represents 4 to 5 per cent of the total nitrogen of the urine, ammonia standing next to urea in this respect.

Under ordinary conditions most of the ammonia which results from the metabolic processes is transformed into urea. When, how-

ever, acids are present in excess, either from ingestion of mineral acids or from abnormal production of acids within the body (for example, diacetic and oxybutyric acids in diabetes mellitus), ammonia combines with them and is so excreted, the urea of the urine being correspondingly decreased. This is an important part of the mechanism by which the body protects itself against acid intoxication (Acidosis, p. 648). The ammonium salts are not, however, increased in all forms of acidosis, notably not in acidosis of nephritis.

In diabetes mellitus, and other conditions associated with excessive production of diacetic and oxybutyric acids, the output of ammonium salts is a very important *index of the degree of acidosis*. Ammonia elimination in diabetes with mild acidosis may be 1 or 1.5 Gm. daily, rising to 4 or 5 Gm. in severe cases and even to 8 or 10 Gm. in extreme cases. Ammonia is likewise increased in pernicious vomiting of pregnancy, but *not in nervous vomiting;* and in conditions in which the power to synthesize urea is interfered with, notably cirrhosis and other destructive diseases of the liver and conditions associated with deficient oxygenation. Certain drugs have a marked influence upon ammonia elimination; thus, fixed alkalies and salts of organic acids diminish it, while inorganic acids, such as hydrochloric, increase it.

The ammonia referred to in the above paragraphs is in the form of ammonium salts and should not be confused, as it often is, with the accumulation of free ammonia, which is derived from decomposition of urea after the urine is secreted and which leads to volatile alkalinity.

Quantitative Estimation.—*The urine must be fresh, since decomposition increases the ammonia.* The formalin method is entirely satisfactory for clinical work, though subject to some inaccuracies. When carried out without use of lead acetate it includes amino-acids with the ammonia, hence sometimes gives figures that are too high. The Folin and Bell method gives ammonia only and is accurate. The difference between the figures obtained by the two methods therefore represents amino-acids. The aeration and titration method is relatively simple, and is most convenient if urea determinations are being made at the same time by the Van Slyke and Cullen method. The technic of this method (Folin-Macallum) is given on page 86.

Ronchese-Malfatti Formalin Method.—This depends upon the fact that when formalin is added to the urine the ammonia combines with it, forming hexamethylenamine. The acids with which the ammonia was combined are set free, and their quantity, ascertained by titration with sodium hydroxide, indicates the amount of ammonia.

Take 10 c.c. of the urine in a beaker or evaporating dish, add 50 c.c. water and 10 drops of 0.5 per cent alcoholic solution of phenolphthalein.

Neutralize by adding a weak sodium hydroxide or sodium carbonate solution until a permanent pink color appears. To 5 c.c. formalin add 15 c.c. water and neutralize in the same way. Pour the formalin into the urine. The pink color at once disappears owing to liberation of acids. Now add decinormal sodium hydroxide solution from a buret until the pink color just returns. Each cubic centimeter of the decinormal solution used in this titration corresponds to 0.0017 Gm. of NH_3. This must be multiplied by 10 to obtain the percentage from which the twenty-four-hour elimination of ammonia is calculated.

The method is more complicated, but distinctly more accurate, when carried out as suggested by E. W. Brown: Treat 60 c.c. of urine with 3 Gm. of basic lead acetate, stir well, let stand a few minutes, and filter. This removes certain interfering nitrogenous substances. Treat the filtrate with 2 Gm. neutral potassium oxalate, stir well, and filter. Take 10 c.c. of the filtrate, add 50 c.c. water and 15 Gm. neutral potassium oxalate, and proceed with the ammonia estimation as above outlined.

Permutit Method of Folin and Bell.—*Reagents Required.*—(*a*) Permutit powder.

(*b*) Sodium hydroxide, 10 per cent aqueous solution.

(*c*) Nessler's reagent and standard ammonium sulfate solution as described on page 364.

(*d*) Ammonia-free distilled water must be used throughout.

Method.—1. Place about 2 Gm. of permutite powder in a 200-c.c. volumetric flask, add 5 c.c. of water, and mix.

2. Add an exactly measured quantity of urine from the mixed twenty-four-hour specimen, usually 1 or 2 c.c., rinse down the urine with 1 to 5 c.c. of water, and shake gently for five minutes.

3. Rinse the powder from the sides of the flask to the bottom with 25 to 40 c.c. of water and decant, leaving all the powder.

4. Wash the powder by adding about 50 c.c. water, shaking gently, and decanting. In cases of urine rich in bile the washing should be repeated.

5. Add a little water to the powder and then add 5 c.c. of 10 per cent sodium hydroxide. Mix well, add about 140 c.c. of water, and shake for a few seconds.

6. At this point prepare the standard as follows: In a 200-c.c. volumetric flask place 10 c.c. of the ammonium sulfate solution, representing 1 mg. nitrogen, add 5 c.c. of 10 per cent sodium hydroxide solution, and about 140 c.c. of water.

7. To each flask add 10 c.c. of Nessler's reagent in the manner described for urea on page 81. Dilute to the 200 c.c. marks with water and mix well.

8. Compare the unknown with the standard in a colorimeter. Details of the calculation depend upon the type of colorimeter used. With the Duboscq and Denison Laboratory colorimeters the following formula may be used, V representing the number of cubic centimeters of urine employed:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \frac{100}{V} = \text{mg. ammonia nitrogen in 100 c.c. urine.}$$

which have just been discussed. Among them are: proteins, sugars, the acetone bodies, bile, urobilin, hemoglobin, hematoporphyrin, and the diazo substances. Most of these are present in negligible traces normally. The detection of drugs in the urine will also be discussed under this head.

1. Proteins.—Of the proteins which may appear in the urine, serum albumin and serum globulin are the most important. Mucin, proteose, and a few others are found occasionally, but are of less interest.

(1) **Serum Albumin and Serum Globulin.**—These two proteins constitute the so-called "urinary albumin." They usually occur together, have practically the same significance, and both respond to all the ordinary tests for "albumin."

Their presence, or *albuminuria*, is probably the most important pathologic condition of the urine, and also the most frequent. It may be either *accidental* or *renal*. The physician can make no greater mistake than to regard all cases of albuminuria as indicating kidney disease.

Accidental or *false albuminuria* is due to admixture with the urine of albuminous fluids, such as pus, blood, and vaginal discharge. The microscope will usually reveal its nature. It occurs most frequently in pyelitis, cystitis, and chronic vaginitis, and the quantity of albumin is usually small.

Renal albuminuria refers to albumin which has passed from the blood into the urine through the walls of the kidney tubules or the glomeruli.

Albuminuria sufficient to be readily recognized by the ordinary clinical methods probably never occurs as a physiologic condition, the so-called *physiologic albuminuria* appearing only under conditions which must be regarded as abnormal. Among these may be mentioned excessive muscular exertion in those unaccustomed to it; excessive ingestion of proteins (dietetic albuminuria); prolonged cold baths; the later stages of pregnancy, and childbirth. In these conditions the albuminuria is ordinarily slight and transient. Albumin is frequently present in the urine of infants, possibly because the young kidney is particularly sensitive to irritants.

There are certain other forms of albuminuria which have still less claim to be called physiologic, but which are not always regarded as pathologic. Among these is *cyclic*, *orthostatic*, or *postural albuminuria*. This appears at certain periods of the day, which may vary in different cases, and disappears with rest in bed. Most frequently the maximal output of albumin occurs late in the afternoon. This

form of albuminuria occurs, for the most part, in neurasthenic subjects during adolescence and is by no means rare. In some cases at least it is associated with curvature of the spine (lordotic albuminuria). The phenolsulfonephthalein test usually shows normal excretion. Tube casts may or may not be present. A considerable amount of protein precipitable by acetic acid in the cold has been noted in many cases. It is noteworthy in this connection that nephritis sometimes begins with a cyclic albuminuria.

In pathologic conditions, and in most, at least, of the "functional" conditions just enumerated, renal albuminuria may be referred to one or more of the following causes. In nearly all cases it is accompanied by tube casts.

(*a*) *Circulatory changes in the kidney*, either anemia or congestion, as in excessive exercise, chronic heart disease, severe general anemia, and pressure upon the renal veins, as in the later stages of pregnancy. The quantity of albumin is usually but not always small. Its presence is constant or temporary, according to the cause. Most of the causes, if continued, will lead to degenerative changes in the kidney and even to nephritis.

(*b*) *Irritation of the Kidneys.*—Here there is slight damage to glomeruli or renal cells, with cloudy swelling or even more serious degeneration, but without definite nephritis. The amount of albumin is generally small and the condition transitory. This is probably the chief factor in toxic and febrile albuminuria, and it is at least a contributing factor in the albuminuria of pregnancy. Among drugs which in toxic doses may cause albuminuria are mercury, cantharides, turpentine, mustard, arsenic, lead, ether, etc. In febrile conditions, particularly in the acute infectious diseases, small or moderate amounts of albumin are frequently found in the urine, owing chiefly to the irritant effect of bacterial toxins. This is especially true of diphtheria, scarlet fever, pneumonia, typhoid fever, and acute streptococcic infections, in any of which the renal condition may develop into true nephritis, with coincident increase in the amount of albumin.

(*c*) *Organic Changes in the Kidney.*—These include the inflammatory and degenerative changes commonly grouped together under the name of nephritis, and also renal tuberculosis and neoplasms. The amount of albumin eliminated in these conditions varies from minute traces to 20 Gm., or even more, in the twenty-four hours, and, except in acute processes, bears little relation to the severity of the disease. In acute and chronic parenchymatous nephritis and in syphilis of the kidney the quantity is usually very large, from 1 to 2 per cent or more. In chronic interstitial nephritis it is small—fre-

quently no more than a slight trace. It is variable in renal tuberculosis and neoplasms. In amyloid disease of the kidney the quantity is usually small, and serum globulin may be present in especially large proportion or even alone. Roughly distinctive of serum globulin is the appearance of an opalescent cloud when a few drops of the urine are dropped into a glass of distilled water.

Detection of albumin depends upon its precipitation by chemicals or coagulation by heat. There are many tests, but none is entirely satisfactory, because other substances as well as albumin are precipitated. The most common source of error is mucin. When any considerable amount of mucin is present it can be removed by acidifying with acetic acid and filtering. Urine voided early in the evening or a few hours after a meal is most likely to contain albumin.

It is very important that urine to be tested for albumin be rendered clear by filtration or centrifugation. This is too often neglected in routine work. When ordinary methods do not suffice, it can usually be cleared by shaking up with a little purified talc, infusorial earth, or animal charcoal, and filtering. This will remove a part of the albumin by absorption, but the remainder is more easily detected. If the urine be alkaline, sufficient acetic acid should be added to make it acid to litmus. Vaughan has called attention to the fact that if bacteria be abundant in an alkaline urine, some of the bacterial proteins may go into solution and give the tests for albumin. In extremely concentrated urine certain of the urinary salts may interfere with the test for albumin. In such cases dilution of the urine will render the test more definite, even though the concentration of albumin is thereby reduced.

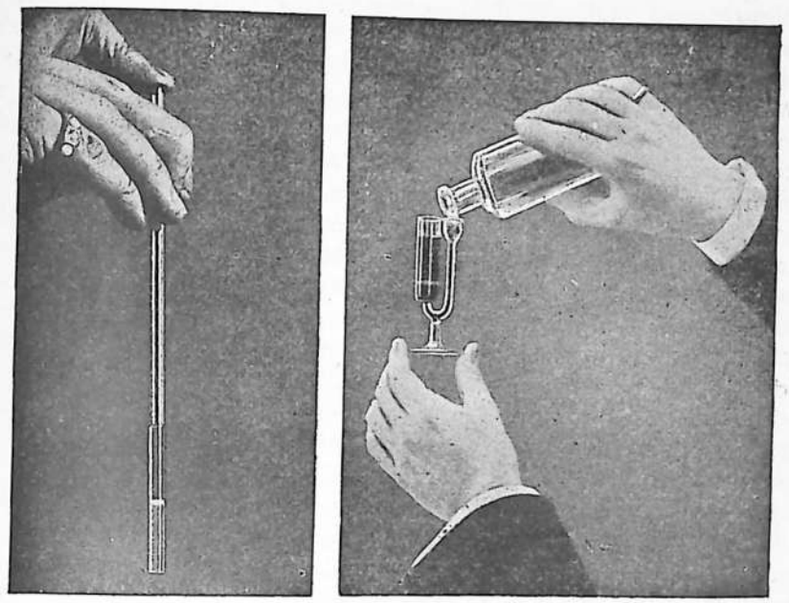
Albuminous urine foams markedly on shaking and the foam remains a long time. This gives a rough indication of the presence of albumin before the tests are made.

Technic of Ring or Contact Tests.—Since this simple and widely useful method of testing is best known in connection with the detection of albumin a general description is given at this place:

Take a few cubic centimeters of the heavier fluid in a conical test glass, hold the glass in an inclined position, and run the lighter fluid gently down the inside of the glass by means of a medicine dropper so that it will form a layer on top of the other without mixing. In the case of the urine, which must be filtered before testing, it may be run in directly from the stem of the funnel by touching this against the wall of the test glass. Monroe[1] advises filtering the urine into the test glass and introducing the reagent

[1] Personal communication of Dr. R. T. Monroe, Harvard Medical School, to the author.

under the urine with a pipet or medicine dropper, thus obtaining better layering than by pouring. He has found it advantageous also to adopt a standard time of one minute as the optimum time for observation of the ring. If the test be positive, a sharply defined white or colored ring will appear where the two fluids come into contact. According to its color the ring is seen most clearly if viewed against a white or a black background, as the case may be; and one side of the test glass may be painted half white, half black, for this purpose. It is better, however, to use clear glass and to have the white or black background at a distance of some feet.



A B

Fig. 38.—Showing two methods of performing the contact test for albumin: A, Boston's method, which is convenient for routine testing of a large number of urines, but is less accurate than the use of a conical test glass as recommended in the text (Boston). B, Horismascope, adding the reagent. The white ring at the zone of contact is seen in the wider arm. This is an extremely satisfactory method for the office laboratory.

Boston brings the fluids into contact in a glass pipet, which is immersed first in the lighter fluid and then (after wiping the outside of the pipet) in the heavier (Fig. 38). This is convenient for the routine testing of a large number of urines, but cannot be recommended for accuracy, owing to the small diameter of the column of fluid. Substitution of a medicine dropper in place of the pipet renders Boston's method more convenient, but no more accurate. For those who do only a little testing the "horismascope" (Fig. 38) will be found very convenient and satisfactory. The instrument is, however, fragile and somewhat expensive.

The albumin tests here given are widely used and can be recommended for clinical purposes. They make no distinction between serum albumin and serum globulin.

1. **Exton's sulfosalicylic acid method** has proved to be very satisfactory. To make the reagent dissolve 200 Gm. sodium sulfate in about 750 c.c. of water with the aid of heat, cool, add 50 Gm. sulfosalicylic acid, and dilute with water to 1000 c.c. The test is performed by mixing equal parts of the urine and reagent in a test tube and warming gently. Boiling is not necessary or desirable. A white cloud shows the presence of albumin or globulin. The reaction is read while the fluid is warm, since secondary proteoses will cause a clouding when it cools. The Bence-Jones protein causes a heavy precipitate which clears partially or wholly upon boiling.

2. **Roberts' Test.**—The reagent consists of pure nitric acid 1 part, and saturated aqueous solution of magnesium sulfate 5 parts. It is applied by the "ring" or "contact" method above described.

Albumin gives a white ring, which varies in density with the amount present, and when traces only are present may not appear for two or three minutes. A similar white ring may be produced by Bence-Jones' protein, primary proteose, thymol, and resinous drugs. White rings or cloudiness in the urine *above* the zone of contact may result from excess of urates or mucus. Colored rings near the junction of the fluids may be produced by iodides, urinary pigments, bile, or indican, but these are not so frequent as with Heller's test.

Roberts' test is one of the best for routine work, although the various rings are apt to be confusing to the inexperienced. It is a little more sensitive than the widely used **Heller's test**, which is performed in exactly the same way, using pure concentrated nitric acid; and it has the additional advantage that the reagent is not so corrosive. If in Heller's test the ring appears only after two minutes the amount of albumin is less than 0.0005 per cent.

3. **Purdy's Heat Test.**—Take a test tube two thirds full of urine, add about one sixth its volume of saturated solution of sodium chloride, and 5 to 10 drops of 50 per cent acetic acid. Mix, and boil the upper inch, holding the tube with the fingers near the bottom. A white cloud in the heated portion shows the presence of albumin. A faint cloud is best seen when viewed against a black background at a distance of 2 or 3 feet.

This is a valuable test for routine work. It is simple, sufficiently accurate for clinical purposes, and has practically no fallacies. *Addition of the salt solution by raising the specific gravity prevents precipitation of mucin.* Bence-Jones' protein may produce a white cloud, which disappears upon boiling and reappears upon cooling.

4. **Heat and Nitric Acid Test.**—This is one of the oldest of the albumin tests, and, if properly carried out, one of the best. Boil about 5 c.c. of filtered urine in a test tube and add 1 to 3 drops of concentrated nitric acid. The tube may be held with a test tube clamp, or simply with a strip of

muslin or filter paper, the center of which is folded once around the neck of the tube. A white cloud or flocculent precipitate (which usually appears during the boiling, but if the quantity be very small only after addition of the acid) denotes the presence of albumin. A similar white precipitate, which disappears upon addition of the acid, is due to earthy phosphates. The acid should not be added before boiling, and the proper amount should always be used; otherwise, part of the albumin may fail to be precipitated or may be transformed to acid albumin and redissolved. Resinous drugs might give a white cloud with this test, but this will disappear upon addition of alcohol. A white cloud which appears only after cooling may be due to Bence-Jones' protein or to primary proteose. Effervescence upon addition of the acid is generally due to carbonates from the food, notably lemonade.

A decided advantage of this test is the fact that it allows a rough estimation of the amount of albumin from the volume of the sediment after standing overnight. When the entire fluid solidifies the albumin amounts to 2 to 3 per cent. Sediments reaching to one half, one third, one fourth, and one tenth the height of the column of urine correspond respectively to about 1, 0.5, 0.25, and 0.1 per cent albumin. When there is only a slight cloudiness the albumin does not exceed 0.01 per cent.

Scheme for Recording Results of Tests.—It is customary to record qualitative albumin tests in such a way as to convey a rough idea of the quantity of albumin. The plan is very useful clinically, but it must be remembered that other factors than the quantity of albumin—notably the salt content of the urine—affect the amount and character of the clouding or precipitate. Also the interpretation of the terms used will vary greatly with different persons. The following scheme is widely used. It is given both for the heat tests, such as heat and nitric acid, and for contact tests, such as Heller's.

1. *Trace.*—The cloudiness or ring can just be seen, usually best against a black background.
2. *Small Amount.*—Heat test: Cloud is distinct and granular without definite flocculles. Precipitate settles to about one tenth height of column of urine in twenty-four hours. Contact test: The ring is dense, but not wholly opaque when viewed from above. Represents about 0.1 per cent of albumin.
3. *Moderate Amount.*—Heat test: Cloud is dense with very marked flocculation. Contact test: The ring is heavy, wholly opaque, sometimes curdy. Represents about 0.2 to 0.3 per cent of albumin.
4. *Large Amount.*—Heat test: Precipitate is very heavy and curdy and may become a solid mass. Contact test: The ring is very dense. Represents more than 0.5 per cent albumin.

The result can be recorded "Albumin 1, 2, 3, or 4," according to the estimated amount of albumin.

Quantitative Estimation.—Accurate estimation of albumin is seldom necessary in routine clinical work. Ordinarily the information obtainable from properly conducted qualitative tests will suffice.

When more definite figures are required, the following simple methods are available. Of these, Exton's method is by far the most accurate.

1. **Esbach's Method.**—The urine must be clear, of acid reaction, and not concentrated. Always filter before testing, and, if necessary, add acetic acid and dilute with water, making allowance for the dilution in the final calculation. Esbach's tube (Fig. 39) is essentially a test tube with a mark U near the middle, a mark R near the top, and graduations ½, 1, 2, 3, etc., near the bottom. Fill the tube to the mark U with urine and to the mark R with the reagent. Close with a rubber stopper, invert slowly several times, and set aside in a cool place. At the end of twenty-four hours read off the height of the precipitate. This gives the amount of albumin in *grams per liter, and must be divided by 10 to obtain the percentage.*

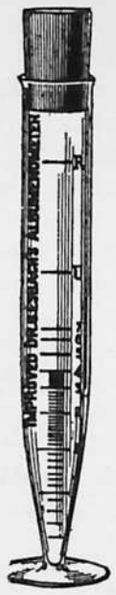


Fig. 39.—Esbach's albuminometer, improved form.

Lenk advises addition of a small quantity of powdered charcoal, pumice, or kaolin after adding Esbach's reagent. This hastens sedimentation, which is complete in ten minutes to half an hour. Andresen uses 0.1 to 0.2 Gm. of barium sulfate.

Esbach's reagent consists of picric acid, 1 Gm.; citric acid, 2 Gm., and distilled water, to make 100 c.c.

2. **Tsuchiya's Method.**—This is carried out in the same manner as the Esbach method, using the following reagent:

Phosphotungstic acid	1.5 Gm.
Alcohol (96 per cent)	95.0 c.c.
Concentrated hydrochloric acid	5.0 "

The urine should be diluted to a specific gravity not exceeding 1.008. The method is said to be much more accurate than the original Esbach method, particularly with small quantities of albumin.

3. **Exton's Quantitative Method.**—This requires a sulfosalicylic acid reagent. It is turbidimetric, and the quantity of albumin is read directly by comparison with a set of standard tubes representing, 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mg. protein for each 100 c.c. It may be necessary to dilute urine containing a large amount of albumin so that a comparison can be made with the standard tubes. All tubes must be scrupulously clean.

Add 2 or 3 c.c. of reagent[1] to an equal amount of urine and allow to stand for at least five minutes. Warm the urine by passing the tube slowly through the flame several times, but do not boil. Make all suspensions homogeneous in the standard tubes by gently inverting.

[1] *Formula of Reagent:* Dissolve 50 Gm. of sulfosalicylic acid and 10 Gm. of sodium sulfate in 800 c.c. distilled water; add 25 c.c. of 0.40 per cent aqueous solution of bromphenol blue. Make up to 1 liter and filter through acid-washed paper.

A somewhat similar method now used by the Metropolitan Life Insurance Company has been devised by Kingsbury and his assistants.[1] They use as a reagent 3 per cent sulfosalicylic acid, adding 7.5 c.c. to 2.5 c.c. of urine. The artificial quantitative standards are made from dilutions of *formazin* held in suspension in gelatin, and are for sale by the Fales Chemical Company, Cornwall Landing, N. Y.

(2) **Mucin.**—Traces of the substances (mucin, mucoid, nucleoprotein, and so forth) which are loosely classed under this name are present in normal urine; increased amounts are observed in irritations and inflammations of the mucous membrane of the urinary tract or the vagina. They are of interest chiefly because they may be mistaken for albumin in most of the tests. If the urine be diluted with water and acidified with acetic acid without heating, the appearance of a white cloud indicates the presence of mucin.

Mucin and mucoid are glycoproteins, and upon boiling with an acid or alkali, as in Fehling's test, yield a carbohydrate substance which reduces copper.

(3) **Bence-Jones' Protein.**—The protein known by this name was originally classed as an albumose, but its protein nature is now well established. It was formerly regarded as practically pathognomonic of multiple myeloma, but has been found in a number of cases of chronic leukemia, of both lymphatic and myelogenous types, in osteomalacia, and, along with albumin, in some cases of chronic nephritis with high blood pressure and edema, and also in certain seemingly healthy young persons with high blood pressure (Miller and Baetjer).

To detect Bence-Jones' protein the urine is slightly acidified with acetic acid and gently heated in a water bath. If this substance be present, the urine will begin to be turbid at about 40° C. and a precipitate will form at about 60° C. As the boiling point is reached the precipitate wholly or partially dissolves. It reappears upon cooling. It may easily be overlooked in the presence of albumin. If the urine be filtered while at or near the boiling temperature, the albumin may be removed, leaving the Bence-Jones' protein.

As a confirmatory test the protein may be precipitated by adding nitric acid at room temperature. This precipitate wholly or partially clears up on boiling and reappears on cooling. The protein may also be precipitated by alcohol, and if the precipitate be collected *at once* by centrifugation it readily dissolves in water. For complete identification of the protein the reader is referred to the more comprehensive works upon biochemistry.

[1] Kingsbury, F. B., Clark, C. P., Williams, Gertrude, and Post, Anna L.: The Rapid Determination of Albumin in Urine, Jour. Lab. and Clin. Med., *11*:981–989 (July), 1926.

(4) **Proteoses.**—These are intermediate products in the digestion of proteins and are frequently, although incorrectly, called albumoses. Two groups are generally recognized: *primary proteoses*, which are precipitated upon half-saturation of their solutions with ammonium sulfate; and *secondary proteoses*, which are precipitated only upon complete saturation.

The proteoses appear in the urine whenever a considerable amount of tissue or exudate is being autolyzed and absorbed, as in febrile and malignant diseases and chronic suppurations, during resolution of pneumonia, and in many other conditions, but their clinical significance is indefinite. In pregnancy, albumosuria may be due to absorption of amniotic fluid and, later, to involution of the puerperal uterus.

The proteoses are not coagulable by heat, but are precipitated by such substances as trichloracetic acid, sulfosalicylic acid, and phosphotungstic acid. The primary proteoses alone are precipitated by adding concentrated nitric acid. This precipitate disappears on heating and reappears on cooling.

Proteoses may be detected by acidifying the urine with acetic acid, boiling, filtering while hot to remove mucin, albumin, and globulin, and testing the filtrate by the trichloracetic acid test. As above indicated, the nitric acid test, and half and complete saturation with ammonium sulfate, will separate the two groups.

2. Sugars.—Various sugars may at times be found in the urine. Dextrose is by far the most common, and is the only one of much clinical importance. Levulose, lactose, and some others are occasionally met.

(1) **Dextrose (Glucose).**—Traces of glucose and other sugars too small to respond to the ordinary tests are present in the urine in health. The presence of glucose in appreciable amount constitutes *glycosuria* or *glycuresis*, and is the result of increase of dextrose in the blood (hyperglycemia), or of lowered renal threshold for sugar, or both. These are discussed on page 387.

Transitory glycosuria is unimportant, is generally slight, and may occur in many conditions, as after general anesthesia and administration of certain drugs, in some cases of hyperthyroidism, in pregnancy, and following shock and head injuries. Recently attention has been directed to glycosuria following strong emotions (anger, fear, anxiety), due, according to Cannon, to increased adrenal secretion leading to sudden mobilization of dextrose which has been stored as glycogen. The urine of a considerable percentage of a class of students will give positive tests for sugar following a long and hard examination. The possibility that a trace of sugar found in a patient's urine after a physical examination may be due to his anxiety, must be kept in mind. Glycosuria may also occur after eating excessive amounts of carbohydrates (alimentary glycosuria). The "assimilation limit" varies with different individuals and with different conditions of exercise. It also depends upon the kind of carbohydrate. The normal for pure dextrose is generally given as about 100 to 200 Gm., but more recent work has shown that in many individuals glycuresis cannot be induced by much larger amounts even up to the maximum (400 to 500 Gm.) which the stomach will tolerate. Glycuresis following ingestion of 100 Gm. or less is definitely abnormal, indicating lowered renal threshold, diminished capacity of the liver to store glucose as glycogen, or disturbance of carbohydrate metabolism. Excretion lasts for a period of four or five hours.

Persistent, although not necessarily continuous, glycosuria has been noted in brain injuries involving the floor of the fourth ventricle and in renal glycosuria (p. 187), of which it is the essential symptom. As a rule, however, persistent glycosuria is diagnostic of diabetes mellitus, and this is by far its most important indication. The amount of glucose eliminated in diabetes is usually considerable, and is sometimes very large, reaching 500 Gm., or even more, in twenty-four hours, but it does not bear any uniform relation to the severity of the disease. Glucose may, on the other hand, be almost or entirely absent as a result of careful restriction of the diet, and in mild cases it may appear only about two or three hours after ingestion of considerable quantities of carbohydrate. Diabetes is discussed more fully on page 187.

Detection of Dextrose.—Albumin, if present in any considerable quantity, interferes with precipitation of copper in the copper tests, and should be removed by acidifying with acetic acid, boiling, and filtering.

1. **Benedict's Test.**—The reagent will detect 0.15 to 0.2 per cent of dextrose and is thus several times as sensitive as Haines' or Fehling's solutions. It is not reduced by uric acid, creatinine, chloroform, or the aldehydes. It consists of:

Copper sulfate (pure crystallized)	17.3 Gm.
Sodium or potassium citrate	173.0 "
Sodium carbonate (crystallized)	200.0 "
(or 100 Gm. of anhydrous sodium carbonate)	
Distilled water, to make	1000.0 c.c.

Dissolve the citrate and carbonate in 700 c.c. of water, with the aid of heat, and filter. Dissolve the copper sulfate in 100 c.c. of water and pour

slowly into the first solution, stirring constantly. Cool, and make up to 1 liter. The reagent keeps indefinitely. *It cannot be used for quantitative estimations.*

Take about 5 c.c. of this reagent in a test tube, heat to boiling to make sure that none of the copper is precipitated by heat alone, and add *8 or 10 drops* (*not more*) of the urine. Heat to vigorous boiling, keep at this temperature for one or two minutes, and allow to cool slowly. In the presence of glucose the entire body of the solution will be filled with a precipitate, which may be red, yellow, or green in color. When traces only of glucose are present, less than 0.3 per cent, the precipitate may appear only upon cooling. In the absence of glucose the solution remains clear or shows only a faint *bluish* precipitate, due to urates. The long boiling is inconvenient, especially when there is much bumping in the tube. It will therefore generally be found more satisfactory, particularly when a large number of urines must be tested, to place the tubes in a beaker of water which is kept at the boiling point for five minutes.

2. **Haines' Test.**—Take about 4 c.c. of Haines' solution in a test tube, boil, examine carefully for a precipitate, and, if none is present, add 6 or 8 drops of urine while keeping the reagent hot but not boiling. A yellow or red precipitate, which settles readily to the bottom, shows the presence of sugar. Neither precipitation of phosphates, as a light, flocculent sediment, nor simple decolorization of the reagent should be mistaken for a positive reaction.

This is one of the best of the copper tests, all of which depend upon the fact that in strongly alkaline solutions glucose reduces cupric hydrate to cuprous hydrate (yellow) or cuprous oxide (red). They are somewhat inaccurate because they make no distinction between glucose and less common forms of sugar; because certain normal substances when present in excess, especially mucin, uric acid, and creatinine, may reduce copper, and because many drugs—for example, chloral, chloroform, copaiba, acetanilid, benzoic acid, morphine, sulfonal, salicylates, aspirin—are eliminated as copper-reducing substances. To minimize these fallacies *dilute the urine, if it be concentrated; do not add more than the specified amount of urine, and do not boil after the urine is added.* If chloroform has been used as a preservative, it should be removed by boiling the urine before making the test.

Haines' solution is prepared as follows: Completely dissolve 2 Gm. pure copper sulfate in 16 c.c. distilled water, and add 16 c.c. pure glycerin; mix thoroughly, and add 156 c.c. of 5 per cent potassium hydroxide. The solution keeps well.

3. **Fehling's Test.**—Two solutions are required—one containing 34.64 Gm. pure crystalline copper sulfate in 500 c.c. distilled water; the other, 173 Gm. Rochelle salt and 100 Gm. potassium hydroxide in 500 c.c. distilled water. Mix equal parts of the two solutions in a test tube, dilute with 3 or 4 volumes of water, and boil. Add the urine a little at a time, heating, but not boiling, between additions. In the presence of dextrose a heavy red

or yellow precipitate will appear. The quantity of urine should not exceed that of the reagent. The fallacies mentioned under Haines' test apply equally to this.

4. **Phenylhydrazine Test.**—*Kowarsky's Method.*—The following directions include certain modifications which have been worked out by C. S. Bluemel at Boulder, Colorado: In a wide test tube take 5 drops pure phenylhydrazine, 10 drops glacial acetic acid, and 1 c.c. saturated solution of sodium chloride. A curdy mass results. Add 3 or 4 c.c. of the urine and 4 or 5 c.c. of water. Boil vigorously for two or three minutes. The annoying bumping can be reduced or obviated by shaking continually or, much better, by placing in the test tube a number of pieces of glass tubing, varying in length from $1\frac{1}{2}$ to 3 inches, so as to produce an organ-pipe effect. The volume of fluid remaining after boiling should be 2 to 3 c.c. Set aside to cool or, if the

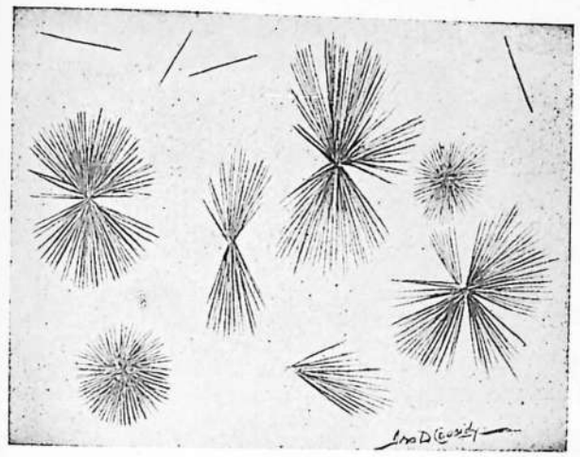


Fig. 40.—Crystals of phenylglucosazone (yellow) from diabetic urine—Kowarsky's test (× 500).

glass tubes were used, pour the fluid into another hot test tube and allow to cool. Examine the sediment with the microscope, using a 16-mm. objective. If dextrose be present, characteristic crystals of phenylglucosazone will be seen. These are yellow, needle-like crystals arranged mostly in clusters or in sheaves (Fig. 40). When traces only of glucose are present the crystals may not appear for one-half hour or more. The best crystals are obtained when the fluid is cooled very slowly and is not agitated during cooling. The test tubes and pieces of tubing can be cleaned when necessary by boiling in a solution of caustic soda or acetic acid.

This is an excellent test for clinical work. Bluemel finds that when applied as above directed, with the tubing to prevent bumping, it will readily detect 0.025 per cent of dextrose in urine, the crystals appearing in three to four hours. The test has practically no fallacies except the reaction caused by levulose, which is a fallacy for all the ordinary tests.

Other carbohydrates which are capable of forming crystals with phenylhydrazine are extremely unlikely to do so when the test is applied directly to the urine. Even if not used routinely, this test should always be resorted to when the copper tests give a positive reaction in doubtful cases.

5. **Fermentation Test.**—This is simple and reliable, but owing to the time required it is not much used in routine work, except as an aid in distinguishing dextrose from other forms of sugar. It is carried out in the same manner as the quantitative test (p. 102). Improvised devices which answer well for the purpose are shown in Fig. 41. If the fermenting urine be placed in the incubator at 40° to 45° C. the result should be definite within an hour or two; and it can be still further hastened by increasing the amount of yeast.

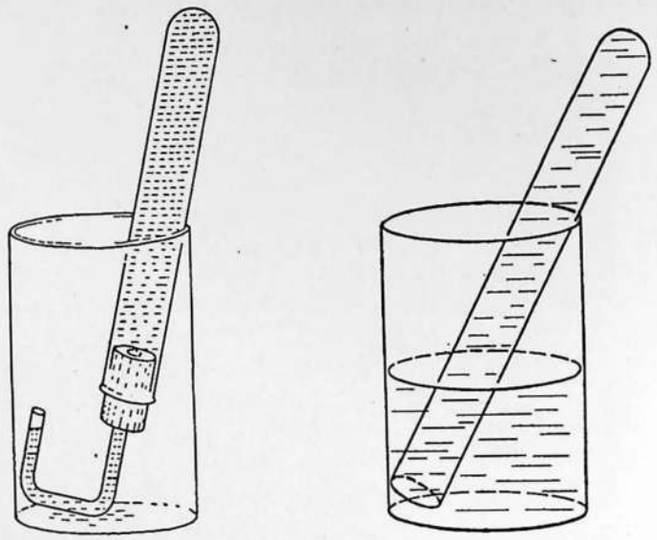


Fig. 41.—Simple devices for fermentation test for dextrose.

Scheme for Recording Results of Tests.—Unless a quantitative estimation is made sugar is generally reported simply as "present" or "absent." If it be desired, when recording a positive qualitative test, to convey some notion of the quantity of sugar present, the following scheme is suggested when Benedict's solution is employed:

1. S. T., *slight trace.* No reduction is evident during two minutes' boiling with 8 or 10 drops of urine, but it appears upon cooling.
2. T., *trace.* With 8 drops of urine the reaction occurs after about one minute's boiling.
3. M. A., *moderate amount.* With 8 or 10 drops of urine the reaction occurs after ten or fifteen seconds' boiling.
4. L. A., *large amount.* Reduction occurs almost immediately after adding 2 drops of urine to the boiling reagent.

Owing to the many variable factors, it is impossible to set down with any degree of accuracy the percentages of dextrose covered by the four terms

Quantitative Estimation.—In quantitative work Fehling's solution, for so many years the standard, has been largely displaced by Benedict's quantitative solution, which appears to be more exact and more satisfactory than any other titration method available for sugar work. The older method is still preferred by some and both are therefore given.

Should the urine contain much glucose, it must be diluted before making any quantitative test, allowance being made for the dilution in the subsequent calculation. Albumin, if present, must be removed acidifying a considerable quantity of urine with acetic acid, boiling, and filtering. Any water lost during the boiling should be replaced before filtering.

A rough but sometimes useful approximation of the amount of sugar in the urine of a diabetic patient can be made by estimating the total solids (p. 72), subtracting what may be regarded as normal for the individual, and regarding the remainder as sugar.

1. **Benedict's Method.**—The following modification of his copper solution has been offered by Benedict for quantitative estimations.

The reagent consists of:

Copper sulfate (pure crystallized)	18.0 Gm.
Sodium carbonate (crystallized)	200.0 "
(or 100 Gm. of anhydrous sodium carbonate)	
Sodium or potassium citrate, C. P.	200.0 "
Potassium sulfocyanate, C. P.	125.0 "
Potassium ferrocyanide solution (5 per cent)	5.0 c.c.
Distilled water, to make	1000.0 "

With the aid of heat dissolve the carbonate, citrate, and sulfocyanate in about 700 c.c. of the water and filter. Dissolve the copper in 100 c.c. of water and pour slowly into the other fluid, stirring constantly. Add the ferrocyanide solution, cool, and dilute to 1000 c.c. Only the copper sulfate need be accurately weighed. This solution is of such strength that 25 c.c. are reduced by 0.05 Gm. glucose. It keeps well.

To make a sugar estimation take 25 c.c. of the reagent in a small flask, add 10 to 20 Gm. of sodium carbonate crystals (or one half this weight of anhydrous sodium carbonate) and a small quantity of powdered pumice-stone or talcum. Heat to boiling, and add the urine a little at a time, but fairly rapidly, from a buret until a chalk-white precipitate forms and the blue color of the reagent begins to fade. After this point is reached, add the urine a drop at a time until the last trace of blue just disappears. This end-point is easily recognized. During the whole of the titration the mixture must be kept vigorously boiling. Loss by evaporation must be made up by adding water. Note the quantity of urine required to discharge the blue

color; this contains exactly 0.05 Gm. dextrose, and the percentage is easily calculated.

Benedict's solution may be employed in the simplified method given for Fehling's solution below by using 2.5 c.c. of Benedict's quantitative solution, without addition of water, 1 Gm. of anhydrous sodium carbonate, and a small piece of cotton to prevent bumping during the boiling.

2. **Fehling's Method.**—Take 10 c.c. of Fehling's solution (made by mixing 5 c.c. each of the copper and alkaline solutions described on p. 98) in a flask or beaker, add 3 or 4 volumes of water, boil, and add the urine very slowly from a buret until the solution is completely decolorized, heating but not boiling after each addition.

Fehling's solution is of such strength that the copper in 10 c.c. will be reduced by exactly 0.05 Gm. of dextrose. Therefore, the amount of urine required to decolorize the test solution contains just 0.05 Gm. dextrose, and the percentage is easily calculated.

The chief objection to Fehling's method is the difficulty of determining the end-point. The use of an "outside indicator," however, obviates this. When reduction is thought to be complete, a few drops of the solution are filtered through a fine-grained filter paper on to a porcelain plate, quickly acidified with acetic acid, and mixed with a drop of 10 per cent potassium ferrocyanide. Immediate appearance of a reddish-brown color shows the presence of unreduced copper.

A somewhat simpler application of this method, which is accurate enough for most clinical purposes, is as follows: Take 1 c.c. of Fehling's solution in a large test tube, dilute with about 5 c.c. of water, heat to boiling, and, while keeping the solution hot but not boiling, add the urine, drop by drop, from a medicine dropper until the blue color is entirely gone. Toward the end add the drops very slowly, not more than 4 or 5 a minute. Divide 10 by the number of drops required to discharge the blue color; the quotient will be the percentage of glucose. If 20 drops were required, the percentage would be $10 \div 20 = 0.5$ per cent. It is imperative that the drops be of such size that 20 of them will make 1 c.c. Test the dropper with urine, not water, and hold it always at the angle which will give the right sized drop. If the drops are too large, draw out the tip of the dropper; if too small, cut off the tip. The method is, of course, more reliable if an accurate 1-c.c. pipet, graduated in tenths, be used. In this case the calculation consists in dividing 5 by the number of tenths of a cubic centimeter of urine required.

3. **Fermentation Method.**—This is convenient and satisfactory, its chief disadvantage being the time required. It depends upon the fact that glucose is fermented by yeast with evolution of CO_2. The amount of gas evolved is an index of the amount of glucose. No preservative must have been added to the urine. Einhorn's saccharimeter is the simplest apparatus available (Fig. 42). The urine must be so diluted as to contain not more than 1 per cent of glucose. A fragment of fresh yeast-cake about the size of a pea is mixed with a definite quantity of the urine measured in the tube which

accompanies the apparatus. The exact amounts of yeast and urine are unimportant. It should form an emulsion free from lumps or air bubbles. A pinch of tartaric acid may be added to prevent bacterial fermentation. The long arm of the apparatus and about half the bulb are then filled with the mixture, all bubbles being carefully discharged by tipping the instrument with the thumb over the opening, and the instrument is stood in a warm place, preferably an incubator. At the end of fifteen to twenty-four hours at room temperature, or about three hours in an incubator, fermentation will be complete, and the percentage of glucose can be read off upon the side of the tube. The result must then be multiplied by the degree of dilution. Since yeast itself sometimes gives off gas, a control test must be carried out with normal urine and the amount of gas evolved must be subtracted from that of the test. A control should also be made with a known glucose solution to make sure that the yeast is active. It has been shown that yeast can split

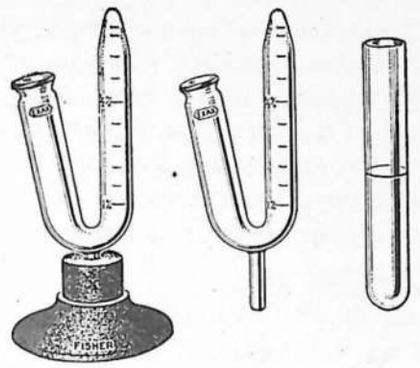


Fig. 42.—Einhorn fermentation saccharimeter.

off carbon dioxide from amino-acids, so that the results with the fermentation method may sometimes be a little high.

The method is not applicable to urine which is undergoing ammoniacal fermentation.

(2) **Levulose,** or **fruit sugar,** is seldom present in urine except in association with dextrose, and has about the same significance. According to von Noorden, its appearance in diabetes indicates an advanced case. Its name is derived from the fact that it rotates polarized light to the left.

Detection of Levulose.—Levulose responds to all the tests above given for dextrose. It may be distinguished from dextrose by the following:

Borchardt's Test.—Mix about 5 c.c. each of the urine and 25 per cent hydrochloric acid (concentrated HCl, 2 parts; water, 1 part) in a test tube

and add a few crystals of resorcinol. Heat to boiling and boil for not more than one half minute. In the presence of levulose a red color appears. Cool in running water, pour into a beaker, and render slightly alkaline with solid sodium or potassium hydroxide. Return to the test tube, add 2 or 3 c.c. of acetic ether, and shake. If levulose be present, the ether will be colored yellow. A similar yellow color will follow administration of rhubarb and senna.

If indican be present the test must be modified as follows: Perform Obermayer's test and extract the indican with chloroform. Reduce the acidity of the indican-free urine by adding one third its volume of water, add a few crystals of resorcinol, and proceed with Borchardt's test.

Quantitative Estimation of Levulose.—The methods are the same as for dextrose (p. 101); 25 c.c. of Benedict's quantitative solution are reduced by 0.053 Gm. levulose.

(3) **Lactose, or milk sugar,** is sometimes present in the urine of nursing women and in that of women who have recently miscarried. It is of interest chiefly because it may be mistaken for glucose. *It is not fermented by yeast but reduces copper*, 0.0676 Gm., being equivalent to 25 c.c. of Benedict's quantitative solution. In strong solution it can form crystals with phenylhydrazine, but is extremely unlikely to do so when the test is applied directly to the urine.

Rubner's Test.—To about 10 c.c. of the urine add 2 or 3 Gm. of lead acetate, shake well, and filter into a test tube. Boil the filtrate, add 1 or 2 c.c. strong ammonia and heat again. If lactose be present, the solution turns brick-red and a red precipitate will separate. The precipitate is the criterion. Glucose gives a red solution with yellow precipitate.

(4) **Maltose and cane sugar** are of little or no clinical importance. Maltose has been found along with dextrose in diabetes. It reduces copper, 0.074 Gm., being equivalent to 25 c.c. of Benedict's solution. Cane sugar (sucrose) is sometimes added to the urine by malingering patients. It does not reduce copper, and hence when it has been added passes unrecognized by the physician who uses the copper tests only. Both sugars are fermentable by yeast.

(5) **Pentoses.**—These sugars are so named because the molecule contains 5 atoms of carbon. Vegetable gums form their chief source. They reduce copper strongly but slowly, and give crystals with phenylhydrazine, but do not ferment with yeast.

Pentosuria is uncommon. It has been noted after ingestion of large quantities of pentose-rich substances, such as cherries, plums, and fruit juices, and is said to be fairly constant in habitual use of morphine. It sometimes accompanies glycosuria in diabetes. An

obscure chronic form of pentosuria without clinical symptoms has been observed most commonly in the Hebrew race. The pentose excreted in these cases is believed to be optically inactive arabinose, although ribose may be present in some cases at least.

Bial's Orcinol Test.—Dextrose is first removed by fermentation. About 5 c.c. of Bial's reagent is heated in a test tube, and after removing from the flame the urine is added drop by drop, not exceeding 20 drops in all. The appearance of a green color denotes pentose.

The reagent consists of:

Hydrochloric acid (30 per cent)	500 c.c.
Ferric chloride solution (10 per cent)	25 drops
Orcinol	1 Gm.

3. Acetone Bodies.—This is a group of closely related substances—acetone, diacetic acid, and beta-oxybutyric acid—whose chief source is in faulty catabolism of fats whereby the fatty acids fail to be completely oxidized. The underlying cause of this failure is not necessarily always the same, but is most frequently associated with defective carbohydrate metabolism. Normally, the metabolism of the carbohydrates furnishes the oxygen which is utilized for oxidation or "burning" of the fats of the body, hence it is said that "the fats burn in the flame of the carbohydrates." The amount of fat which can be completely oxidized bears a definite ratio to the amount of carbohydrate burned. When the supply of carbohydrates is deficient, as in starvation, or when their metabolism is defective, as in diabetes mellitus, there is insufficient oxygen available to properly burn the fats, and "the fire smokes." The result is an excessive production of the acetone bodies which are the products of this incomplete combustion. Formerly beta-oxybutyric acid was held to be the mother substance, but it is now believed that diacetic acid is first formed and that the others are derived from it.

To what extent these substances are toxic is unsettled, but it is quite clear that excessive production of diacetic and oxybutyric acids within the body leads, by virtue of their acid nature, to the condition known as acid intoxication. Since acetone bodies are ketones, this form of acidosis is sometimes given a special name—*ketosis*. The existence of this condition or, rather, the tendency toward it, since there may be moderate acetonuria without definite acidosis, is shown by the presence of acetone bodies in the urine. When the condition is very mild, acetone occurs alone; as it grows more marked, diacetic acid and beta-oxybutyric acid are also found.

Ketogenic Diet.—Gradual limitation of carbohydrates and increase

of fats have been advocated by Wilder[1] and others[2, 3] for the treatment of epilepsy. This diet has also been tried in the treatment of other conditions, and has been employed with some success in the treatment of urinary infections, especially those which are the result of *Escherichia coli*. Fuller[4] has demonstrated that beta-oxybutyric acid is the principal ketone body which is present in the urine after the administration of a ketogenic diet. For efficacy as an antiseptic, the urine must become acid and must have a *p*H of 5.3 to 4.6.

(1) **Acetone.**—Minute traces, too small for the ordinary tests, may be present in the urine under normal conditions. Larger amounts are not uncommon when the intake of carbohydrate is limited, and in fevers, gastro-intestinal disturbances, and certain nervous disorders. A notable degree of acetonuria has likewise been observed in cachectic conditions, in pernicious vomiting of pregnancy, in eclampsia, and in the serious and often fatal toxic condition which is now recognized as a not infrequent late effect of anesthesia, particularly of chloroform anesthesia. This postanesthetic toxemia seems to be more likely to appear and to be more severe when the urine contains any notable amount of acetone before operation, which suggests the importance of routine testing for acetone in surgical cases. It is not to be assumed that all of the toxic conditions enumerated are primarily acid intoxications nor that the symptoms are necessarily due to acidosis. It is more likely that the acetone bodies are formed secondarily as a result of the action of other toxic substances. Indeed, in many cases there may be little or no acidosis as measured by blood tests.

Acetonuria finds its chief clinical importance in connection with diabetes mellitus. It occurs intermittently in some mild cases, fairly regularly in most advanced cases, although much depends upon the diet, and is always present in severe cases. Tests for acetone are fully as important as tests for sugar in diabetes. A progressive increase —as measured by the strength of qualitative tests—is a grave prognostic sign, since acidosis due to the acetone bodies is probably the chief cause of the dreaded diabetic coma. Acetonuria can be diminished temporarily by more liberal allowance of carbohydrates in the diet.

Acetonuria from any cause is apt to be especially marked in

[1] Wilder, R. M.: The Effect of Ketonemia on the Course of Epilepsy, Bull. Mayo Clinic, *2*:No. 307 (July 27), 1921.

[2] Helmholz, H. F., and Keith, H. M.: Ten Years' Experience in the Treatment of Epilepsy with Ketogenic Diet, Arch. Neurol. and Psychiat., *29*:808–812 (April), 1933.

[3] Clark, A. L.: The Ketogenic Diet in the Treatment of Urinary Infections, Jour. Urol. *31*:193–204 (Feb.), 1934.

[4] Fuller, A. T.: The Ketogenic Diet; Nature of Bactericidal Agent, Lancet, *1*:855–856 (April 22), 1933.

children, and this doubtless plays an important part in acute and chronic diseases of childhood, especially in those requiring a restricted diet. In fact, the urine of a considerable percentage of young children shows acetone in appreciable quantities under normal conditions.

According to Folin, acetone is usually present in only small amounts in the above-mentioned conditions, the substance shown by the usual tests, particularly after distillation of the urine, being really diacetic acid. In this connection Frommer's test is to be recommended, since it does not require distillation, and does not react to diacetic acid unless too great heat is applied.

Owing to the marked and variable loss of acetone through the lungs a quantitative estimation is not of much value. In a case of diabetes, after the existence of an acidosis has been established by the detection of acetone bodies, it is better to rely upon an estimation of ammonia as a measure of its severity. The best measure of acidosis, applicable to all forms, is, however, the determination of the CO_2 combining power of the blood (p. 397).

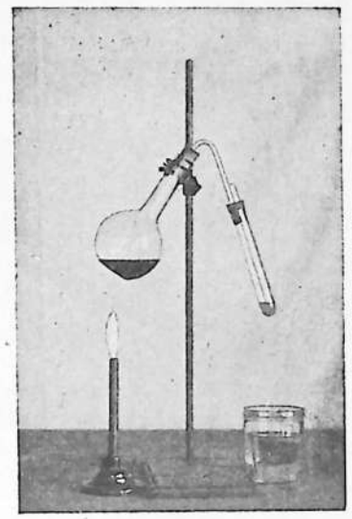


Fig. 43.—A simple distilling apparatus. The longer the delivery tube, the better will the vapor condense. Condensation may also be facilitated by immersing the test tube n a glass of cold water.

Detection of Acetone.—The urine may be tested directly, but it is much better to distil it after adding a little phosphoric or hydrochloric acid to prevent foaming, and to test the first few cubic centimeters of distillate. A simple distilling apparatus is shown in Fig. 43. The test tube may be attached to the delivery tube by means of a two-hole rubber cork as shown, the second hole serving as an air vent, or, what is much less satisfactory, it may be tied in place with a string. Should the vapor not condense well, the test tube may be immersed in a glass of cold water. If a sufficiently long delivery tube be used (2 feet), there will be little difficulty about condensation.

When diacetic acid is present, a considerable proportion will be converted into acetone during distillation.

1. **Gunning's Test.**—To about 5 c.c. of urine or distillate in a test tube add 5 drops of strong ammonia, and then Lugol's solution in sufficient quantity to produce a black cloud which does not immediately disappear. This cloud will gradually clear up and, if acetone be present, iodoform,

usually crystalline, will separate out. The iodoform can be recognized by its odor, especially upon heating (there is danger of explosion if the mixture be heated before the black cloud disappears), or by detection of the crystals microscopically. The latter, alone, is dependable, unless one has an acute sense of smell. The odor of iodine, which is also present, is often confusing. Iodoform crystals are yellowish six-pointed stars or six-sided plates (Fig. 44).

This modification of Lieben's test is less sensitive than the original, but is sufficient for all clinical work; it has the advantage that alcohol does not cause confusion, and especially that the sediment of iodoform is practically always crystalline. When it is applied directly to the urine, phosphates are precipitated and may form large, feathery, star-shaped crystals which are confusing to the inexperienced (Fig. 53). Albumin prevents formation of the iodoform crystals, and when it is present, the urine must be distilled for the test.

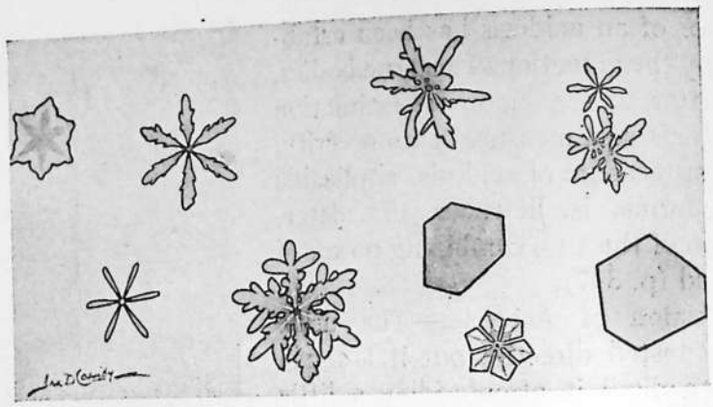


Fig. 44.—Iodoform crystals obtained in several tests for acetone by Gunning's method (× about 600).

2. **Lange's Test.**—This is a modification of the well-known Legal test. It is more sensitive and gives a sharper end-reaction. To a small quantity of urine add about one twentieth its volume (1 drop for each cubic centimeter) of glacial acetic acid and a few drops of fresh concentrated aqueous solution of sodium nitroprusside, and gently run a little ammonia upon its surface. If acetone be present, a reddish-purple ring will form within a few minutes at the junction of the two fluids.

A similar test, which is preferred by some, is known as **Rothera's test:** To 5 or 10 c.c. of urine add about a gram of ammonium sulfate and 2 or 3 drops of fresh concentrated sodium nitroprusside solution and overlay with strong ammonia. A reddish-purple ring shows the presence of acetone.

3. **Frommer's Test.**—This test has proved very satisfactory. The urine need not be distilled. Alkalinize about 10 c.c. of the urine with 2 or 3 c.c. of 40 per cent caustic soda solution, add 10 or 12 drops of 10 per cent alcoholic solution of salicylous acid (salicyl aldehyde), heat the upper portion to

about 70° C. (it should not reach the boiling point), and keep at this temperature five minutes or longer. In the presence of acetone an orange color, changing to deep red, appears in the heated portion. A yellow to brown color may appear in the absence of acetone.

The test can be made more definite by adding the caustic soda in substance (about 1 Gm.), and before it goes into solution adding the salicyl aldehyde and warming the lower portion of the tube.

(2) **Diacetic (aceto-acetic) acid** occurs in the same conditions as acetone, but has more serious significance. In diabetes its persistent presence is a grave symptom and often forewarns of approaching coma. It rarely or never occurs without acetone.

Detection.—The urine should be fresh. If a preservative must be used, toluene is best.

Gerhardt's Test.—To a few cubic centimeters of the urine add 10 per cent aqueous solution of ferric chloride, drop by drop, until the phosphates are precipitated; filter and add more of the ferric chloride. If diacetic acid be present, the urine will assume a Bordeaux-red color. The test is somewhat more definite if applied by the contact or "ring" method (p. 90). Very small amounts of diacetic acid may develop a brown color instead of the characteristic reddish-brown color.

Similar, although not exactly identical, colors may be produced by other substances, as phenol, salicylates, antipyrine, and sodium bicarbonate. To exclude these fallacies it is necessary, whenever the test gives a red or violet color, to repeat it upon a fresh portion of the urine as follows: To about 5 c.c. of urine in a test tube add an equal volume of water and boil down to the original volume. Cool and add the ferric chloride as before. Boiling drives off diacetic acid, hence if the color appears in this second test it was not originally due to diacetic acid. It is not sufficient to boil the urine after the color has been brought out by the ferric chloride, as is sometimes advised, since the color caused by certain of the drugs will then disappear as well as that caused by diacetic acid. Some prefer to acidify the urine with sulfuric acid, shake out with a little ether, and apply Gerhardt's test to the ether. Diacetic acid and certain drugs give red colors, but that due to diacetic acid fades within twenty-four to forty-eight hours. Whenever the reaction is doubtful, the urine should be distilled and the distillate tested for acetone, or Lindemann's test may be tried.

Lindemann's Test.—To about 10 c.c. of urine add 5 drops of 30 per cent acetic acid, 5 drops of Lugol's solution, and 2 or 3 c.c. of chloroform, and shake gently. The chloroform does not change color if diacetic acid be present, but becomes reddish violet in its absence. The test is claimed by its advocates to be more sensitive and more reliable than Gerhardt's. Uric acid also decolorizes iodine, and if much is present double the amount of Lugol's solution should be used.

(3) **Beta-oxybutyric acid** has much the same significance as diacetic acid, but is of more serious import. It is seldom or never present without acetone and diacetic acid which are more easily detected.

Hart's Test.—Remove acetone and diacetic acid by diluting 20 c.c. of urine with 20 c.c. of water, adding a few drops of acetic acid, and boiling down to 10 c.c. To this add 10 c.c. of water, mix, and divide between two test tubes. To one tube add 1 c.c. of hydrogen peroxide, warm gently, and cool. This transforms beta-oxybutyric acid to acetone. Now apply Lange's test for acetone (p. 108) to each tube. A positive reaction in the tube to which hydrogen peroxide has been added shows the presence of beta-oxybutyric acid in the original sample of urine.

Osterberg's[1] Test for Beta-oxybutyric Acid.—This simple test will indicate whether the concentration of beta-oxybutyric acid in a given sample of urine is greater or less than 0.5 per cent.

Reagents.—(*a*) Five per cent solution of sodium nitroprusside, freshly prepared; (*b*) concentrated ammonium hydroxide solution, specific gravity 0.88; (*c*) indicator, made by adding 2.8 c.c. of 0.04 per cent solution of bromthymol blue to 4 c.c. of 0.04 per cent solution of phenol red (Clark indicator solutions); (*d*) phosphate buffer solution of *p*H 8, 5 c.c. of fifteenth normal secondary sodium phosphate and 95 c.c. of fifteenth molar primary potassium phosphate solutions (see p. 836).

Method.—1. Prepare a standard by placing 4 c.c. of indicator (*c*) and 46 c.c. of buffer solution (*d*) in a tall Nessler comparison tube.

2. In a similar tube graduated at 50 c.c. place 800 mg. of ammonium sulfate, add 3 drops (0.15 c.c.) of concentrated ammonium hydroxide solution (*b*), then add 2 drops (0.1 c.c.) of solution (*a*).

3. Add 1 c.c. of urine.

4. After six minutes dilute the mixture with distilled water to the 50 c.c. mark.

5. Compare immediately with the standard (1). If the color is deeper than the standard, the concentration of beta-oxybutyric acid is greater than 0.5 per cent; if the color is lighter, the concentration is less than 0.5 per cent.

4. Bile.—The pigment of bile has its origin in the never-ceasing destruction of red blood corpuscles within the body.

The significance of bile in the urine is practically the same as that of bile-staining of the tissues, known as icterus or jaundice. Small amounts of bile may, however, be found in the urine when the disturbance is not severe enough to produce recognizable jaundice

[1] Osterberg, A. E., and Helmholz, H. F.: Determination whether Ketonurine Has Bactericidal Action. A Simple Technic for Clinical Use, Jour. Am. Med. Assn., *102*: 1831–1832 (June 2), 1934.

or, in other cases, before the jaundice supervenes. The usual cause of icterus is some obstruction to the outflow of bile from the liver, which may be in the nature of foreign bodies or new growths inside or outside of the bile passages, or inflammatory swelling of the walls with narrowing of the lumen. Jaundice may also occur when there is excessive destruction of red blood corpuscles from any cause, notably in the disease known as hemolytic jaundice or congenital familial icterus. This leads to excessive formation of a bile which is more inspissated than normal and thus tends to block the bile capillaries. The formation of bile in the general circulation in these conditions is also recognized. Another, less frequent, cause of jaundice is rapid destruction of liver cells, as in acute yellow atrophy and phosphorus poisoning. Both bile pigment and bile acids may be found. They generally occur together, but the pigment is not infrequently present alone.

Of the several pigments, bilirubin alone occurs in freshly voided urine, the others (biliverdin, bilifuscin, and so forth) being produced from this by oxidation as the urine stands. The bile acids, which occur chiefly as sodium salts, are not often present without the pigments, and are, therefore, seldom tested for clinically. Crystals of bilirubin (hematoidin) (Fig. 45, 4, p. 129) may be deposited in heavily bile-charged urine.

Detection of Bile Pigment.—Bile pigment gives the urine a greenish-yellow, yellow, or brown color, which upon shaking is imparted to the foam. Cells, casts, and other structures in the sediment may be stained brown or yellow. This, however, should not be accepted as proving the presence of bile without further tests.

1. **Smith's Test.**—Overlay the urine with tincture of iodine diluted with nine times its volume of alcohol. An emerald green ring at the zone of contact shows the presence of bile pigments. It is convenient to use a conical test glass one side of which is painted white.

2. **Gmelin's Test.**—This consists in bringing slightly yellow nitric acid into contact with the urine. A play of colors, of which green and violet are most distinctive, denotes the presence of bile pigment. Blue and red may be produced by indican and urobilin, violet by iodides. Colorless nitric acid will become yellow upon standing in the sunlight. The test may be applied in various ways: By overlaying the acid with the urine; by bringing a drop of each together upon a porcelain plate; by filtering the urine through thick filter paper, and touching the paper with a drop of the acid; and, probably best of all, by precipitating with lime water, filtering, and touching the precipitate with a drop of the acid. In the last method, bilirubin is carried down as an insoluble calcium compound which concentrates the pigment and avoids interfering substances.

Detection of Bile Acids.—Hay's test is simple, sensitive, and fairly reliable, and will, therefore, appeal to the practitioner. It depends upon the fact that bile acids lower surface tension. Other tests require isolation of the acids for any degree of accuracy. Oliver has recently shown that with Heller's test bile acids give a white ring just above that due to albumin.

Hay's Test.—Upon the surface of the urine, *which must not be warm*, sprinkle a little finely powdered sulfur ("flowers of sulfur"). If it sinks at once, bile acids are present to the amount of 0.01 per cent or more, if only after gentle agitation, 0.0025 per cent or more. If it remains floating, even after gentle shaking, bile acids are absent. It is said that urobilin when present in large amount also reduces surface tension.

5. Urobilin.—Traces of this pigment, too small for detection by the ordinary tests, are present under normal conditions. It is now regarded as identical with hydrobilirubin, the principal coloring matter of the feces. It is excreted as a chromogen, *urobilinogen*, which is changed into urobilin through the action of light within a few hours after the urine is voided. A great excess gives the urine a dark brown color suggesting the presence of bile, but does not color the foam so deeply as does bile. Small amounts cause no perceptible change in color.

The mode of formation of urobilin is not yet clearly understood. According to the generally accepted view it is a decomposition product of bilirubin, formed chiefly in the intestine through the action of bacteria. Upon the other hand, the formation of small amounts in the liver itself under certain conditions cannot be denied. Urobilinogen is first formed. Under normal conditions a portion of this is absorbed from the intestine, carried to the liver in the portal blood, and there reconverted into bilirubin. When the liver cells are deranged, this transformation into bilirubin does not take place and urobilinogen reaches the general circulation and is excreted by the kidneys. The remainder in the intestine, changed largely into urobilin, passes out with the feces. The pigment and the chromogen have exactly the same significance in the urine, and the name "urobilin" is commonly used to cover both.

Owing to the many unknown factors it is impossible to ascribe definite clinical significance to urobilinuria. Certain statements, however, seem justified. Whenever, owing to excessive destruction of blood corpuscles, there is excessive formation of bilirubin, and hence an increase of urobilin in the feces, there is also a marked increase in the urine. This is especially important in pernicious anemia and malaria.

When not due to excessive blood destruction, urobilinuria usually points toward functional incapacity of the liver. Its recognition is very simple and has considerable practical usefulness, as, for example, in the diagnosis of hepatic cirrhosis; in judging the amount of damage done to the liver parenchyma by poisons and the chronic congestion of poorly compensated heart disease; and in differentiating anemias associated with excessive blood destruction (for example, pernicious anemia) from those due to other causes (carcinoma, hemorrhage). Urobilinuria is frequent in acute infectious diseases, especially in scarlet fever and pneumonia, and usually means either hemolysis or damage to the liver. In severe nephritis urobilin may fail to be excreted even when other conditions favor its appearance in the urine. It is nearly or entirely absent in obstructive jaundice.

To be of value, tests for urobilin should be made upon several successive days, because for some unknown reason it may be absent for a day or two, and it is advisable to make a simultaneous study of the urobilin of the feces.

1. **Ehrlich's Test for Urobilinogen.**—To a few cubic centimeters of the urine in a test tube add a few crystals of para-dimethyl-amino-benzaldehyde and make definitely acid with hydrochloric acid. In the presence of pathologic amounts of urobilinogen a cherry-red color appears. This is best seen by viewing the tube from the top over a sheet of white paper. Normal amounts will cause the red color only when the urine is heated.

2. **Schlesinger's Test for Urobilin.**—To about 10 c.c. of the urine in a test tube add a few drops of Lugol's solution to transform the chromogen into the pigment. Now add about 10 c.c. of a saturated alcoholic solution of zinc acetate or zinc chloride and filter. A greenish fluorescence, best seen when the tube is viewed in bright sunlight against a black background and when the light is concentrated upon it with a lens, shows the presence of urobilin. The fluorescence becomes more marked after an hour or two. Bile pigment, if present, should be previously removed by adding about one-fifth volume of 10 per cent calcium chloride solution and filtering.

Quantitative Estimation.—The clinically satisfactory, indirect method of Wilbur and Addis which is given in detail on page 477 may be applied to the urine as follows:

1. To a 10 c.c. portion of the mixed twenty-four-hour urine, which has been preserved with a crystal of thymol and kept in darkness, add 10 c.c. of a saturated alcoholic solution of zinc acetate and filter.

2. To 10 c.c. of the filtrate (representing 5 c.c. of urine) add 1 c.c. of Ehrlich's reagent, the formula for which is as follows:

Para-dimethyl-amino-benzaldehyde	10 Gm.
Concentrated hydrochloric acid	75 c.c.
Water	75 "

3. Let stand in the dark for one to three hours, not longer.
4. Examine with a small direct-vision spectroscope and dilute with tap water until absorption bands disappear. Calculate the total dilution value for the twenty-four-hour quantity of urine as described for urobilin in feces, *basing the calculation upon the 5 c.c. of urine used.*

Example.—If the twenty-four-hour urine amounts to 1200 c.c. and it is necessary to dilute the 10 c.c. filtrate to 50 c.c. to get rid of the absorption bands (supposing that they disappear together), then the combined dilution value for urobilin and urobilinogen in the 5 c.c. of urine would be 10 + 10 = 20; and the twenty-four-hour value would be 20 × 240 = 4800.

The Wilbur and Addis units do not represent accurate quantitative values. If a more accurate method is desired, the technic developed by Watson[1] should be followed. This method depends on the reduction of all of the urobilin excreted in a twenty-four-hour sample of urine to urobilinogen with ferrous hydroxide. Combining the urobilinogen with paradimethylaminobenzaldehyde (Ehrlich's reagent) and a saturated solution of sodium acetate develops a red solution which can be compared colorimetrically with a phenolsulfonphthalein standard and the amount of urobilinogen in milligrams calculated according to Watson's formula.

The normal adult excretes from 0 to 4 mg. of urobilinogen daily in urine. In diffuse hepatic disease there is usually a marked increase of urobilinogen in the urine. In complete obstruction to the outflow of bile, there is a slight trace or no trace of urobilinogen in the urine.

6. Hemoglobin.—The presence in the urine of hemoglobin, or pigments directly derived from it, accompanied by few, if any, red corpuscles, constitutes *hemoglobinuria*. It is a comparatively rare condition, and must be distinguished from *hematuria*, or *blood* in the urine, which is common. In both conditions chemical tests will show hemoglobin, but in the latter the microscope will reveal the presence of red corpuscles. Urines which contain notable amounts of hemoglobin have a reddish or brown color, and may deposit a sediment of brown, granular pigment.

Hemoglobinuria occurs when there is such extensive destruction of red blood cells within the body that the liver cannot transform all the hemoglobin set free into bile pigment. The most important

[1] Watson, C. J.: Studies of Urobilinogen. I. An Improved Method for the Quantitative Estimation of Urobilinogen in Urine and Feces, Am. Jour. Clin. Path., *6*:458–475 (Sept.), 1936.

II. Urobilinogen in the Urine and Feces of Subjects Without Evidence of Disease of the Liver or Biliary Tract, Arch. Int. Med., *59*:196–205 (Feb.), 1937.

III. The Per Diem Excretion of Urobilinogen in the Common Forms of Jaundice and Disease of the Liver, Arch. Int. Med., *59*: 206–231 (Feb.), 1937.

examples are seen following extensive burns; in poisoning, as by mushrooms and potassium chlorate; in malignant malaria (black-water fever); and in the obscure condition known as "paroxysmal hemoglobinuria." This last is characterized by the appearance of large quantities of hemoglobin at intervals, usually following exposure to cold, the urine remaining free from hemoglobin between the attacks. See page 754 for the Donath-Landsteiner test for autohemolysins in the serum of a patient who has paroxysmal hemoglobinuria. Another form of paroxysmal hemoglobinuria is nocturnal hemoglobinuria, and this is accompanied by hemolytic anemia. (See the discussion of this disease, which is known as Marchiafava-Micheli syndrome, on page 315.)

Detection.—Teichmann's test may be applied to the precipitate after boiling and filtering, but this is not very satisfactory, and the guaiac or benzidine test will be found more convenient in routine work. For further discussion of blood tests, including spectroscopic methods, see page 353.

Guaiac Test.—Mix a few cubic centimeters each of "ozonized" turpentine and a fresh 1 : 60 alcoholic solution of gum guaiac. The guaiac solution may be freshly prepared by dissolving a pocket-knife-pointful of powdered guaiac in 4 or 5 c.c. of alcohol. Make the urine strongly acid with acetic acid, and carry out the test by the "ring" or "contact" method described on page 90. A bright blue ring will appear at the zone of contact within a few minutes if hemoglobin be present in considerable amount; a green ring if traces only are present. It may be difficult to get a sharp line of contact, but this is no hindrance since the test is nearly as sharp when the fluids are mixed. The guaiac should be kept in an amber-colored bottle. Fresh turpentine can be "ozonized" by allowing it to stand a few days in an open vessel in the sunlight. Instead of turpentine, hydrogen peroxide may be used.

This test is very sensitive, and a negative result proves the absence of hemoglobin. Positive results are not conclusive, because numerous other substances—few of them likely to be found in the urine—may produce the blue color. That most likely to cause confusion is pus, but the blue color produced by it will appear equally well if the turpentine be omitted, and does not appear if the urine be previously boiled. The thin film of copper often left in a test tube after testing for sugar may give the reaction, as may also the fumes from an open bottle of bromine. Bromides and iodides likewise give the reaction. Most sources of error can be avoided by extracting the hemoglobin with ether as described on page 353.

Benzidine Test.—The reagents employed are hydrogen peroxide and a saturated solution of benzidine in glacial acetic acid. The benzidine labeled "For blood tests" should be employed. The reagents are mixed in equal parts and a few cubic centimeters are added to a like amount of the

urine. A blue color appears in the presence of hemoglobin. The test has the same fallacies as the guaiac test, but is more sensitive and, in general, more satisfactory.

The benzidine test may be simplified by use of the tablets devised by Roberts and put upon the market by the firm of E. R. Squibb & Sons. These contain benzidine and sodium perborate. A tablet is thoroughly moistened with the fluid to be tested and is then touched with a drop of glacial acetic acid, the appearance of a blue color indicating blood. The test is less delicate than those given above.

Spectroscopic Method.—This is discussed on page 355. It is more reliable than the preceding tests, but less sensitive. Render the urine slightly acid, dilute if very highly colored, and examine with a small direct-vision spectroscope. The usual bands seen are those of oxyhemoglobin or methemoglobin.

To detect traces of blood with the spectroscope proceed as described in Section 2 on page 355. This method will easily detect 2 drops of blood in 8 ounces of urine.

7. Hemosiderin.—This is a dark yellow pigment which contains iron. It may be deposited in the tissues of the body as a result of destruction of the blood, which occurs in such conditions as pernicious anemia. In hemochromatosis and siderosis of the kidney, cells which contain this pigment may be excreted in the urine; many free granules of hemosiderin may also be excreted. Rous[1] has described a simple test for the detection of hemosiderin in urine; the technic is as follows: Centrifugalize a fresh sample of urine in the usual manner. Pour off the supernatant urine and examine a portion of the sediment with a microscope; search for brown granules, especially within the cells. Suspend the rest of the sediment in a fresh mixture of 5 c.c. of a 2 per cent solution of potassium ferrocyanide and 5 c.c. of a 1 per cent solution of hydrochloric acid. Allow to stand for ten minutes; then centrifugalize; and examine the sediment with the microscope. A cover glass should be placed over the preparation. Granules of hemosiderin appear blue in this preparation.

8. Porphyrins.—Porphyrins are substituted tetrapyrril methenes. In small quantities some of these pigments may be found in normal urine. Urine containing large amounts of porphyrin may be dark red or a "port wine" color. In clinical conditions in which porphyrinuria may be suspected and in which the freshly passed urine is light colored, darkening of the urine on standing for twenty-four hours or more suggests the advisability of an investigation for these substances.

[1] Rous, Peyton: Urinary Siderosis. Hemosiderin Granules in the Urine as an Aid in the Diagnosis of Pernicious Anemia, Hemochromatosis, and Other Diseases Causing Siderosis of the Kidney, Jour. Exper. Med. *28*:645–658 (Nov.), 1918.

For a discussion of porphyrins in relation to disease, the reader should consult the references to the work of Watson[1] or Dobriner and Rhoads.[2]

Porphyrins have been studied in the urine and feces in febrile conditions, liver disease, hemolytic diseases of the blood, deficiency diseases, diseases of the skin and in toxic states due to various drugs or poisons. *Congenital porphyria*, a rare disease, is usually found in young males. There is a marked sensitiveness of the skin to sunlight. *Acute porphyria* occurs more often in adult females with unexplained abdominal pain, flaccid paralysis or other nervous manifestations.[3] There is usually little photosensitivity of the skin.

If the urine is a dark color and other symptoms suggest porphyria, a sample of urine should be extracted with ether and acetic acid. To 25 c.c. of urine in a separatory funnel add 10 c.c. of glacial acetic acid. Extract this mixture twice with 50 c.c. portions of ether and combine the extracts. Wash the combined extracts with 10 c.c. of 5 per cent hydrochloric acid. Examine the washings under ultraviolet light. If there is a strong red fluorescence, a large amount of coproporphyrin is present and is suggestive of porphyria. Examine the urine residue after ether extraction under ultraviolet light. A discernible red fluorescence is evidence of uroporphyrin. This should be confirmed spectroscopically. Usually, the urine will show 2 absorption bands, characteristic of the zinc complex of uroporphyrin. These bands are very similar in their location to those of oxyhemoglobin. Add 1 to 2 c.c. of concentrated hydrochloric acid to 5 c.c. of the urine residue. This decomposes the zinc complex and the three bands of uroporphyrin can be recognized (Fig. 185, page 359). If porphyrins are present, separation and identification must be carried out by means of special methods. Porphyrins are identified by the strength of hydrochloric acid in which they may be extracted, by the characteristic absorption bands (Fig. 185, page 359) or by the crystal forms and the melting points of the methyl esters.[4]

Watson and Schwartz[5] found that in acute idiopathic porphyria, even though the freshly voided urine is normal in color, a simple

[1] Watson, C. J.: The porphyrins and their relation to disease; porphyria. Oxford Medicine. Vol. 4, Part 2, Chapter 9A, pp. 228 (1–34).

[2] Dobriner, Konrad and Rhoads, C. F.: The porphyrins in health and disease. Physiological Reviews. *20*:416–468 (July), 1940.

[3] Nesbitt, Samuel and Watkins, C. H.: Acute porphyria. Am. J. Med. Sci., *203*:74–83 (Jan.), 1942.

[4] Dobriner, Konrad: Urinary porphyrins in disease. Jour. Biol. Chem., *113*:1–10 (Feb.), 1936.

[5] Watson, C. J. and Schwartz, Samuel: A simple test for urinary porphobilinogen. Proc. Soc. Exp. Biol. and Med., *47*:393–394 (June), 1941.

test for porphobilinogen is useful. The demonstration of a chloroform-insoluble compound with Ehrlich's aldehyde reagent is pathognomonic for this disease. Mix in a test tube equal parts of urine and Ehrlich's reagent.[1] Add an equal volume of saturated solution of sodium acetate. Add a few cubic centimeters of chloroform and mix thoroughly. The aldehyde compound of urobilinogen will be carried down by the chloroform, while that of porphobilinogen, insoluble in chloroform, will remain in the supernatant solution, coloring it red.

9. Alkapton Bodies.—The name "alkaptonuria" has been given to a condition in which the urine turns reddish brown to brownish black upon standing and strongly reduces copper (but not bismuth), owing to the presence of certain substances which result from imperfect protein metabolism. The chief of these is homogentisic acid. The change of color takes place quickly when fresh urine is alkalinized, hence the name, *alkapton bodies*.

Alkaptonuria is unaccompanied by other symptoms, and has little clinical importance. Only a few cases, mostly congenital, have been reported. The change in color of the urine and the reduction of copper, with no reduction of bismuth nor fermentation with yeast, would suggest the condition.

Abbott[2] has reported the occurrence of alkaptonuria in two children of a Negro family. These are the first cases of this error of metabolism reported in an American Negro. This author stated that at the time of the report only twenty-one of the total number of recorded cases had been reported in the United States, but he also felt that the condition, which is most often recognized by accident, probably exists more commonly than the records show.

10. Melanin.—Urine which contains melanin likewise darkens upon exposure to the air, assuming a dark brown or black color. This is due to the fact that the substance is eliminated as a chromogen—melanogen—which is later converted into the pigment. It does not reduce copper.

Melanuria occurs in most, but not all, cases of melanotic tumor. Its diagnostic value is lessened by the fact that it has been observed in other diseases, although rarely. It is apparently the result of a type of protein destruction which may occur at times in widely different clinical conditions.

[1] See page 479. Modified Ehrlich's reagent. 0.7 gram paradimethylaminobenzaldehyde, 150 c.c. concentrated hydrochloric acid and 100 c.c. distilled water.

[2] Abbott, L. D. F., Jr.: Alkaptonuria in a negro family. Science, *94*:365–366 (Oct. 17), 1941.

Tests for Melanin.—1. Addition of a few drops of a solution of ferric chloride to 10 c.c. of urine gives a gray precipitate which blackens on standing.

2. Bromine water added to urine in equal proportions causes a yellow precipitate which gradually turns black.

11. Diazo Substances.—Certain imperfectly known substances sometimes present in the urine give a characteristic color reaction—the "diazo reaction" of Ehrlich—when treated with diazo-benzolsulfonic acid and ammonia. This reaction has considerable clinical value, *provided its limitations be recognized.* It is at best an empirical test and must be interpreted in the light of clinical symptoms. Although it has been met with in a considerable number of diseases, its usefulness is practically limited to typhoid fever, tuberculosis, and measles.

(1) **Typhoid Fever.**—Practically all cases give a positive reaction, which varies in intensity with the severity of the disease. It is so constantly present that it is sometimes said to be "negatively pathognomonic": If negative upon several successive days *at a stage of the disease when it should be positive*, typhoid is almost certainly absent. Upon the other hand, a reaction when the urine is highly diluted (1 : 50 or more) has much positive diagnostic value, since the dilution prevents the reaction in most conditions which might be mistaken for typhoid; but it should be noted that mild cases of typhoid may not give it at this dilution. Ordinarily the reaction appears about the fourth or fifth day of the disease. It begins to fade about the end of the second week, and soon thereafter entirely disappears. An early disappearance is a favorable sign. It reappears during a relapse, and thus helps to distinguish between a relapse and a complication, in which it does not reappear.

(2) **Tuberculosis.**—The diazo reaction has been obtained in many forms of the disease. It has little or no diagnostic value. Its continued presence in pulmonary tuberculosis is, however, a grave prognostic sign, even when the physical signs are slight. After it once appears it generally persists more or less intermittently until death, the average length of life after its appearance being about six months. The reaction is often temporarily present in mild cases during febrile complications, and has then no significance.

(3) **Measles.**—A positive reaction is usually obtained in measles, and may help to distinguish this disease from German measles, in which it does not occur. It generally appears before the eruption and remains about five days.

Technic.—Although the test is really a very simple one, careful attention to technic is imperative. Many of the early workers were very lax in this regard. Faulty technic and failure to record the stage of the disease in which the tests were made have probably been responsible for the bulk of the conflicting results reported. Reactions more or less closely simulating the diazo reaction have been observed after administration of naphthalin, opium, and its alkaloids, atophan, salol, creosote, phenol, and the iodides. Tannic acid and its compounds prevent the reaction.

The reagents are:

(1) Sulfanilic acid	1.0 Gm.
Concentrated hydrochloric acid	10.0 c.c.
Water	200.0 "
(2) Sodium nitrite	0.5 Gm.
Water	100 0 c.c.
(3) Strong ammonia.	

Mix 100 parts of (1) and 1 part of (2).[1] In a test tube take equal parts of this mixture and the urine, and pour 1 or 2 c.c. of the ammonia upon its surface. If the reaction be positive, a garnet ring will form at the junction of the two fluids, and, upon shaking, a distinct pink color will be imparted to the foam. The color of the foam is the essential feature. If desired, the mixture may be well shaken before the ammonia is added: The pink color will then instantly appear in that portion of the foam which the ammonia has reached, and can be readily seen. The color varies from eosin pink to deep crimson, depending upon the intensity of the reaction. *It is a pure pink or red; any trace of yellow or orange denotes a negative reaction.* A doubtful reaction should be considered negative.

Weisz' Urochromogen Test.—Weisz[2] believed the diazo reaction to be due principally to urochromogen, which, because of the effect of certain toxins upon metabolism, fails of conversion into urochrome; and he offered this permanganate reaction as a more satisfactory test, both for urochromogen and for an antecedent substance which has the same significance as urochromogen, but which the diazo fails to detect. This test has been studied chiefly in its relation to prognosis in tuberculosis, in which it appears to have about the same value as the diazo, with the differences that it is more frequently noted and is less intermittent in a given case and probably has less serious import.

In a test tube mix 2 c.c. of urine and 4 c.c. distilled water, and add 3 drops of 1 : 1000 aqueous solution of potassium permanganate. The appearance of a yellow color denotes a positive reaction. The color is best judged by comparison with a tube of diluted urine to which no permanganate has been added, the two tubes being viewed from the top over a sheet of white

[1] These proportions are recommended by Greene, and are now generally used. Ehrlich used 40 parts of (1) and 1 part of (2).

[2] Weisz, Moriz: Die Bedeutung des Urochromogens für die Prognose und Therapie der Lungentuberkulose, München. med. Wchnschr., *58*:1348–1352 (June 20), 1911.

paper. The color of a genuine reaction is a canary yellow. A yellow color, usually not so bright, and tending more toward brown, may be produced by urobilin and other substances, but these false reactions fade quickly, usually within thirty seconds, while the color of a true reaction remains a longer time.

12. Drugs.—The effect of various drugs upon the color of the urine has been mentioned (p. 66). Most poisons are eliminated in the urine, but their detection is more properly discussed in works upon toxicology. A few drugs which are of interest to the practitioner, and which can be detected by comparatively simple methods, are mentioned here.

Acetanilid and Phenacetin.—The urine is evaporated by gentle heat to about half its volume, boiled for a few minutes with about one fifth its volume of strong hydrochloric acid, and shaken out with ether. The ether is evaporated, the residue dissolved in water, and the following test applied: To about 10 c.c. are added a few cubic centimeters of 3 per cent phenol followed by a weak solution of chromium trioxide (chromic acid) drop by drop. The fluid assumes a red color, which changes to blue when ammonia is added. If the urine be very pale, extraction with ether may be omitted.

Antipyrine.—This drug gives a dark red color when a few drops of 10 per cent ferric chloride are added to the urine. The color does not disappear upon boiling, which excludes diacetic acid.

Arsenic.—*Reinsch's Test.*—Add to the urine in a test tube or small flask about one seventh its volume of hydrochloric acid, introduce a piece of bright copper foil about 3 mm. square, and boil for several minutes. If arsenic be present, a dark gray film is deposited upon the copper. The test is more delicate if the urine be concentrated by slow evaporation. This test is well known and is widely used, but is not so reliable as the following:

Gutzeit's Test.—In a large test tube place a little arsenic-free zinc, and add 5 to 10 c.c. pure dilute hydrochloric acid and a few drops of iodine solution (Gram's solution will answer), then add 5 to 10 c.c. of the urine. At once cover the mouth of the tube with a filter paper cap moistened with saturated aqueous solution of silver nitrate (1 : 1). If arsenic be present, the paper quickly becomes lemon yellow, owing to formation of a compound of silver arsenide and silver nitrate, and turns black when touched with a drop of water. To make sure that the reagents are arsenic free, the paper cap may be applied for a few minutes before the urine is added.

Electrolytic Gutzeit Test.—The best tests for arsenic are now performed with some type of apparatus converting the arsenic into

arsine by electrolysis. Osterberg[1] has described one of the simplest of these pieces of apparatus with a quantitative method especially applied to the estimation of the amount of arsenic in urine.

Atropine will cause dilatation of the pupil when a few drops of the urine are placed in the eye of a cat or rabbit.

Bromides can be detected by acidifying about 10 c.c. of the urine with dilute sulfuric acid, adding a few drops of fuming nitric acid and a few cubic centimeters of chloroform, and shaking. In the presence of bromine the chloroform, which settles to the bottom, assumes a yellow color.

Chloral hydrate appears in the urine chiefly as urochloralic acid, which reduces the copper solutions used for sugar tests. To detect it, evaporate about 500 c.c. of the urine to about one fourth its volume, make decidedly acid with hydrochloric acid, add about 50 c.c. of ether, shake thoroughly, and separate the ether. Now evaporate the ether and dissolve the residue in a little water. If urochloralic acid be present, this aqueous solution will respond to Fehling's test.

Formaldehyde is discussed under Hexamethylenamine.

Hexamethylenamine.—Interest in this drug centers chiefly in its value as a urinary antiseptic, which depends upon its decomposition in acid urine with liberation of formaldehyde. According to a number of recent workers formaldehyde can be detected in the urine of only about 50 per cent of patients who are taking hexamethylenamine. A test for formaldehyde is, therefore, necessary in order to know whether the object in administering the drug is being accomplished.

Jorissen's Test for Formaldehyde.—To 1 or 2 c.c. of the urine or other fluid to be tested add about 0.5 c.c. of a 1 per cent solution of phloroglucinol (Merck's "reagent") in 10 per cent sodium hydroxide. The appearance of a bright red color shows the presence of free formaldehyde. The test is said to detect 1 part in 10,000,000.

Iodine, from ingestion of iodides or absorption from iodoform dressings, is tested for in the same way as the bromides, the chloroform assuming a pink to reddish-violet color; or Obermayer's reagent may be used in the same way as described for indican (p. 77). To detect traces a large quantity of urine should be rendered alkaline with sodium carbonate and greatly concentrated by evaporation before testing.

For accurate quantitative estimation of the iodine content of

[1] Osterberg, A. E.: A Modification of the Electrolytic Gutzeit Apparatus for the Estimation of Arsenic in Biological Material, Jour. Biol. Chem., *76:*19–22 (Jan.), 1928.

urine Kendall's[1] method for the determination of iodine in biologic material is recommended. As applied to urine this involves the concentration to small bulk and the fusing with sodium hydroxide. After the destruction of the organic matter the iodine is retained as sodium iodide. This is oxidized in an acid solution to iodic acid with bromine. The iodic acid liberates iodine from potassium iodide. The amount of iodine liberated, which is six times the amount of iodine originally present in the sample, is then determined by titrating with suitable strength sodium thiosulfate solution using soluble starch as an indicator. With 0.005 normal solution (N/200) of sodium thiosulfate 1 c.c. is equivalent to 0.106 mg. of iodine originally present.

Lead.—No simple method is sufficiently sensitive to detect the traces of lead which occur in the urine in chronic poisoning. Of the more sensitive methods, that of Fairhall is very satisfactory. For an interesting symposium on lead poisoning the reader is referred to the reports of Lanza[2]; Aub[3]; Kehoe, Thamann, and Cholak[4]; Jones[5]; Gray[6]; and Belknap.[7] A very complete bibliography on the subject will be found in these papers.

Fairhall's[8] Method for Lead in Urine.—*Reagent.*—The only carefully standardized reagent required is 0.005 normal (N/200) sodium thiosulfate. Thoroughly dissolve 25 Gm. of sodium thiosulfate with 18 liters of freshly distilled water. Great care is necessary in keeping all glassware chemically clean, and in keeping the carbon dioxide of the air away from the solution. The solution must be kept very accurately standardized by any of the recognized quantitative procedures.

[1] Kendall, E. C.: The Determination of Iodine in Connection with Studies in Thyroid Activity, Jour. Biol. Chem., *19:*251–256, 1914. Kendall, E. C.: Determination of Iodine in Connection with Studies in Thyroid Activity, Jour. Biol. Chem., *43:*149–159 (Aug.), 1920. Kendall, E. C. and Richardson, F. S.: Determination of Iodine in Blood and in Animal Tissues, Jour. Biol. Chem., *43:*161–170 (Aug.), 1920.

[2] Lanza, A. J.: Epidemiology of Lead Poisoning, Jour. Am. Med. Assn., *104:*85–87 (Jan. 12), 1935.

[3] Aub, J. C.: The Biochemical Behavior of Lead in the Body, Jour. Am. Med. Assn., *104:*87–90 (Jan. 12), 1935.

[4] Kehoe, R. A., Thamann, Frederick, and Cholak, Jacob: Normal Absorption and Excretion of Lead, Jour. Am. Med. Assn., *104:*90–92 (Jan. 12), 1935.

[5] Jones, R. R.: Symptoms in Early Stages of Industrial Plumbism, Jour. Am. Med. Assn., *104:*195–200 (Jan. 19), 1935.

[6] Gray, Irving: Recent Progress in the Treatment of Plumbism, Jour. Am. Med. Assn., *104:*200–205 (Jan. 19), 1935.

[7] Belknap, E. L.: Control of Lead Poisoning in the Worker, Jour. Am. Med. Assn., *104:*205–210 (Jan. 19), 1935.

[8] Fairhall, L. T.: Lead Studies: I. The Estimation of Minute Amounts of Lead in Biological Material, Jour. Ind. Hyg., *4:*9–20 (May), 1922. Fairhall, L. T.: Lead Studies. XI. A Rapid Method of Analyzing Urine for Lead, Jour. Biol. Chem., *60:*485–488 (July), 1924. Aub, J. C., Fairhall, L. T., Minot, A. S., and Reznikoff, P.: Lead Poisoning, Medicine, *4:*1–250 (Feb.–May), 1925.

Method.—Add ammonium hydroxide to the urine until it is strongly ammoniacal. Allow the mixture to stand until the supernatant fluid is clear, or if necessary for twenty-four hours. Filter out the gelatinous mass containing the alkaline earth phosphates which entrain any lead phosphate present. Decant the clear supernatant fluid and centrifugate out the precipitate. Dissolve all of the precipitate in from 10 to 15 c.c. of 1 : 1 hydrochloric acid; then wash out the centrifuge tube with water. Heat the solution to boiling and filter while hot. Neutralize carefully with ammonium hydroxide. Add hydrochloric acid until just acid to methyl orange, or to Congo red.[1] Saturate the cold solution with hydrogen sulfide and allow to stand for several hours, and then filter, or centrifugate. Wash the precipitate and dissolve it in from 2 to 5 c.c. of concentrated nitric acid. Boil to expel the hydrogen sulfide. Cool, filter, and make slightly alkaline with sodium hydroxide. Acidify again with a slight excess of acetic acid. Add 2 or 3 drops of a saturated solution of potassium chromate. Boil for a few minutes. Allow to stand for several hours, and filter or centrifugate out the precipitate. Wash all of the soluble chromate from the filter paper, or the centrifuge tube by successive washings with water. Dissolve the precipitate with from 2 to 5 c.c. of 1 : 1 hydrochloric acid solution followed by a few cubic centimeters of water. Add an excess of potassium iodide solution, and titrate the free iodine with 0.005 normal sodium thiosulfate solution, using a drop of 5 per cent soluble starch as an indicator. One cubic centimeter of 0.005 normal sodium thiosulfate is equivalent to 0.3451 mg. of metallic lead.

Mercury (*Method of Vogel and Lee*).—This will detect 1 mg. of mercury in 100 c.c. of urine, gastric contents, or feces.

To about 150 c.c. of the urine add about 5 c.c. of concentrated hydrochloric acid and boil down to 25 or 30 c.c. Add about 2 c.c. of hydrochloric acid to replace that lost in boiling and enough potassium chlorate, usually about 2 Gm., to oxidize the organic matter, as shown by a change of color to pale yellow. Dilute to about 60 c.c. and boil a few minutes to drive off chlorine.

Secure a piece of copper wire of about 18 gage and 4 cm. long, bend it several times upon itself, clean by boiling a few minutes in dilute hydrochloric acid, and with the aid of forceps place it in the concentrated urine, where it should remain for two hours. In the presence of mercury it will become coated with a silvery film. Next remove the wire with the aid of forceps, rinse in water, dry, and place it in the bottom of a slender test tube, made by sealing one end of a piece of glass tubing about 15 cm. long and 3 to 5 mm. in diameter. Also insert in the tube a plug of gold leaf and push it down to about 2 mm. from the wire. The gold leaf is that used in dentistry.

[1] Dr. Osterberg states that Congo red precipitating in the acid solution entrains the lead sulfide.

Holding the tube horizontally, gently heat the end containing the wire, but avoid heating the plug of gold leaf. Examine the gold leaf frequently during the heating, preferably with a hand lens. If mercury be present, it will form a silvery patch of amalgam on the end of the gold plug toward the wire. Vogel and Lee recommend that in important cases the tube and its contents be kept as a permanent record after sealing the open end.

For confirmation the gold plug may be removed and suspended in a test tube containing a crystal of iodine which is gently heated. The silvery deposit of mercury is changed to red iodide of mercury.

Morphine.—Add sufficient ammonia to the urine to render it distinctly ammoniacal, and shake thoroughly with a considerable quantity of ether. Separate the ether and evaporate to dryness. To a little of the residue in a watch glass or porcelain dish add a few drops of formaldehyde-sulfuric acid, which has been freshly prepared by adding 1 drop of formalin to 1 c.c. pure concentrated sulfuric acid. If morphine be present, this will produce a purplish-red color, which changes to violet, blue violet, and finally nearly pure blue.

Phenol.—As has been stated, the urine following phenol poisoning turns olive green and then brownish black upon standing. Tests are of value in recognizing poisoning from ingestion and in detecting absorption from carbolized dressings.

The urine is strongly acidified with hydrochloric acid and distilled. To the first few cubic centimeters of distillate is added 10 per cent solution of ferric chloride, drop by drop. The presence of phenol causes a deep amethyst-blue color, as in Uffelmann's test for lactic acid (p. 453).

Phenolphthalein, which is now widely used as a cathartic, gives a bright pink color when the urine is rendered alkaline.

Quinine.—A considerable quantity of the urine is rendered alkaline with ammonia and extracted with ether; the ether is evaporated, and a portion of the residue dissolved in about 20 drops of dilute alcohol. The alcoholic solution is acidulated with dilute sulfuric acid, 1 drop of an alcoholic solution of iodine (tincture of iodine diluted about ten times) is added, and the mixture is warmed. Upon cooling, an iodine compound of quinine (herapathite) will separate out in the form of a microcrystalline sediment of green plates.

The remainder of the residue may be dissolved in a little dilute sulfuric acid. This solution will show a characteristic blue fluorescence when quinine is present.

Resinous drugs cause a white precipitate like that of albumin when strong nitric acid is added to the urine. This is dissolved by alcohol.

Salicylates, salol, aspirin, and similar drugs give a bluish-violet color upon addition of a few drops of 10 per cent ferric chloride solution to the urine, which must previously be boiled to drive off any diacetic acid that may be present. When the quantity of salicylates is small, the urine may be acidified with hydrochloric acid and extracted with ether, the ether evaporated, and the test applied to an aqueous solution of the residue.

Tannin and its compounds appear in the urine as gallic acid, and the urine becomes greenish black (inky if much gallic acid be present) when treated with a solution of ferric chloride.

III. MICROSCOPIC EXAMINATION

A careful microscopic study should be a part of every routine urine examination. It will often reveal structures of diagnostic importance in urine which seems perfectly clear, and from which only very slight sediment can be obtained with the centrifuge. Upon the other hand, cloudy urines with abundant sediment are often shown by the microscope to contain no structures of clinical significance.

Since the nature of the sediment soon changes, the urine must be examined while fresh, preferably within six hours after it is voided. If it must be kept for a much longer period some preservative should be added, preferably 4 drops of formalin, or 5 grains of boric acid, or 1 c.c. of toluene for each 4 ounces of urine. When possible it should be placed on ice. The sediment is best obtained by means of the centrifuge. If a centrifuge is not available, the urine may be allowed to stand in a conical test glass for six to twenty-four hours.

A small amount of the sediment should be transferred to a slide by means of a pipet. It is very important to do this properly. The best pipet is a simple glass tube, 7 or 8 inches long, which has been drawn out at one end to a tip with a 1 or 1.5 mm. opening. The centrifuge tube containing the sediment is held on a level with the eye, the larger end of the pipet is closed with the index finger, which must be dry, and the tip is carried down into the sediment. By carefully loosening the finger, but not entirely removing it, a small amount of the sediment is then allowed to run slowly into the pipet. Slightly rotating the pipet will aid in accomplishing this, and at the same time will serve to loosen any structures which cling to the bottom of the tube. After wiping off the urine which adheres to the outside, a drop from the pipet is placed upon a clean slide. A hair is then placed in the drop and a large cover glass applied. The correct size of the drop can be learned only by experience. It should not be so large as to float the cover glass about, or so small as to leave unoccupied

space beneath the cover. Many workers use no cover. This offers a thicker layer and larger area of urine, the chance of finding scanty structures being proportionately increased. It has the disadvantage that any jarring of the room (as by persons walking about) sets the microscopic field into vibratory motion and makes it impossible to see anything clearly; and, since it does not allow satisfactory use of high-power objectives, one cannot examine details as carefully as one often wishes to do. It is true that a cover can be applied later, but any structure which one has found with the low power and wishes to study with the high is sure to be lost when the cover is applied. A cover glass (about 22 mm. square) with a hair beneath it avoids these disadvantages, and gives enough urine to find any structures which are present in sufficient number to have clinical significance, provided other points in the technic have been right. It is best, however, to examine several drops, and, when the sediment is abundant, drops from the upper and lower portions should be examined separately. The hair is dispensed with in routine work, but its use should be required in class exercises, since it is a useful aid in teaching the correct thickness of the layer of urine, and the hair serves as a conspicuous object upon which to focus when few structures are to be found.

In examining urinary sediments microscopically no fault is so common or so fatal to good results as improper illumination (Fig. 6, p. 5), and none is so easily corrected. The light should be central and very subdued for ordinary work, but oblique illumination, obtained by swinging the mirror a little out of the optical axis, will be found helpful in identifying certain delicate structures like hyaline casts. The 16-mm. objective should be used as a finder, while the 4-mm. is reserved for examining details. An experienced worker will rely almost wholly upon the lower power.

It is well to emphasize that *the most common errors which result in failure to find important structures, when present, are, (a) lack of care in transferring the sediment to the slide; (b) too strong illumination, and (c) too great magnification.*

In order to distinguish between similar structures it is often necessary to watch the effect upon them of certain reagents. This is especially true of the various unorganized sediments. They very frequently cannot be identified from their form alone. With the structures still in focus, a drop of the reagent may be placed at one edge of the cover glass and drawn underneath it by the suction of a piece of blotting paper touched to the opposite edge; or, better, a small drop of the reagent and of the urine may be placed close together upon a

slide and a cover gently lowered over them. As the two fluids mingle, the effect upon various structures may be seen.

A common error is the attempt to identify objects in urine which has dried upon the slide. Satisfactory examination is impossible under such conditions. Not only are the delicate organized structures distorted beyond recognition, but there is a confusing deposit of urinary salts. After a little experience one recognizes at a glance from the peculiar refraction of the structures seen that the urine has dried.

The record of the microscopic examination should not merely state that particular structures are present, but should give an approximate idea of their number. The best plan is to record the average number seen in a field of the low-power objective, although the number will vary greatly with the thoroughness of centrifugation, and especially with the care with which the sediment is transferred to the slide. The approximate amount of sediment in the centrifuge tube should also be recorded.

Urinary sediments may be studied under three heads: A. Unorganized sediments. B. Organized sediments. C. Extraneous structures.

A. Unorganized Sediments

In general, these have little diagnostic or prognostic significance. Most of them are substances normally present in solution, which have been precipitated either because present in excessive amounts, or, more frequently, because of some alteration in the urine (as in reaction, concentration, and so forth) which may be purely physiologic, depending upon changes in diet or habits. Various substances are always precipitated during decomposition, which may take place either within or without the body.

Unorganized sediments may be classified according to the reaction of the urine in which they are *most likely* to be found. This classification is useful, but is not accurate, since the characteristic sediments of acid urine may remain after the urine has become alkaline, while the alkaline sediments may be precipitated in a urine which is still acid.

In Acid Urine.—Uric acid, amorphous urates, sodium urate, calcium oxalate, leucine and tyrosine, cystine, and fat globules may be found. Uric acid, the urates, and calcium oxalate are the common deposits of acid urines; the others are less frequent, and depend less upon the reaction of the urine.

In Alkaline Urine.—Phosphates, calcium carbonate, and ammonium biurate may be deposited.

Other crystalline sediments (Fig. 45) which are rare and require

no further mention are: Calcium sulfate, cholesterol, hippuric acid, hematoidin, fatty acids, and indigo.

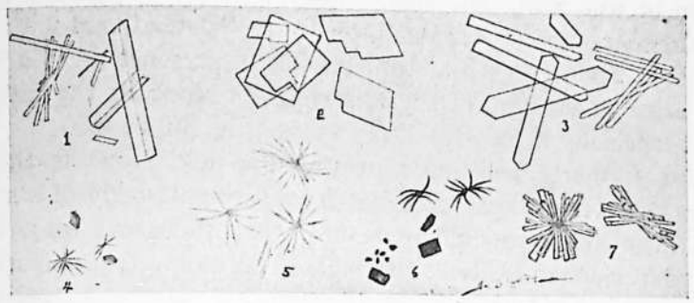


Fig. 45.—Unusual urinary crystals (drawn from various authors): 1, Calcium sulfate (colorless); 2, cholesterol (colorless); 3, hippuric acid (colorless); 4, hematoidin (brown); 5, fatty acids (colorless); 6, indigo (blue); 7, sodium urate (yellowish).

The following brief table will aid the student in identifying the chemical sediments which one meets every day:

	In acid urine.	In alkaline urine.
Yellow crystals.....	Uric acid — dissolve in NaOH.	Ammonium biurate—dissolve in HCl.
Colorless crystals.....	Calcium oxalate — dissolve in HCl.	Phosphate crystals — dissolve in acetic acid.
Amorphous material.....	Urates — dissolve with heat.	Amorphous phosphates—dissolve in acetic acid.

1. In Acid Urine.—(1) **Uric-acid Crystals.**—These crystals are the red grains—"gravel" or "red sand"—which are often seen adhering to the sides and bottom of a vessel containing urine. Microscopically, they are yellow or reddish-brown crystals, which differ greatly in size and shape. The color is due to urinary pigments, chiefly uro-erythrine. The most characteristic forms (Plate III and Fig. 46) are "whetstones"; roset-like clusters of prisms and whetstones; and rhombic plates, which have usually a paler color than the other forms and are sometimes colorless. A very rare form is a colorless hexagonal plate resembling cystine. Recognition of the crystals depends less upon their shape than upon their color, the reaction of the urine, and the facts that they are soluble in sodium hydroxide solution and insoluble in hydrochloric or acetic acid. When ammonia is added, they dissolve and crystals of ammonium urate appear.

A deposit of uric acid crystals has no significance unless it occurs before or very soon after the urine is voided. Every urine, if kept

acid, will in time deposit its uric acid. Factors which favor an early deposit are high acidity, diminished urinary pigments, and excessive excretion of uric acid. The chief clinical interest of the crystals lies in their tendency to form calculi, owing to the readiness with which they collect about any solid object. Their presence in the freshly voided urine in clusters of crystals suggests stone in the kidney or bladder, especially if blood is also present (Fig. 86, p. 185).

It was formerly believed that the uric acid stone is the most common form of renal calculus, but from a recent study of a series of calculi Kahn and Rosenbloom believe that the great majority are composed of calcium oxalate, although all contain a trace of uric acid.

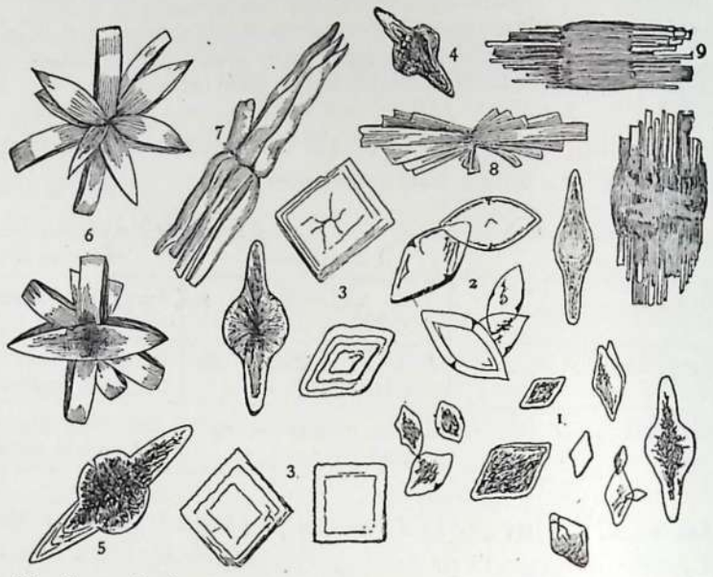


Fig. 46.—Forms of uric acid: 1, Rhombic plates; 2, "whetstone" forms; 3, 3, quadrate forms; 4, 5, prolonged into points; 6 8, rosets; 7, pointed bundles; 9, barrel forms precipitated by adding hydrochloric acid to urine. (Ogden.)

(2) **Amorphous Urates.**—These are chiefly urates of sodium and potassium which are thrown out of solution as a yellow or red "brick-dust" deposit. In pale urines this sediment is almost white. It disappears upon heating. A deposit of amorphous urates is very common in concentrated and strongly acid urines, especially in cold weather, and has no clinical significance. It is particularly frequent in febrile conditions. Under the microscope it appears as fine yellowish granules, sometimes almost colorless (Plate III). Often they are so abundant as to obscure all other structures. In such cases the urine should be warmed before examining. The granules have a tendency to collect upon tube casts, strands of mucus, and other structures. Amorphous

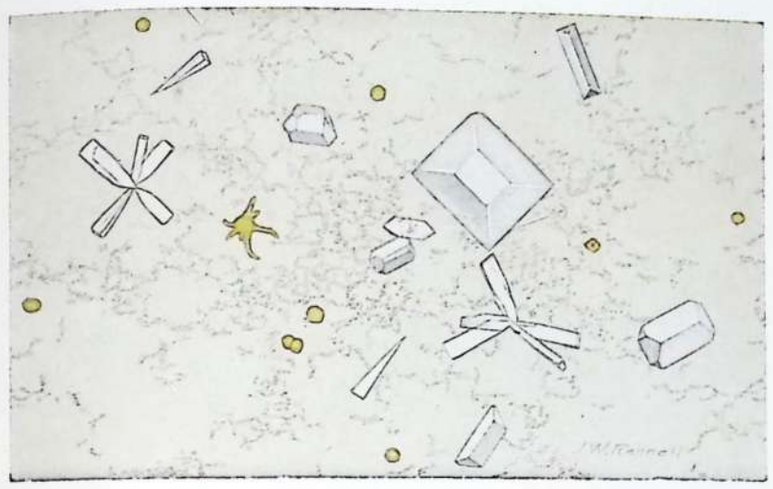


Fig. 1.—Common sediments of alkaline urine: Triple phosphate crystals, calcium phosphate crystals, ammonium urate crystals, and amorphous phosphates. (× 150.)

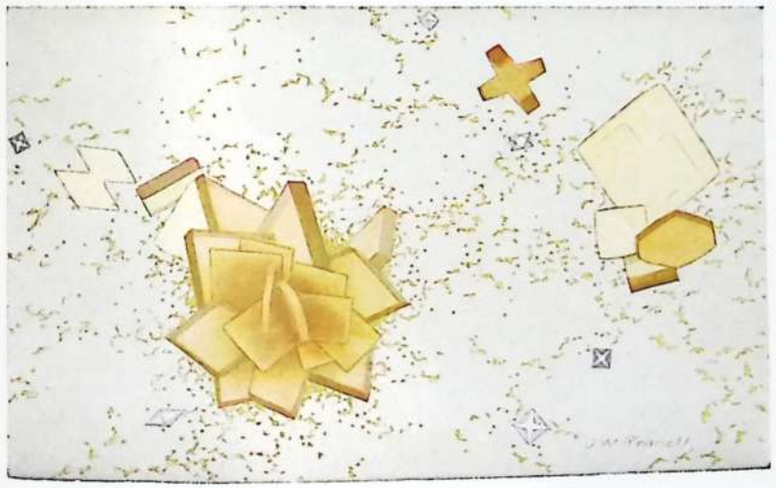


Fig. 2.—Common sediments of acid urine: Uric-acid crystals, calcium oxalate crystals, and amorphous urates. (× 150.)

urates are readily soluble in caustic soda solutions. When treated with hydrochloric or acetic acid, they slowly dissolve and rhombic crystals of uric acid appear in ten to twenty minutes.

Rarely sodium urate occurs in crystalline form—slender prisms, arranged in fan- or sheaf-like structures (Fig. 45).

(3) **Calcium Oxalate.**—Characteristic of calcium oxalate are colorless, glistening, octahedral crystals, giving the appearance of small squares crossed by two intersecting diagonal lines—the so-called "envelope crystals" (Fig. 47). They vary greatly in size, being sometimes so small as to seem mere points of light with medium-power objectives. Unusual forms are colorless dumb-bells, spheres, and variations of the octahedra (Fig. 47). The spheres might be mistaken

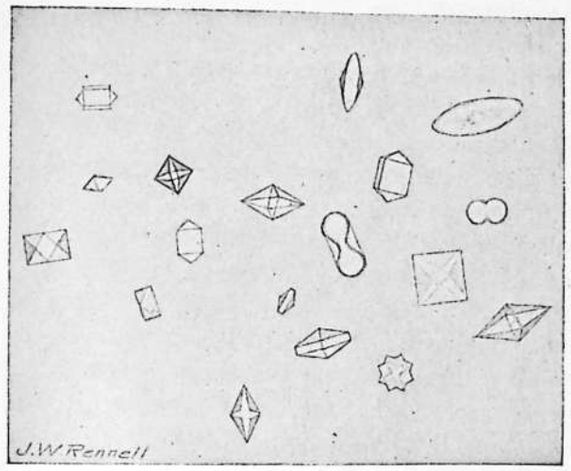


Fig. 47.—Various forms of calcium oxalate crystals from urine. The majority are the typical octahedra seen in different positions (× 450).

for globules of fat or red blood corpuscles. Crystals of calcium oxalate are insoluble in acetic acid or caustic soda. They are dissolved by strong hydrochloric acid, and recrystallize as octahedra upon addition of ammonia. They are sometimes encountered in alkaline urine.

The crystals are commonly found in the urine after ingestion of vegetables rich in oxalic acid, as tomatoes, spinach, asparagus, and rhubarb. They have no definite significance pathologically. They often appear in digestive disturbances with fermentation of carbohydrates, in neurasthenia, and when the oxidizing power of the system is diminished. When abundant they are generally associated with a little mucus; and, in men, frequently with a few spermatozoa. Their chief clinical interest lies in their tendency to form calculi, and their presence in fresh urine, together with evidences of renal or cystic irrita-

tion, should be viewed with suspicion, particularly if they are clumped in small masses.

Sulkowitch Test.—A very simple test for the amount of calcium excreted in the urine has been devised by Sulkowitch.[1]

Reagent.—The only reagent required is oxalic acid, 2.5 Gm.; ammonium oxalate, 2.5 Gm.; glacial acetic acid, 5 c.c. Make up to 150 c.c. with distilled water.

Method.—The patient should be kept on a neutral, low-calcium diet before the test is begun. Save a twenty-four-hour specimen of urine. Take equal parts of the Sulkowitch reagent and urine, mix thoroughly and allow to stand for two to three minutes. The calcium in the urine will come down almost immediately as a fine, white precipitate of calcium oxalate. If there is no precipitate, there is no calcium, and the serum calcium level is probably from 5 to 7.5 mg. per 100 c.c. If there is a fine, white cloud, there is a moderate amount of calcium and the level of calcium in the serum is in the normal range. If the precipitate is like milk, the danger of hypercalcemia is present. The amount of precipitate may be graded much as precipitate of albumin, 1, 2, 3 and 4. This is a rough quantitative test for the amount of calcium that is excreted in cases of hypoparathyroidism or urinary calculi.

(4) **Leucine and Tyrosine.**—These substances are cleavage products of the protein molecule. They are of comparatively rare occurrence in the urine and generally appear together. In general, their presence indicates autolysis of tissue proteins. Clinically, they are seen most frequently in severe fatty destruction of the liver, such as occurs in acute yellow atrophy and phosphorus poisoning. Crystals are deposited spontaneously only when the substances are present in large amount. Usually they will be deposited when the urine is evaporated to a small volume on a water bath. It is best, however, to separate them from the urine as follows:

Treat 500 to 1000 c.c. of urine, which has been freed from albumin, with neutral, then with basic, lead acetate until a precipitate no longer forms. Filter, precipitate excess of lead with hydrogen sulfide, and filter again. Concentrate to a syrup on a water bath. Extract repeatedly with small quantities of absolute alcohol to remove urea. Treat the residue with hot dilute alcohol to which a little ammonia has been added. Filter and evaporate the filtrate to a small volume and let stand for the leucine

[1] Albright, Fuller: Note on the Management of Hypoparathyroidism with Dihydrotachysterol. J. A. M. A., *112*:2592–2593 (June 24), 1939. Barney, J. D. and Sulkowitch, H. W.: Progress in the Management of Urinary Calculi. J. Urol., *37*:746–762 (June), 1937.

and tyrosine to separate out. The leucine can be separated from the tyrosine by boiling with glacial acetic acid. Leucine dissolves, leaving the tyrosine, and can again be recovered by evaporating the acetic acid.

The crystals cannot be identified from their morphology alone, since other substances, notably calcium phosphate (Fig. 54, p. 138) and ammonium biurate, may take similar or identical forms. It is, therefore, necessary to try out their solubility in various reagents or to apply special tests.

Leucine crystals (Fig. 48) as they appear in the urine do not represent the pure substance. They are slightly yellow, oily looking spheres, many of them with radial and concentric striations. Some may be merged together in clusters. They are not soluble in hydrochloric acid or in ether.

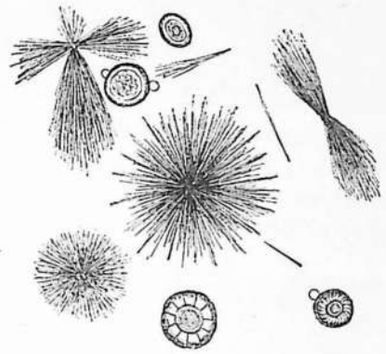


Fig. 48.—Leucine spheres and tyrosine needles (Stengel).

Tyrosine crystallizes in very fine needles, which may appear black and which are usually arranged in sheaves, with a marked constriction at the middle (Fig. 49). It is soluble in ammonia and hydrochloric acid, but not in acetic acid.

Mörner's Test for Tyrosine.—To a small quantity of the crystals in a test tube add a few cubic centimeters of Mörner's reagent (formalin, 1 c.c.; distilled water, 45 c.c.; concentrated sulfuric acid, 55 c.c.). Heat gently to the boiling point. A green color shows the presence of tyrosine.

(5) **Cystine crystals** are colorless, highly refractive, rather thick, hexagonal plates with well-defined edges. They lie either singly or superimposed to form more or less irregular clusters (Fig. 50). Uric acid sometimes takes this form and must be excluded. Cystine is soluble in hydrochloric acid, insoluble in acetic; it is readily soluble in ammonia and recrystallizes upon addition of acetic acid.

Cystine is one of the amino-acids formed in decomposition of the protein molecule, and is present in traces in normal urine. Crystals are deposited only when the substance is present in excessive amount.

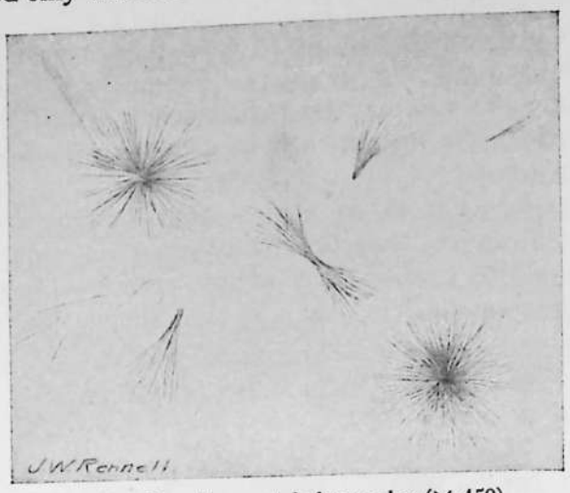


Fig. 49.—Tyrosine crystals from urine (× 450).

Their presence is known as *cystinuria*. It is a rare condition, due to an obscure abnormality of protein metabolism and usually continues

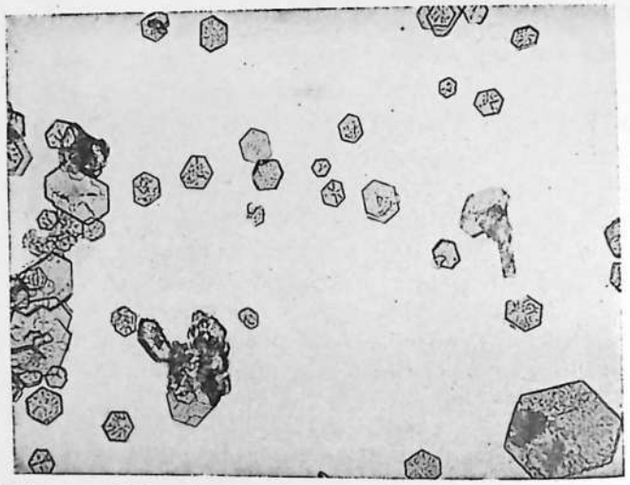


Fig. 50.—Cystine crystals from urine of patient with cystine calculus. The crystals are colorless, with a luster suggesting mother of pearl (photograph; × 200).

throughout life. The amount of cystine can be greatly diminished by a low-protein diet, and the formation of crystals can in some measure be prevented by administration of sodium carbonate (Smillie). There

are rarely any symptoms save those referable to renal or cystic calculus, to which the condition strongly predisposes.

(6) **Sulfanilamide Derivatives.**—Sulfanilamide derivatives may produce typical urinary crystals in acid urine. These are illustrated diagrammatically in Fig. 51.[1]

(7) **Fat Globules.**—Fat appears in the urine as highly refractive globules of various sizes, frequently very small. These globules are easily recognized from the fact that they are stained black by osmic

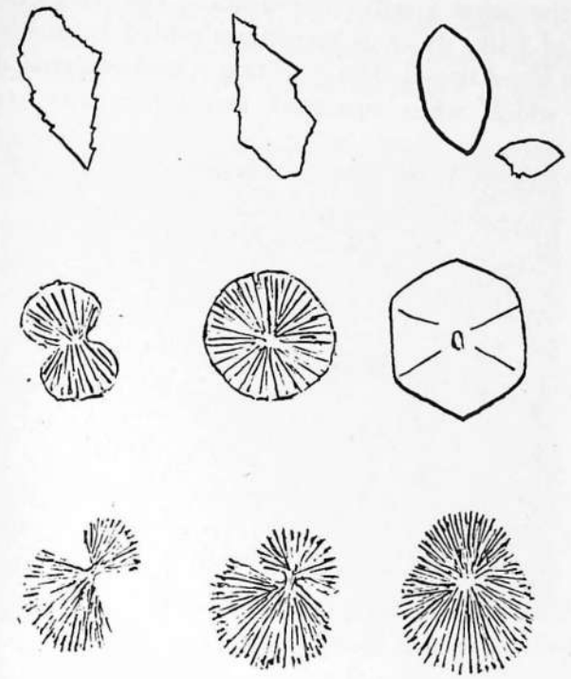


Fig. 51.—Crystals appearing in human urine after administration of: *Sulfapyridine*—arrowheads and whetstones. *Sulfathiazole*—striated dumb-bells (shocks of wheat with central binding), rosettes with radial striations and regular hexagonal platelets (all structures *symmetrical*). *Sulfadiazine*—striated dumb-bells (shocks of wheat with eccentric binding) and cell forms with radial striations (all structures *asymmetrical*) (× 250). (Lehr and Antopol, Science, Vol. 94.)

acid and orange or red by sudan III or scharlach R. The stain may be applied upon the slide, as already described (p. 127). Osmic acid should be used in 1 per cent aqueous solution; formulae for sudan III and scharlach R are given on page 833.

Fat in the urine is usually a contamination from unclean vessels, oiled catheters, or similar sources. A very small amount may be pres-

[1] Lehr, David and Antopol, William: Typical Urinary Crystals of Three Sulfanilamide Derivatives Produced in Vitro. Science, *94*:282–283 (Sept. 19), 1941.

ent after ingestion of large quantities of cod liver oil or other fats. In fatty degeneration of the kidney, as in phosphorus poisoning and chronic parenchymatous nephritis, fat globules are commonly seen, both free in the urine and embedded in cells and tube casts. Fat droplets are common in pus corpuscles and in degenerating cells of any kind.

In *chyluria*, or admixture of chyle with the urine as a result of rupture of a lymph vessel, minute droplets of fat are so numerous as to give the urine a milky appearance. The droplets are smaller than those of milk, which is sometimes added by malingerers. The fluid is often blood tinged. The condition is best recognized by shaking with ether, which, when separated, leaves the urine comparatively

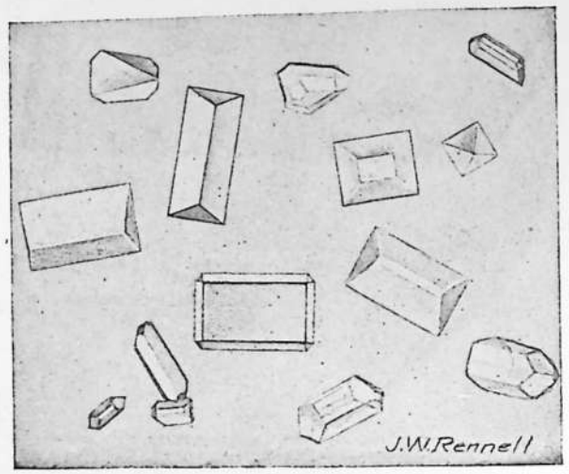


Fig. 52.—Prismatic forms of triple phosphate crystals from urine (× 450).

clear. If, then, the ether be evaporated, a fatty residue remains. Chyluria occurs most frequently as a symptom of infection by filaria (p. 565), the larvae of which can usually be found in the milky urine. In other cases the etiology is obscure.

2. In Alkaline Urine.—(1) Phosphates.—While most common in alkaline urine, phosphates are sometimes deposited in amphoteric or feebly acid urines. The usual forms are: (*a*) Ammoniomagnesium phosphate crystals; (*b*) dicalcium phosphate crystals, and (*c*) amorphous phosphates. All are readily soluble in acetic acid.

(*a*) *Ammoniomagnesium Phosphate Crystals.*—They are the common "triple phosphate" crystals, which are generally easily recognized (Figs. 52, 53, and 87, and Plate III). They are colorless except when bile stained. Their usual form is some modification of the prism, with

oblique ends. Most typical are the well-known "coffin-lid" and "hip-roof" forms. The long axis of the hip-roof crystal is often so shortened that it resembles the envelope crystal of calcium oxalate. It does not, however, have the same luster; this, and its solubility in acetic acid, will always prevent confusion.

When rapidly deposited, as by artificial precipitation, triple phosphate often takes feathery, star-, or leaflike forms (Fig. 53). These gradually develop into the more common prisms. **X**-forms may be produced by partial solution of prisms.

(*b*) *Dicalcium Phosphate Crystals.*—In feebly acid, amphoteric, or feebly alkaline urines dicalcium phosphate is not infrequently deposited in the form of colorless prisms arranged in stars and rosets

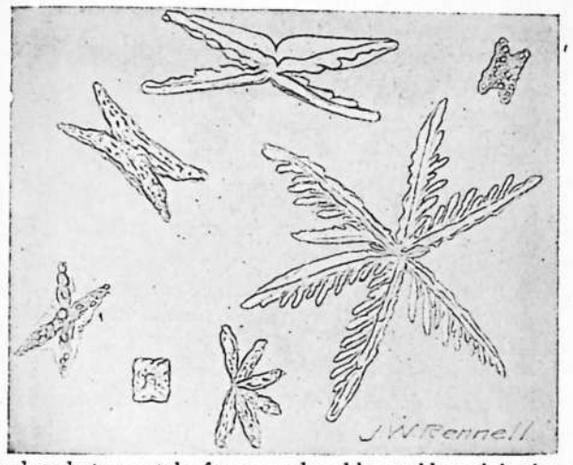


Fig. 53.—Triple phosphate crystals; forms produced by rapid precipitation and by partial solution of prisms (× 450).

(Fig. 54, 1). Because of the shape of the crystals it is sometimes called "stellar phosphate." The individual prisms are usually slender, with one beveled, wedgelike end, but are sometimes needle-like. They may sometimes take forms resembling tyrosine (Fig. 54, 2), calcium sulfate, or hippuric acid, but are readily distinguished by their solubility in acetic acid.

Calcium phosphate often forms large, thin, irregular, usually granular, colorless plates (Fig. 54, 3) which should be easily recognized, although small plates might be mistaken for squamous epithelial cells. These crystals most frequently form a scum upon the surface of the urine. They are regarded by some as magnesium phosphate.

(*c*) *Amorphous Phosphates.*—The earthy phosphates are thrown out of solution in most alkaline and many amphoteric urines as a

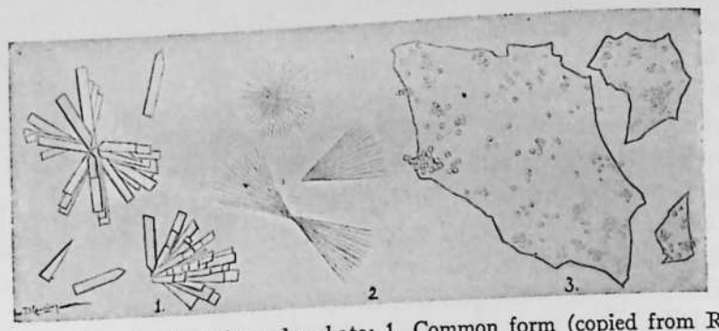


Fig. 54.—Crystals of calcium phosphate: 1, Common form (copied from Rieder's Atlas); 2, needles resembling tyrosine (drawn from nature); 3, large, irregular plates (from nature).

white, amorphous sediment, which may be mistaken for pus macroscopically. Under the microscope the sediment is seen to consist of

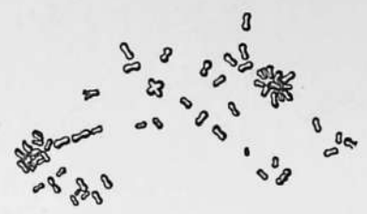


Fig. 55.—Indistinct crystalline sediment (dumb-bell crystals) of calcium carbonate. Similar crystals are sometimes formed by calcium oxalate and calcium sulfate (after Funke).

numerous colorless granules, distinguished from amorphous urates by their color, their solubility in acetic acid, and the reaction of the urine.

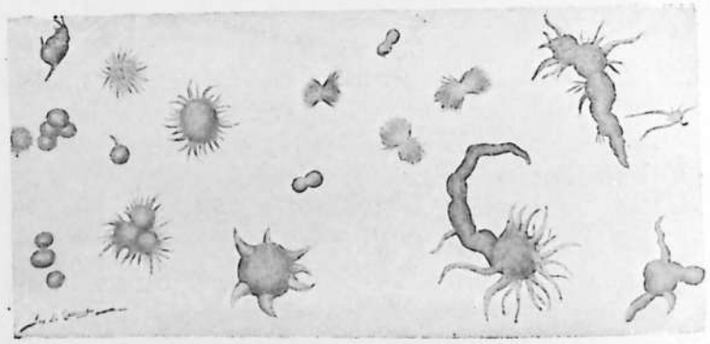


Fig. 56.—Crystals of ammonium biurate (one half of the forms copied from Rieder's Atlas; the others, from nature).

The various phosphatic deposits frequently occur together. They are sometimes due to excessive excretion of phosphoric acid, but

usually merely indicate that the urine has become, or is becoming, alkaline (Phosphates, p. 75).

(2) **Calcium carbonate** may sometimes be mingled with the phosphatic deposits, usually as amorphous granules, or, more rarely, as colorless spheres and dumb-bells (Fig. 55), which are soluble in acetic acid with gas formation.

(3) **Ammonium Biurate Crystals.**—This is the only urate deposited in alkaline urine. It forms opaque yellow crystals, usually in the form of spheres (Plate III and Fig. 87), which are often covered with fine or coarse spicules–"thorn-apple crystals." Sometimes dumb-bells, compact sheaves of fine needles, and irregular rhizome forms are seen (Fig. 56). Upon addition of acetic acid they dissolve, and rhombic plates of uric acid appear.

These crystals occur only when free ammonia is present. They are generally found along with the phosphates in decomposing urine and have no clinical significance.

B. Organized Sediments

The principal organized structures in urinary sediments are: Tube casts; epithelial cells; pus corpuscles; red blood corpuscles; spermatozoa; bacteria, and animal parasites. They are much more important than the unorganized sediments just considered.

1. Tube Casts.—These interesting structures are albuminous casts of the uriniferous tubules. Their presence in the urine (known as *cylindruria*) probably always indicates some pathologic change in the kidney, although this change may be very slight or transitory. Large numbers may be present in temporary irritations and congestions. *They do not in themselves, therefore, imply organic disease of the kidney.* They rarely occur in urine which does not contain, or has not recently contained, albumin, and in a general way have the same clinical significance as renal albuminuria.

While it is not possible to draw a sharp dividing line between the different varieties, casts may be classified as follows:

(1) Hyaline casts:
  (*a*) Narrow.
  (*b*) Broad.
(2) Waxy casts.
(3) Fibrinous casts.
(4) Granular casts:
  (*a*) Finely granular.
  (*b*) Coarsely granular.
(5) Fatty casts.

(6) Casts containing organized structures:
  (*a*) Epithelial casts.
  (*b*) Blood casts.
  (*c*) Pus casts.
  (*d*) Bacterial casts.

As will be seen later, practically all varieties are modifications of the hyaline. Not infrequently two varieties are included in the same cast.

The significance of the different varieties is more readily understood if one considers their mode of formation. Albuminous material, the source and nature of which are not definitely known but which are doubtless not the same in all cases, probably enters the lumen of a uriniferous tubule in a fluid or plastic state. The material has been variously thought to be an exudate from the blood, a pathologic secretion of the renal cells, and a product of epithelial degeneration. In the tubule it hardens into a cast which, when washed out by the urine, retains the shape of the tubule, and contains within its substance whatever structures and débris were lying free within the tubule or were loosely attached to its wall. If the tubule be small and has its usual lining of epithelium, the cast will be narrow; if it be large or entirely denuded of epithelium, the cast will be broad. *A cast, therefore, indicates the condition of the tubule in which it is formed, but does not necessarily indicate the condition of the kidney as a whole.* In any particular case of kidney disease several forms or even all may be found. Their number and the preponderance of certain forms will, as is shown later, furnish a clue to the nature of the pathologic process, but further than this one cannot go with certainty. One cannot rely upon the casts for accurate diagnosis of the histologic changes in the kidney.

At times during the course of a nephritis the urine is suddenly flooded with great numbers of tube casts. Such "showers" may be of serious import, but are not necessarily so. In some cases they may result from a clearing out of the plugged renal tubules coincident with improvement and increased flow of urine.

The search for casts must be carefully made. The urine must be fresh, since hyaline casts soon dissolve when it becomes alkaline. It should be thoroughly centrifugalized. When the sediment is abundant, casts, being light structures, will be found near the top of the deposit. In cystitis, where casts may be entirely hidden by the pus, the bladder should be irrigated to remove as much of the pus as possible and the next urine examined. In order to prevent solution of the casts the urine, if alkaline, must be rendered acid by previous

administration of boric acid or other drugs. Heavy sediments of urates, blood, or vaginal cells may likewise obscure casts and other important structures. The last can be avoided by catheterization. Urates can be dissolved by gently warming before centrifugalizing, care being taken not to heat enough to coagulate the albumin. The aluminum shield of the centrifuge tube may also be heated. Blood can be destroyed by centrifugalizing, pouring off the supernatant urine, filling the tube with water, adding a few drops of dilute acetic acid, mixing well, and again centrifugalizing; this process being repeated until the blood is completely decolorized. Too much acetic acid will dissolve hyaline casts.

In searching for casts the low-power objective should invariably be used, although a higher power may occasionally be desirable in studying details, as, for example, in distinguishing between an epithelial and a pus cast. The casts are perhaps most frequently found near the edge of the cover glass. Their cylindric shape can best be seen by slightly moving the cover glass while observing them, or by pressing upon one edge of the cover with a needle, thus causing them to roll. This little manipulation should be practiced until it can be done satisfactorily. It will prove useful in many examinations.

Various methods of staining casts so as to render them more conspicuous have been proposed. They offer no special advantage to one who understands how to use the substage mechanism of his microscope. The "negative-staining" method is as good as any. It consists simply in adding a little India-ink to the drop of urine on the slide. Casts, cells, and other substances will stand out as colorless structures on a dark background. Some workers tinge the urine faintly with eosin or Lugol's solution, which is taken up by the casts.

(1) **Hyaline Casts.**—Typically, these are colorless, homogeneous, semitransparent, cylindric structures, with parallel sides and usually rounded ends. Not infrequently they are more opaque or show a few granules or an occasional cell or oil globule, either adhering to them or contained within their substance. Generally they are straight or curved, less commonly convoluted (Fig. 67, p. 147). Their length and breadth vary greatly; they are sometimes so long as to extend across several fields of a medium-power objective, but are usually much shorter; in breadth they vary from one to seven or eight times the diameter of a red blood corpuscle (Figs. 6, 57, and 58).

Hyaline casts are the least significant of all the casts, and occur, usually in company with pale very finely granular casts, in many slight and transitory conditions. Small numbers are common following ether anesthesia, in fevers, after excessive exercise, and in con-

gestions and irritations of the kidney. They are always present, and are usually stained yellow when the urine contains much bile. While they are found in all organic diseases of the kidney, they are most important in chronic interstitial nephritis. Here they are seldom abundant, but their persistent presence is a significant sign of the

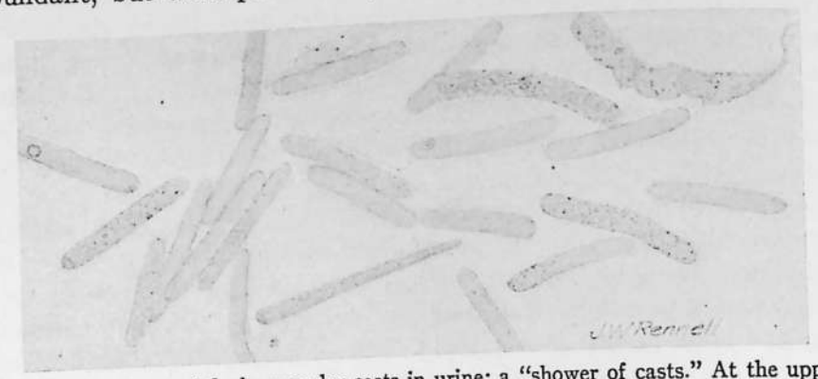


Fig. 57.—Hyaline and finely granular casts in urine; a "shower of casts." At the upper right is a mucous shred. A portion of an actual field (× 100).

disease. Small areas of chronic interstitial change are probably responsible for the few hyaline casts so frequently found in the urine of elderly persons.

Very broad hyaline casts commonly indicate complete desquamation of the tubular epithelium, such as occurs in the late stages of nephritis; or they may originate in relatively normal collecting tubules.

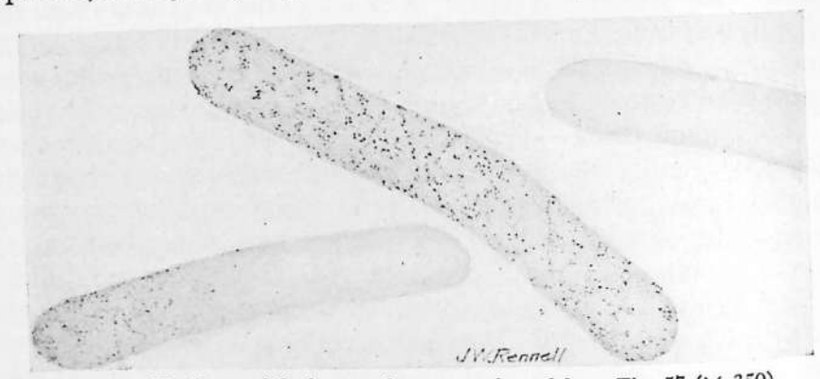


Fig. 58.—Hyaline and finely granular casts enlarged from Fig. 57 (× 350).

(2) **Waxy Casts.**—Like hyaline casts, these are homogeneous when typical, but frequently contain a few granules or an occasional cell. They are much more opaque than the hyaline variety, and are usually shorter and broader, with irregular, broken ends, and sometimes appear to be segmented. They are grayish or colorless, and have a

dull, waxy look, as if cut from paraffin (Figs. 59 and 85, p. 178). They are sometimes composed of material which gives the amyloid reactions. All gradations between hyaline and waxy casts may be found, and doubtless many waxy casts are merely hyaline casts which have lain in the kidney tubules for a long time.

Waxy casts are found in most advanced cases of nephritis, where they are an unfavorable sign. They are perhaps most abundant in amyloid disease of the kidney, but are not distinctive of the disease as is sometimes stated.

(3) **Fibrinous Casts.**—Casts which resemble waxy casts, but have a distinctly yellow color, as if cut from beeswax, are often seen in acute nephritis. They are called fibrinous casts, but the name is inappropriate, as they are not composed of fibrin. They are often classed with waxy casts, but should be distinguished, as their sig-

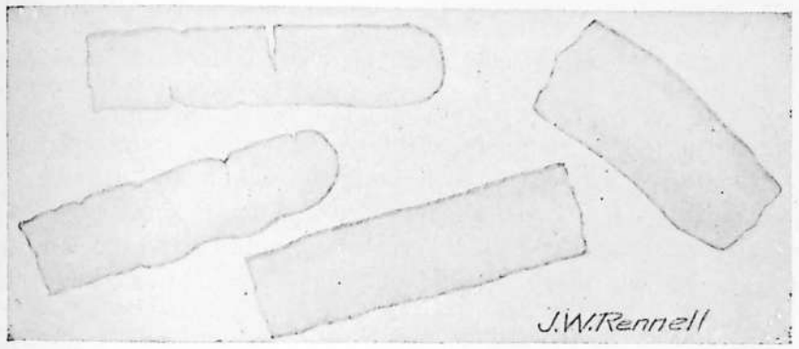


Fig. 59.—Waxy tube casts (× 350).

nificance is much less serious. Their color is probably due to altered blood pigment.

(4) **Granular Casts.**—These are merely hyaline casts in which numerous granules are embedded (Figs. 57, 58, 60, and 61).

Finely granular casts contain many fine granules, are usually shorter, broader, and more opaque than the hyaline variety, and are more conspicuous. Their color is grayish or pale yellow.

Coarsely granular casts contain larger granules and are darker in color than the finely granular, being often dark brown, owing to presence of altered blood pigment. They are usually shorter and more irregular in outline, and more frequently have irregularly broken ends.

(5) **Fatty Casts.**—Small droplets of fat may at times be seen in any variety of cast. Those in which the droplets are numerous are

called fatty casts (Figs. 61 and 85). The fat globules are not difficult to recognize. Staining with osmic acid or sudan III (p. 135) will remove any doubt as to their nature.

The granules and fat droplets seen in casts are chiefly products of epithelial degeneration. Granular and fatty casts, therefore, always

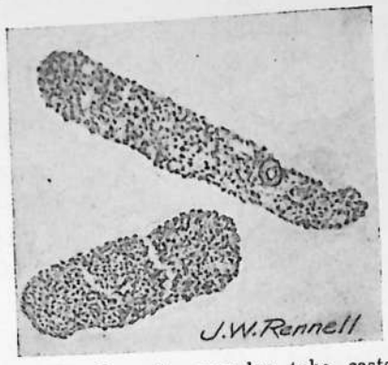


Fig. 60.—Coarsely granular tube casts (× 350).

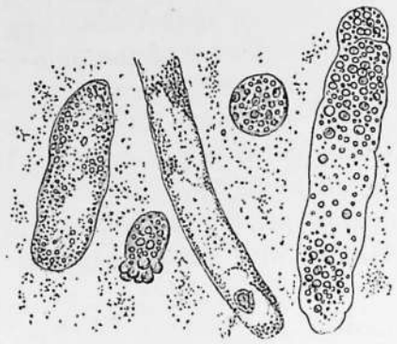


Fig. 61.—Granular and fatty casts and two compound granule cells (Stengel).

indicate partial or complete disintegration of the renal epithelium. The finely granular variety is the least significant, and may be found along with hyaline casts when the epithelium is only slightly and perhaps not seriously affected. Coarsely granular, and especially fatty casts, if present in considerable numbers, point toward a serious

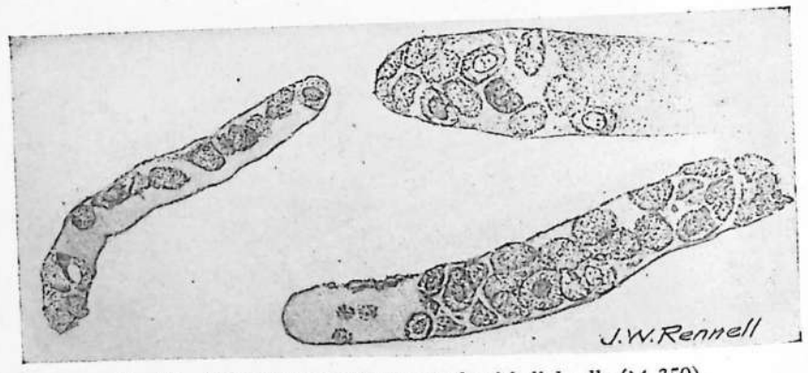


Fig. 62.—Tube casts containing renal epithelial cells (× 350).

parenchymatous nephritis. Brown granular casts are most common in acute nephritis.

(6) **Casts Containing Organized Structures.**—Cells and other structures are frequently seen adherent to a cast or embedded within it. When numerous, they give name to the cast.

(*a*) *Epithelial casts* contain epithelial cells from the renal tubules. The cells vary in size, and are often flattened, oval, or elongated. They may be recognized as epithelial cells by irrigating with dilute acetic acid, which usually brings out the nucleus clearly. Epithelial casts always imply desquamation of epithelium, which rarely occurs

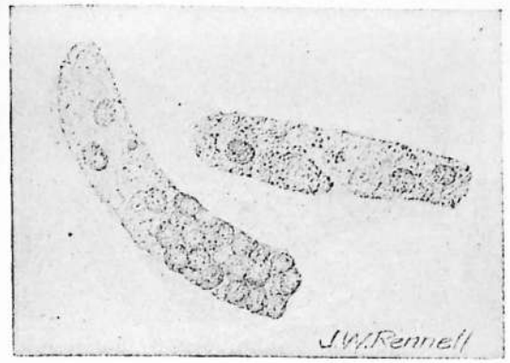


Fig. 63.—Tube casts containing pus corpuscles (× 350).

except in parenchymatous inflammations (Figs. 62, 84, and 85). When the cells are well preserved they point to acute nephritis.

(*b*) *Blood casts* contain red blood corpuscles, usually much degenerated (Figs. 64, 65, and 84). They always indicate hemorrhage into

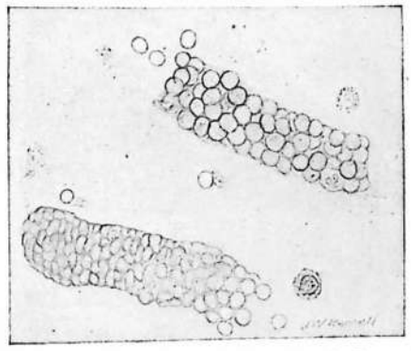


Fig. 64.—Two blood casts, one containing a leukocyte; six free red blood cells; and two renal epithelial cells. From the urine of a child with acute nephritis (× 300).

the tubules, which is most common in acute nephritis or an acute exacerbation of a chronic nephritis.

(*c*) *Pus casts* (Figs. 63 and 86), composed almost wholly of pus corpuscles, are uncommon, and point to a suppurative process in the kidney, usually a pyelonephritis. Casts containing a few pus cor-

puscles, either alone or in combination with epithelial or red blood cells, are common, especially in acute nephritis. In these the pus cells have no special significance.

(*d*) True *bacterial casts* are rare. They indicate a septic condition in the kidney. Bacteria may permeate a cast after the urine is voided.

Fig. 65.—Red blood corpuscles and blood casts (courtesy of Dr. A. Scott) (obj. 4 mm.) (Boston).

Structures Likely to Be Mistaken for Casts.—(1) **Mucous Threads.**—Mucus frequently appears in the form of long strands which slightly resemble hyaline casts (Fig. 66). They are, however, more ribbon-like, have less well-defined edges, and usually show faint longitudinal

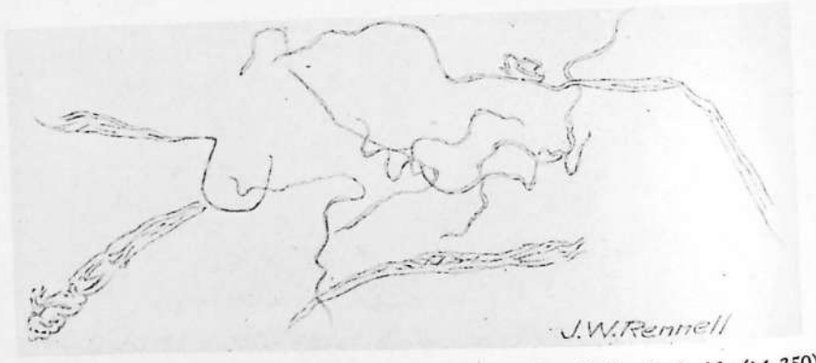


Fig. 66.—Mucous threads in urine. These are often wrongly called cylindroids (× 350).

striations. Their ends taper to a point or are split or curled upon themselves, and are never evenly rounded, as is commonly the case with hyaline casts.

Such threads form a part of the nubecula of normal urine, and are especially abundant when calcium oxalate crystals are present.

When there is an excess of mucus, as in irritations of the urinary tract, every field may be filled with an interlacing meshwork.

Mucous threads are microscopic, and should not be confused with urethral shreds or "gonorrheal threads," which are macroscopic, 0.5 to 1 cm. long, and consist of a matrix of mucus in which many epithelial and pus cells are embedded.

Fig. 67.—Four cylindroids and one convoluted hyaline cast (× 350).

(2) **Cylindroids.**—This name is sometimes given to the mucous threads just described, but is more properly applied to certain peculiar structures more nearly allied to casts. They resemble hyaline casts in structure, but differ in that they taper to a slender tail, which is

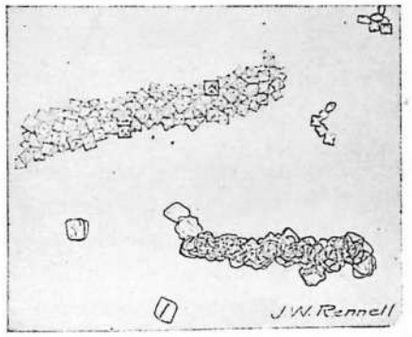


Fig. 68.—Two pseudocasts, one composed of calcium oxalate crystals, one of uric acid (× 300).

often twisted or curled upon itself (Fig. 67). They frequently occur in the urine along with hyaline casts, especially in circulatory disturbances and irritations of the kidney, and have practically the same significance.

(3) **Masses of amorphous urates, or phosphates, or very small crystals** (Fig. 68), which accidentally take a cylindric form, or shreds

of mucus covered with granules, closely resemble granular casts. The application of gentle heat or appropriate chemicals will serve to differentiate them. When urine contains both mucus and granules, large numbers of these "pseudocasts," all lying in the same direction, can be produced by slightly moving the cover glass from side to side. It is possible—as in urate infarcts of infants—for urates to be molded into cylindric bodies within the renal tubules.

(4) **Hairs** and **fibers** of wool, cotton, and so forth. These could be mistaken for casts only by beginners. One can easily become familiar with their appearance by suspending them in water and examining with the microscope (Fig. 81, p. 160).

(5) **Hyphae of molds** are not infrequently mistaken for hyaline casts. Their higher degree of refraction, their jointed or branching

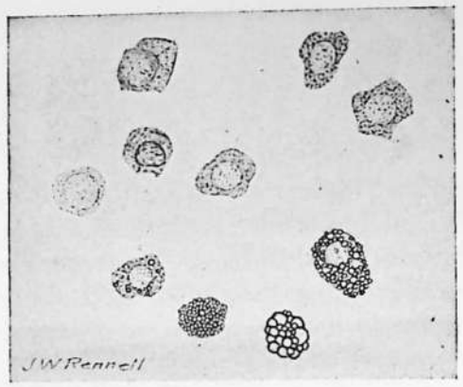


Fig. 69.—Renal epithelial cells from nephritic urine. The four cells below show different grades of fatty degeneration (× 475).

structure, and the accompanying spores will differentiate them (Fig. 82, p. 161).

2. Epithelial Cells.—A few cells from the epithelium of various parts of the urinary tract occur in every urine. A marked increase indicates some pathologic condition at the site of their origin. It is sometimes, but by no means always, possible to locate their probable source from their form, notably in the case of vaginal epithelium. One should, however, be extremely cautious about making any definite statement as to the origin of any individual cell. Most cells are much altered from their original shape, and any may be so granular from degenerative changes that the nucleus is obscured. Many of them contain fat globules. They may be divided into three groups. In reporting their presence at least the group to which they belong should be recorded.

(1) **Small round** or **polyhedral cells** are about the size of pus corpuscles, or more frequently about one third larger, with a single round nucleus. Such cells may come from the deeper layers of any part of the urinary tract. They are uncommon in normal urine. When they are polygonal in shape, rather dark in color, very granular, and contain a comparatively large nucleus (Fig. 69), they probably come from the renal tubules, but their origin in the kidney is not proved unless they are found embedded in casts. In chronic passive congestion of the kidney, in renal infarction, and in hemochromatosis some of these cells may contain yellow granules of altered blood pigment. They are analogous to the "heart-failure cells" of the sputum (p. 40). Renal cells are abundant in parenchymatous nephritis,

Fig. 70.—Caudate epithelial cells from pelvis of kidney (Jakob).

Fig. 71.—Epithelial cells from urethra and bladder: *a*, Squamous cells from superficial layers; *b*, irregular cells from deeper layers (Jakob).

especially the acute form. They are nearly always fatty—most markedly so in chronic parenchymatous nephritis, where their substance is sometimes wholly replaced by fat droplets ("compound granule cells") (Figs. 61, 69, 84, and 85).

(2) The epithelial cells of the second group are larger than the small round cells just described, being two to four times the diameter of a pus corpuscle, and have various forms (Figs. 70 and 71). Commonly they are pear-shaped, spindle-shaped, or round, or have tail-like processes; and they are hence named pyriform, spindle, large round, or caudate cells, respectively. Each contains a round or oval nucleus, which generally stands out distinctly and is smaller in proportion to the size of the cell than is the nucleus of the typical renal cell. These cells are, for the most part, derived from the transitional

epithelium which lines the bladder, ureters, and pelves of the kidneys; hence they may conveniently be grouped together as **transitional cells.** Cells of the same general type may, however, originate in the prostate and seminal vesicles, and, moreover, some of the superficial cells of the bladder are so thin and flat as to be more properly classed as squamous cells. Caudate forms apparently come most commonly from the pelvis of the kidney.

(3) **Squamous** or **pavement cells** are large flat cells, each with a small, distinct round or oval nucleus (Fig. 71, *a*). They are derived from the superficial layers of the urethra or vagina, and when desquamation is active appear in stratified masses. Squamous cells from the vagina are especially large, thin, and angular, and are sometimes rolled into cigar-like cylinders. Great numbers of these vaginal cells,

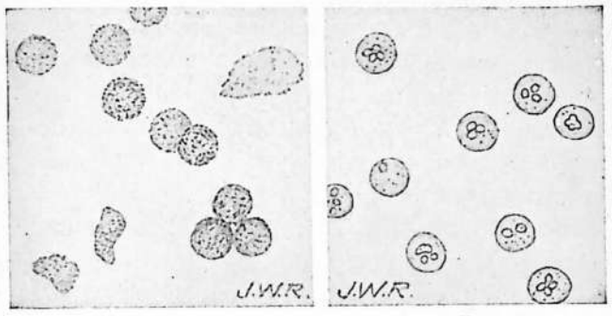
Fig. 72.—Squamous epithelial cells, pus corpuscles, and bacteria in urine; vaginal contamination (× 300).

together with pus corpuscles, may be present when leukorrhea exists (Fig. 72). The most superficial of the cells lining the bladder are also thin and scalelike and may be classed with the squamous cells. They are, however, generally less angular than are the vaginal cells.

3. Pus Corpuscles.—A very few leukocytes are present in normal urine, particularly when mucus is present. They are numerous only as a result of a pathologic process. The cells are then called pus corpuscles and their presence constitutes *pyuria*. Although pus corpuscles are less well preserved than are leukocytes and show more tendency to form small clumps, yet when only a few are present the line of distinction between them must be drawn arbitrarily and is best based upon the number present, although this depends largely upon the care used in preparing the slide. Students should be instructed

to report "a very few pus corpuscles" when they find an average of three or more of these cells to the field of the 6-mm. objective with 5× eyepiece. The great majority of pus corpuscles are the polymorphonuclear leukocytes of the blood.

When at all abundant, pus adds an appreciable amount of albumin to the urine and forms a white sediment resembling amorphous phosphates macroscopically. Under the microscope the corpuscles appear as very granular spheric cells, about 10 to 12 μ in diameter or somewhat larger than red blood corpuscles (Figs. 73 and 87, p. 186). The granules are partly the normal neutrophilic granules, partly granular products of degeneration. In freshly voided urine many exhibit ameboid motion, assuming irregular outlines. Each pus corpuscle contains one irregular nucleus or several small, rounded nuclei. The nuclei are obscured or entirely hidden by the granules, but may



A B

Fig. 73.—Pus corpuscles in urine. A, As ordinarily seen. At the lower left are two ameboid corpuscles. The large structure at the right is a bit of degenerated epithelium; B, when treated with acetic acid (× 475).

be brought clearly into view by running a little dilute acetic acid under the cover glass. This enables one to easily distinguish pus corpuscles from small, round epithelial cells, which resemble them in size, but have a single, rather large, round nucleus. In moderately acid urine the cell structure is generally fairly well preserved. In very strongly acid urine the corpuscles may be shrunken and irregularly shaped, suggesting ameboid forms. When the urine is alkaline they are usually swollen, very granular, often ragged, and have a strong tendency to adhere in clumps; while in decomposing urine they are soon destroyed and converted into a gelatinous substance which gives the urine a mucilaginous consistence.

Pyuria indicates suppuration in some part of the urinary tract—urethritis, cystitis, pyelitis, and so forth—or may be due to contamination from the vagina, in which case many vaginal epithelial

cells will also be present. Of these conditions, chronic cystitis usually gives by far the greatest amount of pus. In general, the source of the pus can be determined only by the accompanying structures (epithelium, casts) or by the clinical signs. A considerable amount of pus, appearing suddenly, usually originates from a ruptured abscess.

A fairly accurate idea of the quantity of pus from day to day may be had by shaking the urine thoroughly and counting the number of corpuscles per cubic millimeter upon the blood-counting slide, but conditions—ingestion of water, and so forth—must be kept as nearly uniform as possible. A drop of the urine is placed directly upon the slide. Dilution is not necessary unless the corpuscles exceed 20,000 for each cubic millimeter. The urine must not be alkaline or the corpuscles will adhere in clumps. In cystitis the number of corpuscles runs from about 5000 for each cubic millimeter in mild cases to 100,000 or 150,000 in severe cases.

Pus always adds a certain amount of albumin to the urine, and it is often desirable to know whether the albumin present in a given specimen is due solely to pus. It has been estimated that 80,000 to 100,000 pus corpuscles for each cubic millimeter add about 0.1 per cent of albumin. If albumin is present in much greater proportion than this, the excess is probably derived from the kidney.

4. Red Blood Corpuscles.—Urine which contains blood is always albuminous. Very small amounts do not alter its macroscopic appearance. Larger amounts alter it considerably. Blood from the kidneys is generally intimately mixed with the urine and gives it a hazy, reddish, or brown "smoky" color. When from the lower urinary tract, it is not so intimately mixed and settles more quickly to the bottom, the color is brighter, and small clots are often present. A further clue to the site of the bleeding may sometimes be gained by having the patient void his urine in three separate portions. If the blood be chiefly in the first portion, the bleeding point is probably in the urethra; if in the last, it is probably in the bladder. If the blood is uniformly mixed in all three portions, it probably comes from the kidney or ureter. Microscopically the presence of tube casts or of considerable numbers of epithelial cells of the renal type would be suggestive, while the presence of blood casts would, of course, point definitely to hemorrhage into the kidney tubules.

Red blood corpuscles usually are not difficult to recognize with the microscope. However, fat, yeasts, or oxalate crystals (see p. 159) may cause confusion, and erythrocytes should be positively identified by examining them with the high dry objective. When very fresh they have a normal appearance, being yellowish disks of uniform

size. They are apt to be swollen in dilute and crenated in concentrated urines. When they have been in the urine any considerable time their hemoglobin may be dissolved out, and they then appear as faint colorless circles or "shadow cells," and are more difficult to see (Fig. 74; also Figs. 64, 65, and 84). The shadow cells are not always uniform in size, and, while usually circular, may be oval, pear-shaped, or irregular in outline. The microscopic findings may be corroborated by chemical tests for hemoglobin, although the microscope may show a few red corpuscles when the chemical tests are negative. When the blood cells are very numerous they are often accompanied by yellowish shreds of fibrin of various sizes.

When not due to contamination from menstrual discharge, blood in the urine, or *hematuria*, is always pathologic, and usually, although

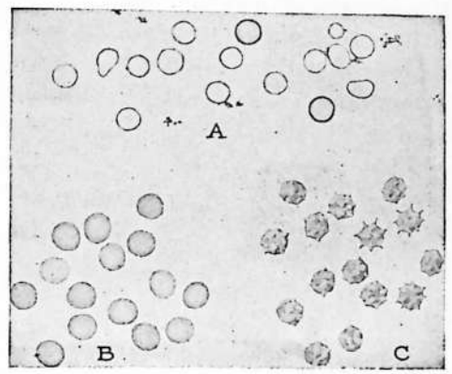


Fig. 74.—Red blood corpuscles in urine: A, Shadow cells from a case of nephritis; B, fresh red corpuscles; C, cremated corpuscles in a urine of high specific gravity (× 475).

by no means always, of serious import. A few red blood corpuscles may be found after strenuous exercise. Blood comes from the *kidney tubules* in severe hyperemia, in acute nephritis and exacerbations of chronic nephritis, and in renal tuberculosis and malignant disease. Renal hematuria may also be a manifestation of the "hemorrhagic diseases"; and an "idiopathic hematuria," probably of nervous origin, has been described. Usually, however, the term "idiopathic hematuria" covers a failure in diagnosis. The urine of healthy infants frequently contains red blood corpuscles for weeks at a time. This has been attributed to slight toxic injury to the kidneys, which are particularly sensitive in infancy. Blood comes from the *pelvis of the kidney* in the presence of renal calculus, of which hematuria is the classical and most constant symptom. The bleeding in this condition is usually intermittent, small in amount, and accompanied by a little

pus and perhaps crystals of the substance forming the stone. Considerable hemorrhages from the *bladder* may occur in vesical calculus, tuberculosis, and new growths. Small amounts of blood generally accompany acute cystitis. In Africa the presence of *Schistosoma haematobium* in the veins of the bladder is a common cause of hemorrhage (Egyptian hematuria).

Addis Cast and Cell Count Method.—Addis,[1] and Addis and Oliver[2] in the description of a method for enumerating the casts and cells in a twelve-hour sample of urine, held that while an appreciable number of these elements may be present in normal urine, wide variations occur in different types of nephritis.

Method.—1. Save a twelve-hour sample of urine, which must be measured accurately within ± 2 c.c.

2. Transfer 10 c.c. of urine to a special graduated centrifuge tube (Fig. 72). The narrow tip is graduated for the measuring of small amounts of sediment.

Fig. 75.—Addis graduated centrifuge tube.

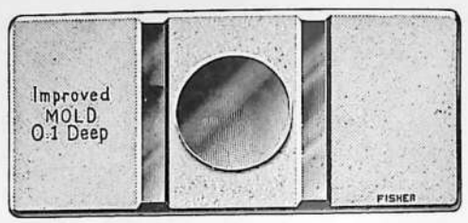


Fig. 76.—Exton counting chamber.

3. Centrifugalize for five minutes at 1800 revolutions per minute.

4. Pour off the supernatant urine and adjust the volume of the remaining fluid with salt solution (1 to 5 c.c.) so that the sediment from the 10 c.c. of urine can be well distributed in the fluid by mixing thoroughly with a fine pipet.

5. Place a drop of the mixture of sediment in a hemocytometer and count the number of casts in the total ruled area, which represents the number in 0.0009 c.c. Also count the erythrocytes, and the leukocytes that are found in the same ruled area. Repeat this procedure two to ten times and add the number of cells and casts that have been counted.

Calculation.—The following formula is self-explaining: Let V = volume of urine, expressed in cubic centimeters, for twelve hours; 10 = number of cubic centimeters of urine centrifugalized; s = volume in cubic centimeters

[1] Addis, Thomas: A Clinical Classification of Bright's Disease, Jour. Am. Med. Assn., *85*:163–167 (July 18), 1925.

[2] Addis, Thomas, and Oliver, Jean: The Renal Lesion in Bright's Disease, New York, P. B. Hoeber, 1931, 631 pp.

of mixed sediment; v = volume in cubic centimeters in which count was made; n = total number of cells or casts counted; N = number of casts, or cells in twelve-hour sample.

$$N = \frac{s}{v} \times n \times \frac{V}{10}$$

Normal Values and Significance.—Hyaline casts may number as high as 5000 normally in the twelve-hour period, while in nephritis casts of various sorts may total 50,000 to 1,000,000. Erythrocytes number normally from 0 to 500,000 (possibly even 1,000,000); in nephritis there may be an enormous increase in the number of erythrocytes in the twelve-hour period (15 to 400,000,000). Leukocytes may be normally present; usually the number of leukocytes does not exceed 1,000,000 in twelve hours. In pathologic conditions the epithelial cells and pus cells may number from 2,000,000 to 50,000,000. It is evident that with such wide variations in normal subjects a close division between normal and abnormal is impossible.

Lyttle[1] has shown that in the urine of children there may normally be slightly more albumin and casts present than in the urine of adults, while the count of erythrocytes and leukocytes in the urine excreted in twelve hours by a normal child will be slightly less on the average than similar counts on samples obtained from adults.

Exton[2] advocated careful estimation of the number of formed elements in the urinary sediment after centrifugation, and has devised a counting chamber (Fig. 76, p. 154) to facilitate accuracy.

5. Spermatozoa are generally present in the urine of men after nocturnal emissions, after epileptic convulsions, and in spermatorrhea. They may be found in the urine of both sexes following coitus. They are easily recognized from their characteristic structure (Fig. 77). The 4-mm. objective should be used, with subdued light and careful focusing.

6. Bacteria.—Under normal conditions urine is free from bacteria in the bladder, but becomes contaminated in passing through the urethra. Various nonpathogenic bacteria, notably *Micrococcus ureae*, which forms chains like those of the streptococci, are present in old or decomposing urine. They are easily seen with the 4-mm. objective in the routine microscopic examination, but ordinarily no attempt is made to identify them. They produce a cloudiness which will not clear upon filtration through paper.

In many infectious diseases the specific bacteria may be eliminated

[1] Lyttle, J. D.: The Addis Sediment Count in Normal Children, Jour. Clin. Investigation, *12*:87–93 (Jan.), 1933.

[2] Exton, W. G.: Quantitative Microscopic Urinalysis, Jour. Lab. and Clin. Med *15*:386–404 (Jan.), 1930.

in the urine without producing any local lesion. Typhoid bacilli are present in the urine in about 30 per cent of the cases, and have been known to persist for months and even years after the attack.

Tubercle bacilli are nearly always present in the urine when tuberculosis exists in any part of the urinary tract, and are often present in general miliary tuberculosis, but may be difficult to find, especially when the urine contains little or no pus.

In local nontuberculous infections of the urinary tract, notably in acute and chronic pyelitis and cystitis, a variety of bacteria have been found. In more than half of the cases the colon bacillus is present alone or in company with others. Next most frequent are the staphylococci. *Bacillus proteus vulgaris*, *Bacillus pyocyaneus*, streptococci, and others have also been encountered.

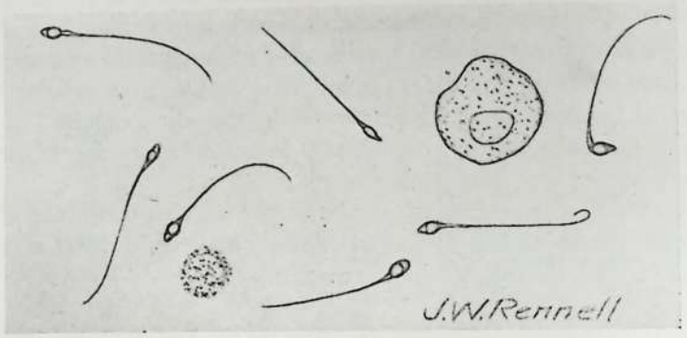


Fig. 77.—Spermatozoa in urine. A pus corpuscle and a transitional epithelial cell are also shown (× 475).

Detection of Tubercle Bacilli in Urine.—In order to avoid the smegma bacillus the urine should be obtained aseptically by catheter after careful cleansing of the parts, or by having the patient void urine in three portions, only the last being used for the examination.

1. Concentrate the tubercle bacilli into a small amount of sediment. This may be done by simply centrifugalizing thoroughly at high speed, or, better, by the method of Petroff, as follows:

(*a*) Acidify 100 c.c. of the urine with 30 per cent acetic acid, add 2 c.c. of 5 per cent tannic acid solution, and mix.

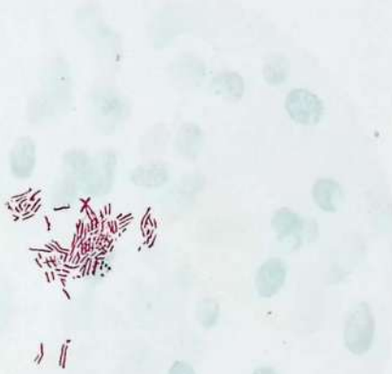
(*b*) Place in the refrigerator for twenty-four hours.

(*c*) Centrifugalize thoroughly at high speed, pipet off the supernatant fluid, and redissolve the sediment with dilute acetic acid.

(*d*) Centrifugalize thoroughly once more.

2. Make thin smears of the sediment, adding a little egg albumen if necessary to make the smear adhere to the glass; dry, preferably in the incubator, for three hours, and fix in the usual way.

PLATE IV



Tubercle bacilli in urinary sediment; × 800. (Ogden.)

3. Stain thoroughly with carbolfuchsin in the usual way (p. 45).

4. Wash in water, and then in 5 per cent nitric acid, until only a faint pink color remains.

5. Wash in water.

6. Soak in alcohol fifteen minutes or longer. This decolorizes the smegma bacillus (p. 51), which is often present in the urine, and might easily be mistaken for the tubercle bacillus. Some strains of the smegma bacillus are very resistant to alcohol. It is therefore best to avoid it altogether by examining only catheterized specimens, in which case this step may be omitted.

7. Wash in water.

8. Apply Loeffler's methylene blue solution for a few seconds. The blue stain should be light.

9. Rinse in water, dry between filter papers, and examine with the oil-immersion objective.

Fluorescent Dye Method.—The method of staining acid-fast organisms with Auramine O and examining with a fluorescence microscope, described on page 47, has proved very effective in demonstrating tubercle bacilli in urine. When there are only a few organisms on the slide, as is often the case in preparations from urinary sediments, they are more easily found with this method than with the carbolfuchsin stain.

A careful search of several smears may be necessary to find the bacilli. They usually lie in clusters (Plate IV). Failure to find them in suspicious cases should be followed by inoculation of guinea-pigs; this is the court of last appeal, and must also be sometimes resorted to in order to exclude the smegma bacillus. Recently culture methods have been devised which may prove useful instead of animal inoculation. (See Corper and Uyei's method, p. 51.)

Detection of Gonococci in Urine.—In acute and chronic gonorrhea gonococci can sometimes be found within pus cells in the sediment, but more commonly in the "gonorrheal threads" or "floaters," which are described on page 147. In themselves these threads are by no means diagnostic of gonorrhea. They are most common in the morning or after massage of the prostate. The floater is fished out, spread upon a slide, dried, and fixed by heat. It is then stained and searched for gonococci as described on page 589. Recognition of the gonococcus in isolated pus cells of the sediment is difficult since these cells are usually much shrunken. The smears should be thin and quickly dried.

Method for Urine Cultures.—The tubercle bacillus and the gonococcus are generally sought by staining methods as above described. Other organisms can be found and identified only by culture.

The urine must be collected in a sterile test tube or bottle with every precaution to avoid contamination. In the case of females this necessitates catheterization. In males it will suffice to wash the glans penis and margins of the urethral orifice with green soap and water followed by mercuric chloride in 1 : 1000 solution. The urine is then voided and most of it is

allowed to escape, only the latter portion being saved. A part of this should be thoroughly centrifugalized (adding alcohol if the specific gravity is high), and stained smears from the sediment should be studied in order to gain approximate knowledge of the number and kind of bacteria present. From the remaining portion of the urine cultures should be made upon plain agar and Endo's medium, and, if streptococci are expected, also upon blood agar or ascitic-fluid agar. The amount of urine to plant in order to secure isolated colonies must be judged from the number of bacteria found in the preliminary microscopic examination. It may be necessary to dilute the urine with several times its volume of nutrient broth or physiologic salt solution before spreading it upon the media. Upon the other hand, when bacteria are very scarce it may be necessary to use the concentrated sediment after long centrifugation, but this, of course, should not be used if alcohol has been added to favor sedimentation.

Fig. 78.—1, Scolex of *Taenia echinococcus*, showing crown of hooklets; 2, scolex and detached hooklets (obj. 4 mm.). (Boston.)

Culture of Tubercle Bacilli.—Centrifugalize a sample of urine for one hour in a 15-c.c. centrifuge tube. Remove the upper 1-c.c. layer with a sterile pipet. Decant the remainder of the supernatant fluid and add the urine in the pipet to the sediment. Mix thoroughly; add 1 c.c. of 5 per cent oxalic acid and treat the mixture as described on page 51 for the culture of tubercle bacilli in sputum according to the method of Corper and Uyei.

7. Animal parasites are rare in the urine. Hooklets and scolices of *Taenia echinococcus* (Fig. 78) and larvae of filariae have been met. In Africa the ova, and even adults, of *Schistosoma haematobium* are common, accompanying "Egyptian hematuria." *Trichomonas hominis* is a not uncommon contamination, usually reaching the urine from the vagina or the rectum. This and other protozoa may be mistaken for spermatozoa by the inexperienced.

A worm which is especially interesting is *Anguillula aceti*, the "vinegar eel." This is generally present in the sediment of table vinegar, and may reach the urine through use of vinegar in vaginal douches, or through contamination of the bottle in which the urine is contained. It has been mistaken for *Strongyloides stercoralis* and for the larval filaria. It somewhat resembles the former in both adult and larval stages. The young larvae have about the same length as the larvae of *Filaria bancrofti*, but are nearly twice as broad, and the intestinal canal is comparatively easily seen (compare Figs. 79 and 264).

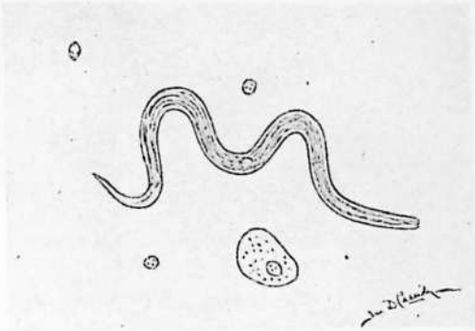


Fig. 79.—Larva of "vinegar eel" in urine, from contamination; length, 340μ; width, 15μ. An epithelial cell from bladder and three leukocytes are also shown.

For fuller descriptions of these parasites the reader is referred to Chapter VII.

C. Extraneous Structures

The laboratory worker must familiarize himself with the microscopic appearance of the more common of the numerous structures which may be present from accidental contamination (Fig. 81).

Yeast cells are smooth, colorless, highly refractive, spheric or ovoid cells. They sometimes reach the size of a leukocyte, but are generally smaller (Fig. 80). They are often mistaken by the inexperienced for red blood corpuscles, and more rarely for fat droplets or the spheric crystals of calcium oxalate, but are distinguished by the facts that they are usually ovoid and not of uniform size; that they tend to adhere in short chains; that small buds may often be seen adhering to the larger cells; and that they do not give the hemoglobin test, are not stained by osmic acid or sudan III, but are colored brown by Lugol's solution, and are insoluble in acids and alkalis. Yeast cells multiply rapidly in diabetic urine, and may reach the bladder and multiply there.

Mold fungi (Fig. 82) are characterized by refractive, jointed, or branched rods (hyphae), often arranged in a network, and by highly

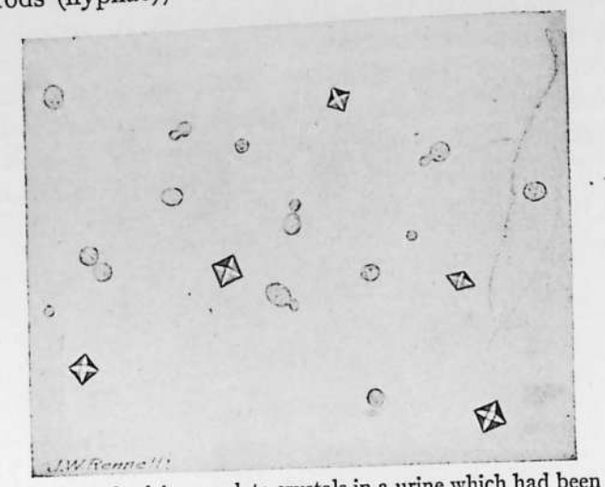


Fig. 80.—Yeasts and calcium oxalate crystals in a urine which had been preserved for two weeks with boric acid. Note the budding forms which are characteristic of the yeasts (× 450).

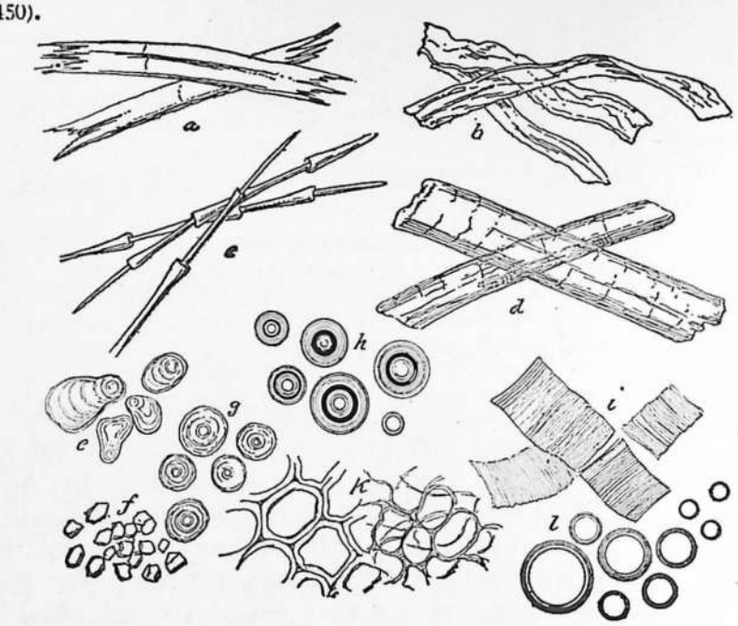


Fig. 81.—Extraneous matters found in urine: *a*, Flax fibers; *b*, cotton fibers; *c*, feathers; *d*, hairs; *e*, potato-starch granules; *f*, rice-starch granules; *g*, wheat-starch granules; *h*, air bubbles; *i*, muscular tissue; *k*, vegetable tissue; *l*, oil globules.

refractive spheric or ovoid spores. They are common in urine which has stood exposed to the air. Not infrequently a spore with a short

hypha growing from it is reported as a spermatozoon. The spores sometimes tend to form short chains.

Fibers of wool, cotton, linen, or silk, often colored, derived from towels, the clothing of the patient, or the dust in the air, are present in almost every urine. They are best identified by comparison with known fibers which are placed in a drop of water on a slide and covered. **Fat droplets** are most frequently derived from unclean bottles or oiled catheters. **Starch granules** may reach the urine from towels, the clothing, or dusting powders. They are recognized by their concentric striations, which, however, are sometimes difficult to see, and by the blue color which they take when treated with weak iodine solution. **Lycopodium granules** (Fig. 13) may also reach the urine from dusting powders. They might be mistaken for the ova of parasites. **Bubbles of air** (Fig. 81, *h*) are often confusing to beginners, but are easily recognized after once being seen. Another common source of confusion, which does not seem to be well enough recognized, is **pollen** from flowers which have been kept in the sick-room. The pollen granules differ in the different species, but are generally clean cut, more or less rounded, light yellow to brown bodies, and bear little resemblance to the ova for which they are sometimes reported. **Diatoms** from the tap water are not infrequently encountered, and certain of the elongated forms have been mistaken for tube casts.

Fig. 82.—Aspergillus from urine (Boston).

Scratches and **flaws** in the glass or cover are often most assiduously studied by beginners, and are not infrequently reported as rare crystals, tube casts or even worms. **Dirt** upon the top of the cover (especially when this is taken directly from the original box without cleaning) is likewise a common source of confusion. It often takes the form of crystals which, because they are more prominent than the structures in the urine beneath the cover, receive the student's whole attention. Fibers of **muscle** (Figs. 81, *i*, and 207) and other particles which are evidently of fecal origin are usually the result of accidental contamination, but may rarely be present in catheterized specimens. They then indicate rectovesical fistula. A suspicion of fecal contamination may be confirmed by a strong reaction to the urobilin test.

IV. TESTS OF KIDNEY FUNCTION

With the growing appreciation that it is usually the altered function of a diseased organ that measures its menace to the patient's health and that the functional disturbance may be the first definite indicator of anatomic changes, much thought has been given within recent years to the development of methods which seek to ascertain whether the kidneys are capable of performing the work which the activities of normal life demand of them, and to measure the degree of insufficiency when they fail in this respect. Such methods afford invaluable aid in the study of kidney disease and of certain extrarenal conditions which interfere with kidney function, and they furnish a useful guide to prognosis and treatment as well.

It must be borne in mind, however, that the tests measure function only, not the extent of anatomic change in the kidney; and these do not necessarily go hand in hand even when definite organic disease exists. As is well known, kidney function may be markedly depressed by conditions which are primarily extrarenal, such as heart disease with poor compensation, severe general anemia, hypertrophied prostate, and cystitis; while, upon the other hand, some of the tests may show exalted functional activity in early stages of kidney disease. Moreover, since the kidney's function is multiple, some phases of its activities may be markedly interfered with while others remain normal; and the different tests, which do not necessarily measure the same function, may sometimes give apparently inconsistent results in a given case. For this reason it is highly desirable that two or more of the tests be employed in every case.

No less than fifty tests of renal function have been proposed, but only a few have received wide acceptance. Even the simple calculation of total solids, described upon a previous page, has much value in this field. Of the more important functional tests the following are perhaps the most useful for clinical work: (1) Phenolsulfonephthalein test; (2) Mosenthal's test meal for renal function; (3) Volhard and Fahr concentration and dilution tests; (4) estimation of nitrogen retention; and (5) estimation of excretion of urea. Of these, the first two are so simple as to be easily carried out in private practice. The other two demand special training in the technic of blood chemistry.

The limitations of these five functional tests and their application to the study of nephritis and other conditions are discussed at some length in the section upon Nephritis. The following summary of their comparative value and special fields of usefulness seems to be warranted:

1. The test meal for renal function affords, particularly in the character of the night urine, the most sensitive indicator of defective kidney function, and is especially useful for early diagnosis of chronic nephritis. It may, indeed, indicate grades of functional disturbance which are so slight that they may be disregarded.

2. The phenolsulfonephthalein test and urea clearance run closely parallel, and are most useful in mild, moderate, and severe grades of insufficiency. The latter is thought by some, but by no means all, of those who have used it to be slightly more reliable; but the former is certainly preferable for practical work because of its simplicity, and may be accepted as our most useful test for clinical purposes.

3. The estimation of nitrogen retention by determination of blood urea finds its chief usefulness in the latter stages of kidney disease, where it is the most reliable prognostic sign. It is also useful in distinguishing passive congestion due to heart disease. Owing to the wide range of normal variations, it fails to yield unmistakable evidence as to the condition of the kidney in slight and moderate grades of insufficiency.

4. Owing to the remarkable reserve power of the kidneys, whereby one kidney can, in the absence of any unusual strain, carry on the work of the two, *bilateral* disturbance may be inferred when these tests indicate a notable degree of renal insufficiency, and particularly when nitrogen is retained. When there is a question of *unilateral* kidney disease a special method must be employed; and, of the tests described here, only the phenolsulfonephthalein test is applicable. Its modification for this purpose is described on page 168.

The table on page 164 shows the values to be expected of the different tests in various grades of kidney insufficiency.

1. Phenolsulfonephthalein Test.—This test, which was offered by Rowntree and Geraghty in 1910, consists in the intramuscular or intravenous injection of a solution of phenolsulfonephthalein, a drug which is eliminated only by the kidneys, and whose amount in the urine is easily estimated by colorimetric methods. The time of its first appearance in the urine and the quantity eliminated within a definite period are taken as a measure of the functional capacity of the kidneys. The test is harmless, extremely simple, and for general purposes the most satisfactory of the functional tests. Other substances—methylene blue, indigo carmine, etc.—have been used in a similar manner, as well as urea and creatinine by mouth, but have not met with general approval.

Technic.—The original procedure, in which the patient was catheterized when the drug was injected and the catheter was left in place

SCALE OF DEGREES OF RENAL INSUFFICIENCY AND COMPARISON OF VALUES YIELDED BY THE MORE IMPORTANT TESTS OF KIDNEY FUNCTION

State of renal function.	Phenol-sulfone-phthalein excretion, per cent.	Nonprotein nitrogen of the blood mg. per 100 c.c.	Urea nitrogen of the blood mg. per 100 c.c.	Urea clearance.		Test meal for renal function:				
						Night urine.		Variations in specific gravity of two-hour specimens when the highest specific gravity is		
				Standard.	Maximal.	Volume.	Specific gravity.	1018.	1017 to 1015.	1014 and 1013.	1012 or less.
Normal	60 or more	40 or less	20 or less	40 to 65	60 to 95	500 c.c. or less	1018 or more	9 or more	6 or more	6 or more	6 or more
Slight impairment	59 to 40	40 to 45	20 to 27	30 to 40	40 to 60	500 c.c. to 750 c.c.	1016 and 1017	8 to 5	5 and 4	4 and 5	5 or less
Moderate "	39 to 25	46 to 65	28 to 44	20 to 30	30 to 40	750 c.c. or more	1015 or less	4 or less	3 or less	3 or less	
Marked "	24 to 11	66 to 90	45 to 64	10 to 20	10 to 30	750 c.c. or more	1015 or less	4 or less	3 or less		
Maximal "	10 to 0	91 or more	65 or more	below 10	below 10	750 c.c. or more	1015 or less	4 or less			

Slightly modified from Mosenthal, H. O. and Lewis, D. S., Jour. Amer. Med. Assn., 67: 933 (Sept. 23), 1916.

until the drug was detected in the urine, is now seldom followed. The catheter is still used if there be obstruction to the outflow of urine, but ordinarily it is dispensed with, and the procedure is as follows:

1. Give the patient 300 to 400 c.c. (about 2 glasses) of water to promote secretion of urine.

2. Twenty minutes afterward have him empty his bladder and discard the urine. Then with a hypodermic syringe inject exactly 1 c.c. of the sterile phenolsulfonephthalein solution[1] intramuscularly, preferably into the deltoid, gluteal, or lumbar muscles. If there be general edema, hindering absorption, it should be injected intravenously.

3. In exactly one hour and ten minutes from the time of the injection have the patient empty his bladder and save all the urine. The ten-minute period represents the usual time which elapses between the injection and the first appearance of the dye in the urine.

4. In two hours and ten minutes after the injection have the patient empty his bladder again, and save all the urine in a separate container. He should be under observation during the two-hour period, else it is difficult to make sure that he carries out his instructions exactly.

5. Estimate the output of phenolsulfonephthalein in each of the two portions of urine separately as described below.

Estimation of Output.—*Record the volume of each of the one-hour portions of urine.* Also embody this information in the report to the clinician, as it may be of importance to him in some instances. If either is very low—less than 40 c.c.—results are not dependable. To each of the two portions add sufficient sodium hydroxide solution to bring out the maximum purplish-red color, and estimate the amount of the drug contained in each by comparing the color with that of an alkalinized standard solution. The result is recorded in terms of the percentage of the amount injected.

In detail, this is done as follows:

1. In a graduate or volumetric flask dilute each of the one-hour specimens of urine with water to about 800 c.c., add about 5 c.c. of 10 per cent sodium hydroxide solution or enough to bring out the maximum purplish-red color, and bring each to 1000 c.c. with water. Mix well.

2. Add 1 c.c. of the phenolsulfonephthalein solution to about 800 c.c. of water, alkalinize with sodium hydroxide, and dilute to 1000 c.c. Since this contains the same amount of the drug as was injected, it may be rated as a 100 per cent standard-color solution. As a rule no more than 100 c.c. of the standard solution will be needed, and there usually will be enough of the solution left in the original ampule to make this amount.

3. Filter each of the diluted and alkalinized specimens of urine and compare with the 100 per cent standard solution in any good colorimeter. The use of colorimeters is described on page 347. The simple and inexpensive Denison laboratory instrument is especially useful for this purpose.

[1] This solution may be obtained of any druggist. It is sold in 1-c.c. ampules, sterilized ready for use; but it should be noted that these ampules contain somewhat more than 1 c.c., hence one should not inject the entire contents.

Results with this and the Duboscq type are most dependable when the unknown solution and the standard have nearly the same depth of color. It is therefore well to use a 50 per cent standard solution made by diluting the 100 per cent standard above recommended with an equal amount of water and mixing well. The Dunning colorimeter (Fig. 83) consists of thirteen sealed ampules containing standard color solutions of different percentages, an open ampule in which the unknown specimen is placed, and a small box in which the specimen is compared with the standards. It is very satisfactory for office work because the physician need not make his own standard solution. We have found the colors to remain for over a year with very little fading when kept in the dark.

4. In the absence of a colorimeter one can easily make up a series of standards, representing 10, 20, 30 per cent, and so forth, by diluting the 100 per cent standard. The unknown is then matched against these in test tubes of the same diameter. The standard solution which matches the unknown indicates the percentage of excretion. If, for example, the first hour's specimen is matched by the 40 per cent standard, and the second

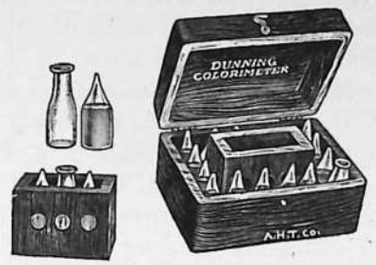


Fig. 83.—Dunning's colorimeter for the phenolsulfonephthalein test of kidney function.

hour's specimen by the 20 per cent standard, then the phenolsulfonephthalein output for the two periods is 40 and 20 per cent, respectively, and for the two hours is 60 per cent.

In order to equalize the slight difference in color due to a highly colored urine, the standard color may be viewed through a faintly yellow-tinted piece of glass, or an amount of normal urine sufficient to give the desired shade may be included in the standard solution. For those who do much work it is convenient to add a few drops of a solution of some yellow dye, such as Echtgelb G or tropeolin OO.

When it is necessary to defer the color comparison for hours or days the urine must be kept acid, as the color fades in alkaline solution. Phosphoric acid is best for this purpose. A standard solution made without urine will remain for some months with only slight fading.

Under normal conditions the drug first appears in the urine in five to eleven minutes after the injection. Within the first hour after its appearance 40 to 50 per cent is eliminated; in the two hours, 60

to 75 per cent. Pathologically, the excretion may be reduced to a trace or even, in extreme cases, to none at all in the two hours. The values to be expected in various grades of kidney insufficiency are indicated in the table on page 164. The test should not be applied when the patient is taking saline cathartics, as they tend to delay excretion.

Time has proved the great usefulness of this test in every-day practice. It will sometimes reveal a serious degree of renal failure when other urinary findings are practically normal, but it must be remembered that it is a test of functional capacity only, not a measure of the extent of anatomic changes in the kidney, and that no one of the functional tests covers the entire range of the kidney's activities. The test is extremely valuable in diagnosis and prognosis of nephritis, and in this connection is discussed more fully on page 179. It is most useful in chronic interstitial nephritis where the phenolsulfonephthalein output runs fairly parallel with the course of the disease. In very early nephritis there may be excessive elimination, owing probably to irritation and overaction of undamaged portions of the kidney. In such cases this test should be supplemented by Mosenthal's test meal for renal function. In acute nephritis the output does not always agree with the clinical and pathologic picture. Particularly is this true in the acute glomerulonephritis of scarlet fever, where the excretion percentage may sometimes be fully up to the normal. Apparently the test speaks less definitely concerning glomerular changes than tubular.

The output is low in heart disease with chronic passive congestion of the kidneys and rises when the congestion is relieved by improved heart action. Low values are also often found in prostatic hypertrophy and in cystitis with retention of urine.

In using the intravenous method, Shaw[1] advocated plotting a curve that would represent the elimination at intervals of fifteen minutes. By using this method Chapman and Halsted[2] studied the elimination in a number of cases of Bright's disease by collecting samples at intervals of fifteen, thirty, sixty, and one hundred and twenty minutes. The total elimination may be normal in cases of acute nephritis, but an elimination of less than 25 per cent of the drug in the first fifteen minutes after its injection may be the earliest evidence of renal disease.

[1] Shaw, E. C.: A Study of the Curve Elimination of Phenolsulphonephthalein by Normal and Diseased Kidneys, Jour. Urol., *13*:575–591 (June), 1925.

[2] Chapman, E. M. and Halsted, J. A.: The Fractional Phenolsulphonephthalein Test in Bright's Disease, Am. Jour. Med. Sc., *186*:223–232 (Aug.), 1933.

Application of the Test to the Two Kidneys Separately.—This requires catheterization of the ureters, the patient being given two glasses of water about one-half hour beforehand and the phenolsulfonephthalein solution being injected intravenously immediately after the catheters are in place.

The urine is received from the catheters directly into two test tubes which contain a few drops of 10 per cent sodium hydroxide solution. The time of first appearance of the dye in the urine, indicated by the appearance of a red color in the tubes, is noted, and the urine is collected for one-half or one hour thereafter in fifteen-minute or half-hour periods. The output of the drug in each of the specimens is then determined as already described.

Under normal conditions the dye first appears in the urine in three to five minutes after intravenous injection, although it may sometimes be delayed for one or both kidneys as a result of reflex inhibition due to the presence of the catheters. The total output for each kidney is more important than the time of appearance. This for the two kidneys together is about 35 to 45 per cent in fifteen minutes, 50 to 60 per cent in the first half hour, and 65 to 80 per cent in the first hour. When one kidney is defective the appearance of the drug in the corresponding urine is greatly delayed and the total elimination from this kidney is reduced. In such cases the other kidney may compensate to greater or less degree by increased output. When the catheters are removed care should be taken to ascertain whether any urine has leaked past them into the bladder. This accident would confuse results.

2. Test-meal for Renal Function.—Following the work of Hedinger and Schlayer and others, Mosenthal has placed upon a practical footing a valuable test of renal function which is based upon the characteristics of the urine—chiefly volume of the night urine and variations in specific gravity of two-hour specimens taken during the day—when the patient is upon a prescribed diet.

When the kidneys are healthy, the urine which they excrete at different periods within the twenty-four hours varies markedly in volume and specific gravity. These variations represent the ready response of normal kidneys to the varying demands for the elimination of water or solids imposed by the necessity of maintaining the concentration of the body fluids at a constant level in spite of the periodic intake of food and water. Diseased kidneys, upon the other hand, lose this adaptive power to greater or less degree, and the limits within which they can vary their activities become greatly narrowed. The urine which they excrete consequently remains of almost uniform concentration from hour to hour. Numerous modifications of this test are now in use, but only the original method need be described here.

Method in Detail.—Mosenthal originally prescribed a definite, weighed and measured test diet, but this is no longer thought essential.

1. Upon the day of the test, and preferably also the day before, place the patient upon a full diet, such as the following: Breakfast of fruit, cereal, bread, butter, and tea, coffee, cocoa, or water at 8 A. M.; dinner of soup, meat, vegetables, bread, butter, dessert, and tea, coffee, or water at noon; and supper of eggs, bread, butter, fruit, and tea or water at 5 P. M. Much latitude is allowable in the choice of foods, and in many cases the ordinary diet to which the patient is accustomed may be used. At least a pint of fluid—tea, coffee, water, etc.—must be taken at each meal; and no food or liquid of any sort may be taken outside of these meals until after 8 o'clock the following morning.

2. Instruct the patient to empty his bladder immediately before breakfast. Collect specimens of urine at 10 A. M.; 12 noon; 2 P. M.; 4 P. M.; 6 P. M.; 8 P. M.; and, finally, at 8 o'clock the following morning. It is essential that the intervals be exact and that the bladder be completely emptied each time. Should the hour for the meals be changed, the times of collecting the samples of urine should be changed accordingly. The last of the two-hour specimens must not be collected less than three hours after the beginning of the evening meal.

3. Measure the night urine (8 P. M. to 8 A. M.) and take its specific gravity with an accurate urinometer.

4. Measure the six two-hour specimens and take their specific gravity, first making sure that they are all at the same temperature, since misleading figures may be obtained if some have been kept on ice and some at room temperature.

Normal Values.—In health the urinary response is as follows:

1. The night urine will be much less than the total day urine. It is usually 250 to 350 c.c., and will seldom exceed 400 to 500 c.c.; 750 c.c. is the maximum. Its specific gravity will usually be 1.018 or above.

2. The highest specific gravity recorded for the two-hour day specimens will exceed 1.018, while the difference between the highest and the lowest will not be less than 8 or 9 points. If, for example, the most concentrated specimen has a specific gravity of 1.020, the most dilute will be 1.011 or less.

Indications of Impaired Renal Function.—One or more of the following changes may be noted:

1. *Nocturnal Polyuria.*—The volume of the night urine exceeds 750 c.c. This is usually one of the first and most definite evidences of impaired kidney function. A volume between 500 and 750 c.c. is suspicious, and usually indicates impairment. As H. W. Jones has pointed out, a determination of the ratio between day and night urine (p. 65) is especially helpful in interpreting these borderline figures.

2. *Low maximal specific gravity* of day urine, the highest of the two-hour specimens falling below 1.018.

3. *Fixation of specific gravity*, that is, lessened variations in the specific gravities of the two-hour specimens. This is a very important sign of renal

insufficiency. In marked cases the difference between the highest and lowest specific gravities may be only one or two points. As a rule, the level at which the specific gravity is fixed becomes lower as the functional impairment increases and the kidneys lose their ability to concentrate urine.

Fixation of specific gravity at a high level, near 1.018 or 1.020, may occur in acute nephritis, chronic parenchymatous nephritis, and passive congestion of the kidney, but is not necessarily an indication of disease, since it may occur when the patient has been taking insufficient water, or when there has been excessive loss of water through perspiration. Absorption and elimination of edema at the time of the test, leading to fixation of specific gravity at a low level, may also confuse the results.

3. Concentration and Dilution Tests.—Volhard and Fahr have suggested two very simple tests for kidney function, one measuring concentrating power, and the other, diluting power. The concentration should be done first and twenty-four hours allowed to elapse before beginning the dilution test.

Concentration Test.—Allow no fluids from the evening before the test until the test is finished, and no food between meals.

8 A. M., Breakfast: Dry cereal with sugar, syrup, or honey; no milk; one egg; toast or bread with butter.

12 Noon, Dinner: Roast beef, steak, or chops; potatoes, boiled, baked, or riced; bread and butter; jam.

5 P. M., Supper: Two eggs; bread and butter; jam.

8 A. M. of same day: Empty bladder. Collect urine in separate containers every three hours thereafter until night, that is, at 11 A. M., 2 P. M., 5 P. M., 8 P. M.; and collect all urine from 8 P. M. to 8 A. M. next morning in one container.

Note the quantity and specific gravity of each three-hour sample and of the twelve-hour sample and plot as a curve. Normally, the specific gravity of at least one sample should be 1.030, or at least 1.025.

Fishberg[1] suggested a simple modification of the concentration test. Have the patient eat the regular evening meal at 6 o'clock with a minimal amount of fluid and a considerable amount of protein. Discard all urine voided during the night. Save the first sample of urine voided in the morning. The patient should rest in bed one hour after awaking, and a second sample of urine should be collected in a separate container. If possible the patient should arise and after one hour's activity a third sample of urine should be collected in another container. All three samples of urine may be taken to the laboratory and examined at about 10 A. M. If the renal function is unimpaired, the specific gravity of at least one sample of urine will be between

[1] Fishberg, A. M.: Hypertension and Nephritis, Ed. 3, Philadelphia, Lea and Febiger, 1934, p. 58.

1.022 and 1.032. In case of very severe impairment the specific gravity may be as low as 1.010, and in intermediary conditions it will be between 1.010 and 1.020.

Dilution Test.—This is also known as the "water test."

Omit breakfast. For dinner and supper give the routine nephritic diet or the diet to which the patient has been accustomed. Permit one glass of water after supper.

8 A. M.: Empty bladder and give 1500 c.c. water.

Collect urine in separate containers at 8.30, 9.00, 9.30, 10.00, 10.30, 11.00, 11.30 A. M., and 12.00 noon, eight specimens in all. Collect all the urine from 12.00 noon to 8.00 next morning in one container.

Note the quantity and specific gravity of each sample and plot as a curve. Normally, the total quantity voided should be 80 to 120 per cent of the intake (1200 to 1800 c.c.). The specific gravity of at least one sample should be as low as 1.003.

The two tests given above, particularly the first, show decreased renal efficiency often before it can be demonstrated with other standard renal tests. They are so simple that they can be done in general practice, and without any elaborate equipment.

4. Nitrogen Retention as Evidence of Kidney Insufficiency.—The nitrogen-containing substances of the blood may conveniently be divided into two classes: (1) Proteins, which are essential and permanent constituents of the blood; and (2) various nonprotein nitrogenous substances whose transportation is one of the chief functions of the blood; partly transformed foodstuffs, which, unless utilized by the tissues, circulate until excreted by the kidneys; partly waste products of metabolism which also must be excreted. It is the nonprotein group which concerns us here. Although the quantity to be eliminated varies greatly from hour to hour, depending upon intake of food or exercise, yet through the adaptability of the kidney the concentration in the blood is kept at a fairly constant level, with a rise for only a short period following meals. When the kidneys are damaged and their capacity to excrete thereby lessened, these substances tend to accumulate in the blood. To this condition, in which chemical analysis of the blood shows abnormally large and sometimes enormous amounts of nonprotein nitrogen, the name "nitrogen retention" is applied; and, other things being equal, the extent to which it occurs may be taken as an index of the degree of disability of the kidneys. Among the retained substances urea holds first place, amounting to about one half of the whole. Others, which need not be considered here, are uric acid and creatinine.

The following figures represent the values obtained in health under ordinary conditions of diet and exercise:

Total nonprotein nitrogen	25 to 30	mg.	per 100 c.c.	of blood
Urea nitrogen[1]	12 to 15	"	"	"
Uric acid	2 to 3	"	"	"
Creatinine	1 to 2	"	"	"

In health and disease the concentration of these substances in the blood is the resultant of three variable factors: Intake of nitrogenous food, activity of metabolism, and excretory ability of the kidneys. To these must be added in certain cases the formation of extensive edema or large exudates which "lock up" a considerable amount of urea.

Owing to the impracticability of fully controlling the diet and accurately measuring the balance of metabolism in clinical work and to the remarkable reserve power of the kidneys, estimations of the nonprotein nitrogenous substances are of comparatively small value for detecting and measuring slight grades of kidney disability. In more advanced grades, upon the other hand, when nitrogen retention is sufficiently marked to be unquestioned, these estimations probably constitute the most helpful of all functional tests, particularly as a guide to prognosis and treatment and as an aid in the differential diagnosis of renal and cardiac disorders, in the latter of which nitrogen does not accumulate in the blood. The figures which may be expected with this and other functional tests in various grades of kidney disability are given in the table on page 164.

To estimate the degree of nitrogen retention some workers determine the total nonprotein nitrogen of the blood, others rely upon urea nitrogen alone. It apparently makes little difference which of the two is determined, since the results run closely parallel under practically all circumstances. Estimation of urea is much simpler and is therefore given preference here.

Further discussion of nitrogen retention in relation to kidney disease, particularly the significance of blood uric acid and creatinine, will be found on page 183. Methods of estimating nitrogen retention are described in the chapter on Blood Chemistry, page 363.

5. Urea Excretion (Ambard's Coefficient; McLean's Index; Urea Clearance).—As has already been stated, the concentration of urea in the blood cannot be unreservedly relied upon as a measure of slight insufficiency of the kidneys, owing to the rather wide variations which are possible as a result of extrarenal causes. From the studies of Ambard and others it appears that a much better measure of the kidney's ability to excrete urea can be found in a comparison of the concentration of urea in the blood with the rate of excretion in the urine, and that it is possible to express this relationship numerically by the so-called "Ambard's coefficient."

[1] The distinction between *urea* and *urea nitrogen* must be kept in mind. One gram of urea nitrogen corresponds to 2.14 Gm. of urea. Blood urea is usually recorded in terms of urea nitrogen.

In the calculation of Ambard's coefficient the following factors are taken into account, and they are represented in the formula by the symbols indicated:

D = *Urea grams excreted in urine in twenty-four hours.* This is obtained as follows: (1) Give the patient 150 to 200 c.c. of water. (2) One-half hour afterward have him empty his bladder and note the time of completion to the *minute.* (3) Discard this urine. (4) At the end of an exactly measured period, preferably two hours, have the patient again empty the bladder completely. (5) Measure this urine exactly and estimate its urea. (6) From this calculate the number of grams of urea which would be excreted in twenty-four hours.

C = *Urea grams in 1000 c.c. of urine.* This is calculated from the above estimation.

Ur = *Urea grams in 1000 c.c. of blood.* The blood is taken from a vein in the middle of the period during which urine is collected, and urea is estimated as described on page 365.

Wt = *Weight of patient, without clothing, in kilograms.*

The above factors are combined in the following formula, in which the figure 70 is the standard normal body weight in kilograms and 25 is the standard amount of urea in grams per 1000 c.c. of urine.

$$\frac{\mathrm{Ur}}{\sqrt{\mathrm{D} \times \frac{70}{\mathrm{Wt}} \times \sqrt{\frac{\mathrm{C}}{25}}}} = \text{Coefficient.}$$

With normal kidneys a coefficient of 0.06 to 0.09 is obtained, regardless of how high the blood nitrogen may rise from diet or other causes. When the coefficient rises above 0.09 an impairment of the power of the kidneys to excrete urea is to be inferred.

McLean's index is based upon Ambard's coefficient and is perhaps more widely used in this country. It expresses the same relationship in a somewhat more satisfactory manner in that it adopts 100 as the normal index, and this falls in proportion to the degree of kidney insufficiency instead of rising, as is the case with Ambard's coefficient. McLean's formula is as follows:

$$\frac{\mathrm{D} \times \sqrt{\mathrm{C}} \times 8.96}{\mathrm{Wt} \times \mathrm{Ur}^2} = \text{Index.}$$

McLean has constructed a slide rule, by means of which it is possible with the given data to work out the index in a few minutes without calculation.

Van Slyke and his colleagues have made complete and accurate studies of urea excretion, with a very simple formula—

$$\text{The standard clearance} = \frac{U}{B}\sqrt{V}.$$

In this formula, U = *urine urea nitrogen, mg. for each 100 c.c.*
B = *blood urea nitrogen, mg. for each 100 c.c.*
V = *urine volume, c.c. for each minute.*

The average rate was found to be approximately 1 c.c. for each minute, and the "augmentation limit" about 2 c.c. for each minute. When the urine volume output is at any point above the augmentation limit, 2 c.c. for each minute, the formula for "maximal clearance" becomes $\frac{UV}{B}$. The average "standard clearance" was found to be 54 c.c., the volume of blood that 1 c.c. of urine clears in one minute, and the mean "maximal clearance" about 75 c.c. of blood. For a discussion of the influence of body weight, size in small children, and a review of the work of other investigators of urea excretion, reference should be made to the complete studies.[1]

Urea Clearance

The procedure for this useful test for kidney function, which is performed while the patient is fasting is as follows:

1. Withdraw blood by venipuncture and determine the blood urea nitrogen (B), as described on page 365.
2. Have the patient void, completely emptying the bladder, immediately after the venipuncture.
3. In exactly one hour, have the patient void again, catheterizing him if necessary, completely emptying the bladder. Measure the quantity of urine exactly in cubic centimeters and divide by 60 to calculate the number of cubic centimeters per minute (V).

[1] Studies in urea excretion. I. Austin, J. H., Stillman, E., and Van Slyke, D. D.: Factors Governing the Excretion Rate of Urea, Jour. Biol. Chem., *46*:91–112 (March), 1921. II. Möller, E., McIntosh, J. F., Van Slyke, D. D.: Relationship Between Urine Volume and the Rate of Urea Excretion by Normal Adults, Jour. Clin. Investigation, *6*:427–465 (Dec.), 1929. III. McIntosh, J. F., Möller, E., Van Slyke, D. D.: The Influence of Body Size on Urea Output, Jour. Clin. Investigation, *6*:467–483 (Dec.), 1929. IV. Möller, E., McIntosh, J. F., Van Slyke, D. D.: Relationship Between Urine Volume and Rate of Urea Excretion by Patients with Bright's Disease, Jour. Clin. Investigation, *6*:485–504 (Dec.), 1929. V. MacKay, E. M.: The Diurnal Variation of Urea Excretion in Normal Individuals and Patients with Bright's Disease, Jour. Clin. Investigation, *6*: 505–516 (Dec.), 1929.

4. Dilute 5 c.c. of the urine to 50 c.c. with distilled water, and determine the urine urea nitrogen plus ammonia nitrogen in 5 c.c. of diluted urine (0.5 c.c. of original urine) by the urease method (see p. 81).
5. Determine the ammonia nitrogen in 5 c.c. of diluted urine (p. 86). Subtract the value for ammonia nitrogen from the value for urine urea nitrogen plus ammonia nitrogen (4); this equals the urine urea nitrogen (U).

Calculation.—The urea clearance equals the number of cubic centimeters of blood cleared of urea per minute. If the quantity of urine voided is more than 2 c.c. per minute, the formula used is that for "maximal clearance":

$$\frac{\text{U (urine urea N, mg. per 100 c.c.)}}{\text{B (blood urea N, mg. per 100 c.c.)}} \times \text{V (c.c. urine per minute)}$$

If the quantity of urine excreted is less than 2.1 c.c. per minute the square root law is applied and the formula for "standard clearance" must be used, as follows:

$$\frac{\text{U (urine urea N per 100 c.c.)}}{\text{B (blood urea N per 100 c.c.)}} \times \sqrt{\text{V}}\ \text{(c.c. urine per minute)}*$$

Significance.—Any figure over 40 c.c. may be considered normal. In nephritis, the clearance may be between 10 and 30 c.c. The clearance is very low in uremia.

* The values for the square root of V may be taken from this table.

V	$\sqrt{V}$	V	$\sqrt{V}$	V	$\sqrt{V}$	V	$\sqrt{V}$
0.2	0.45	0.7	0.84	1.2	1.1	1.7	1.3
0.3	0.55	0.8	0.89	1.3	1.14	1.8	1.34
0.4	0.63	0.9	0.95	1.4	1.18	1.9	1.38
0.5	0.71	1.0	1.00	1.5	1.23	2.0	1.42
0.6	0.78	1.1	1.05	1.6	1.27	2.1	1.45

Clearance tests have also been performed by using other substances than urea as the test substance. There has been considerable study using *inulin*.[1] Ten grams of this substance in 100 c.c. of sterile physiologic salt

[1] Alving, A. S., Rubin, Jack and Miller, B. F.: A Direct Colorimetric Method for the Determination of Inulin in Blood and Urine. J. Biol. Chem., *127*:609–616 (March), 1939. Alving, A. S. and Miller, B. F.: A Practical Method for the Measurement of Glomerular Filtration Rate (Inulin Clearance) with an Evaluation of the Clinical Significance of this Determination. Arch. Int. Med., *66*:306–318 (Aug.), 1940. Miller, B. F., Alving, A. S., and Rubin, Jack: The Renal Excretion of Inulin at Low Plasma Concentrations of this Compound and Its Relationship to the Glomerular Filtration Rate in Normal, Nephritic, and Hypertensive Individuals. J. Clin. Invest., *19*:89–94 (Jan.), 1940. Alving, A. S., Flox, Jack, Pitesky, Isadore and Miller, B. F.: Further Notes on the Colorimetric Determination of Inulin in Blood and Urine. Jour. Lab. and Clin. Med., *27*:115–118 (Oct.), 1941.

solution is injected slowly intravenously. The patient should empty his bladder one hour after injection and this sample should be discarded. The urine is collected at two and three-hour intervals and analyzed for inulin content. At one and a half and two and a half hours after injection, 15 c.c. or more of blood is taken and either the serum or plasma examined for inulin content. There is no claim made that this method should supplant urea clearance, but it may be useful in clinical investigative work, as this substance evidently is of interest in its relationship to glomerular filtration.

Diodrast also has been used for clearance studies. While inulin is filtered out of the blood only by the glomeruli of the kidneys, diodrast is filtered by both the glomeruli and tubules. Smith[1] stated that in one minute the normal kidneys clear no glucose from the blood but may clear urea from as much as 70 c.c. of blood. In the same time 125 c.c. of blood may be cleared of inulin and 700 c.c. of blood may be cleared of diodrast.

V. THE URINE IN DISEASE

In this section the characteristics of the urine in those diseases which produce distinctive urinary changes will be briefly reviewed.

1. Acute Congestion of the Kidneys.—This regularly occurs as an early stage of acute nephritis, but more frequently occurs independently as a result of the temporary irritation of toxins, certain drugs, and so forth. The urine is decreased in volume, highly colored, strongly acid. Albumin is always present, varying from traces to considerable amounts, depending upon the severity of the case. The sediment shows a few hyaline and finely granular casts and occasional red blood corpuscles. In severe acute congestion the urine approaches that of acute nephritis, since there is, in fact, no sharp line to be drawn between the two conditions.

2. Chronic Passive Congestion of the Kidneys.—This occurs most commonly as a part of general venous stasis due to cardiac decompensation. The volume of urine is somewhat low and the color and specific gravity high. As a rule, albumin is present in small amount only; in very marked congestion it becomes more abundant. As the liver is generally damaged in cases of long-continued venous stasis, moderate amounts of urobilinogen may be found in the urine. The sediment contains a few hyaline and finely granular casts; and a few red blood corpuscles and renal epithelial cells may occasionally be found. Estimation of blood urea fails to show any appreciable amount of nitrogen retention in cases of passive hyperemia, whereas the phenolsulfonephthalein test indicates a marked degree of kidney insufficiency which improves when the congestion is relieved.

[1] Smith, H. G.: Studies in the Physiology of the Kidney. Porter Lecture, University of Kansas, 1939, 106 pp.

3. Nephritis.—The various degenerative and inflammatory conditions which are grouped under the name of nephritis occupy an extremely important place in medicine and deserve somewhat extended discussion. Owing to lack of correlation between clinical observations and the microscopic structure of kidneys removed at autopsy there is no classification which is satisfactory alike to clinician and pathologist. The forms which are generally given consideration in clinical work are few in number, namely, acute nephritis; chronic diffuse (parenchymatous) nephritis; chronic interstitial nephritis, which includes the arteriosclerotic type; and pyelonephritis, which represents an upward extension of an inflammation already established in the pelvis of the kidney. In acute diffuse nephritis the injury may be borne chiefly by the glomeruli, as in the nephritis of scarlet fever, or by the epithelium of the convoluted tubules, as in poisoning by mercuric chloride. It should therefore be possible to distinguish an acute glomerulonephritis and an acute tubular form, but the distinction may be difficult or impossible in practice except by consideration of etiology. In a sense, except perhaps at the very outset, nephritis is always diffuse, since glomeruli, tubules, and interstitial tissue are all involved to some extent, regardless of which of them has sustained the primary and chief injury. In the course of a chronic nephritis, now one of these, now another, may dominate the clinical and pathologic picture.

There is a tendency at present to discard the anatomic classification altogether. Some, following Christian, divide nephritis into acute and chronic, with subdivisions of the latter based upon the presence or absence of edema or vascular hypertension. Addis has classified nephritis as **hemorrhagic; degenerative,** with desquamation of renal cells; and **arteriosclerotic,** accompanied by hypertension.

In view of the unfortunate tendency of many physicians to rely solely upon the laboratory report for a diagnosis of nephritis, the fact will bear emphasis that here, as in most conditions, the laboratory observations furnish only a part of the data necessary for a diagnosis. Even with every available aid the diagnosis of nephritis may be difficult and the designation of the exact type impossible.

In all forms of nephritis the urine contains **albumin** and **tube casts.** These are the classical signs, and they remain the most delicate indicators of pathologic changes in the kidneys, although they tell little of its nature, extent, and seriousness. The amount of albumin is extremely variable, depending upon the form and severity of the disease. It ranges from traces so small as to be overlooked in a careless examination in many cases of chronic interstitial nephritis, to as

high as 3 or 4 per cent in exceptional cases of chronic parenchymatous nephritis. Urine containing more than 2 per cent of albumin solidifies upon boiling. Upon the other hand, as has already been emphasized, albumin may be present even in considerable amount in many conditions other than nephritis—a fact not well enough appreciated by the average practitioner. The presence of tube casts has also been popularly ascribed greater significance than is warranted, especially in differentiating the several forms of nephritis. A few tube casts of the hyaline and finely granular varieties are often encountered in routine examinations, and may point to nothing more than some slight temporary irritation or circulatory disturbance in the kidney

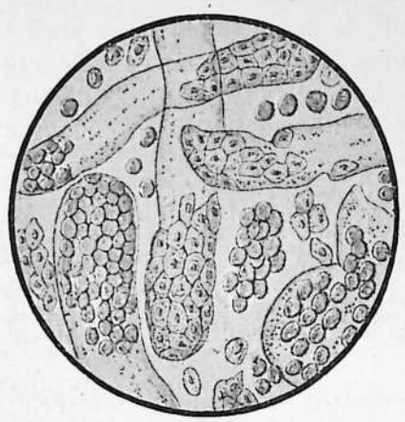


Fig. 84.—Sediment from acute hemorrhagic nephritis: Red blood corpuscles; leukocytes; renal cells not fattily degenerated; epithelial and blood casts (Jakob).

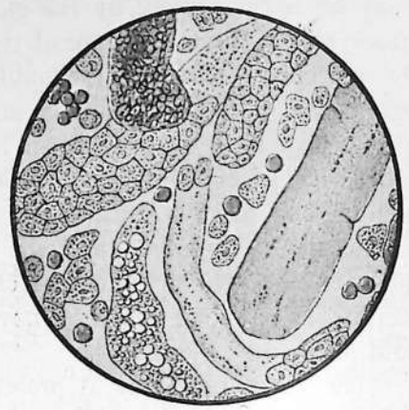


Fig. 85.—Sediment from chronic parenchymatous nephritis: Hyaline (with cells attached), waxy, brown granular, fatty, and epithelial casts; fattily degenerated renal cells, and a few white and red blood corpuscles (Jakob).

or the sclerotic change incident to old age. Nevertheless, the presence of casts always indicates some disturbance in the kidneys, and *their continued presence should arouse strong suspicion of nephritis unless other adequate cause is obvious*. Much more significant of nephritis are coarsely granular, fatty, epithelial, and blood casts.

In addition to casts, the microscope may reveal, according to the variety of nephritis, red blood corpuscles, usually in the form of "shadow cells"; renal epithelial cells, more or less degenerated; and small or moderate numbers of pus corpuscles.

An important feature of nephritis is the failure of the kidneys adequately to excrete **normal solids,** which results in an accumulation of these substances in the body. Chief interest attaches to sodium

chloride and the nitrogenous waste products typified by urea. In some cases urea only is retained, in others sodium chloride, and in still others both these substances. In the case of urea, retention is best measured by the exact methods of blood chemistry and is discussed at another place. Sodium chloride retention is generally associated with edema, and is most typically seen in chronic diffuse (parenchymatous) nephritis in which edema is a common symptom. During a period of elimination of edema the sodium chloride of the urine is strikingly increased. The total volume of the urine likewise demands attention when nephritis is suspected, being diminished in well-defined cases of some forms and much increased in others.

The more characteristic features of the urine in the various types of nephritis which are based on pathologic changes in the kidney, are indicated in the table on page 180, and need not be repeated here, but the data there set down cannot be relied upon implicitly. They show what may be expected in typical cases. The findings are often subject to marked variations at different periods in the course of the same case. For example, even in typical chronic interstitial nephritis there may at times occur marked epithelial degeneration in small groups of tubules, and these may supply the urine with degenerated epithelial cells, coarsely granular, epithelial or fatty casts, and other structures commonly regarded as characteristic of chronic parenchymatous nephritis. Many clinicians prefer a simple clinical classification as mentioned before.

Perhaps the most useful of all laboratory methods which aid in recognizing the disease and in following the progress of individual cases when diagnosed are the various **functional tests.** They should, however, be used with full appreciation that some of the functions may be disturbed, while others remain unaltered, and that the efficiency of the kidneys may be markedly impaired by various extrarenal conditions, the chief of which are listed on page 181. Upon the other hand, owing to the remarkable reserve power of the kidneys, their function is sometimes not appreciably disturbed by actual organic disease until the damage is considerable. Indeed, it is well known that in some early cases of nephritis the functional activity of the kidneys may be temporarily exalted, owing probably to irritation of the uninjured portions. Even after the disease is well established there may be marked divergence between the functional derangement and the recognizable anatomic lesions. This is particularly true in acute nephritis and in the earlier stages of chronic diffuse (parenchymatous) nephritis.

Of all the means of estimating renal function, the *phenolsulfone-*

THE URINE IN NEPHRITIS

	Physical.	Chemical.	Microscopic.
Acute nephritis. (Nephritis of toxemia of pregnancy.)	Quantity diminished, often very greatly. Color dark; may be red or smoky. Specific gravity, 1.020 to 1.030.	Urea and chlorides low. Much albumin: up to 1.5 per cent. Reaction acid.	Sediment abundant, red or brown. Many casts, chiefly granular, blood and epithelial varieties; fatty casts in convalescence. Red blood cells abundant. Numerous renal epithelial cells. Few pus corpuscles.
Nephrotic variation.	Quantity diminished. Color dark. Specific gravity, 1.020 to 1.040.	Urea and chlorides nearly normal, or high. Large quantity of albumin. Reaction acid.	Moderate number of casts, chiefly hyaline and granular. A few red blood cells. Lipoid bodies usually present.
Chronic diffuse (parenchymatous) nephritis. (Large white kidney.)	Quantity usually diminished. Color variable, often pale and hazy. Specific gravity, 1.010 to 1.020.	Urea and chlorides variable. Largest amounts of albumin: up to 3 per cent. Reaction acid.	Sediment rather abundant. Many casts of all varieties; fatty casts and casts of degenerated epithelium most characteristic. Blood present in traces: abundant only in acute exacerbations. Numerous fattily degenerated renal epithelial cells, often free globules of fat, and a few leukocytes.
Chronic interstitial nephritis. (Contracted kidney.)	Quantity markedly increased, especially at night. Color pale, clear. Specific gravity, 1.005 to 1.015.	Urea and chlorides low in advanced cases. Albumin present in traces (often overlooked), increasing in late stages. Reaction acid.	Sediment very slight. Few narrow hyaline and finely granular casts. No blood except in acute exacerbations. Very few renal cells. Uric-acid and calcium-oxalate crystals common.
Pyelonephritis.	Quantity often increased and color pale; slightly or moderately cloudy. Specific gravity normal or low.	Urea and chlorides low in advanced cases. Albumin more abundant than can be explained by pus. Reaction acid.	Variable amount of pus. Many varieties of casts, pus casts most characteristic. Renal and pelvic epithelial cells. Red blood corpuscles occasionally.
Amyloid degeneration of kidney.	Quantity normal or moderately increased. Color pale, clear. Specific gravity, 1.012 to 1.018.	Slight decrease of urea and chlorides. Variable amounts of albumin and globulin.	Sediment slight. Moderate number of hyaline, finely granular, and sometimes waxy casts.

phthalein test has found widest acceptance, partly because it has been so long in use that its reliability is well established, partly because it is easily carried out in office practice without need of special apparatus or unusual skill.

As has already been indicated, the normal kidneys excrete 60 to 75 per cent of the dye in the two-hour period allowed for the test. A fall to 40 per cent indicates impaired function and demands serious attention, although it does not necessarily mean nephritis. Reduction to 20 or 30 per cent is the rule in well-marked cases of nephritis; while excretion of less than 10 per cent may usually be interpreted as forecasting an early fatal termination. Exceptionally, deaths from uremia have occurred when the output was as high as 40 per cent. This, like most of the functional tests, finds its greatest usefulness in the study of chronic interstitial nephritis, the form of kidney disease which is most often met in general practice and in which the urinary changes are least striking. Here the phenolsulfonephthalein output appears to be a very satisfactory index to the extent of the pathologic changes in the kidneys. In some cases a greatly lowered output may be the most striking evidence of a very serious kidney condition.

Despite its unquestioned usefulness in the diagnosis and prognosis of nephritis, the test has certain limitations which are too frequently overlooked in practical work. The chief of these, some of which are shared by the other functional tests, are:

(*a*) Entirely normal or even increased excretion is sometimes noted in acute nephritis, especially in acute glomerulonephritis.

(*b*) In certain other cases of acute nephritis extremely low excretion, reduced almost to zero, which in chronic nephritis would point to an inevitable early fatal outcome, may exceptionally be followed by complete recovery.

(*c*) For a short period at the outset of a chronic nephritis there may be exalted renal activity, with an output of 70 to 85 per cent. There also may be an increased output in liver disease.

(*d*) Low excretion, exceptionally as low as 10 to 15 per cent, is the rule in cardiac decompensation with chronic passive congestion of the kidneys, and this rises rapidly when the congestion is relieved by improved heart action.

(*e*) In chronic diffuse (parenchymatous) nephritis, although the output is significant, it sometimes presents inexplicable variations.

The *test meal for renal function* first advocated in this country by Mosenthal appears to be especially useful for the detection of very early kidney disease, since it may give definite evidence of renal disturbance at a time when the other functional tests still indicate

normal or exalted function. The method is simple enough to be readily carried out in private practice. The principle of the test and the exact procedure, together with the findings under normal conditions, are described on page 168. In chronic interstitial nephritis the findings after the test diet are remarkably constant. The earliest sign is usually nocturnal polyuria—night urine above 600 or 700 c.c.—and this may be noted when there are no other evident signs, or only a trace of albumin and a few hyaline casts in the urine. This is followed somewhat later by a lowering and a fixation of the specific gravity, both of which changes gradually become more marked as the disease advances. In advanced cases the specific gravity of the two-hour specimens is fixed at about 1.010 or lower, with maximum variation of only 1 to 3 points. In chronic diffuse (parenchymatous) nephritis, the urinary findings after the test meal are variable.

The simple concentration and dilution tests mentioned on page 170 are also very valuable in detecting chronic nephritis.

As has been stated in previous pages, the *degree of nitrogen retention* in the blood has come to be relied upon as an index of the functional efficiency of the kidneys. This is of great value in the differential diagnosis of well-established cases, and is especially useful in prognosis since it serves as a direct measure of the tendency to uremia, even though the true nature of uremia and the particular substance which may be responsible for it are alike unknown. The methods used for estimation of nitrogen retention and the normal values have been discussed on page 170; while the figures to be expected in progressive grades of kidney disturbance are given in the table on page 164. While a nonprotein nitrogen concentration above 90 mg. for each 100 c.c. of blood or urea nitrogen above 65 mg. is a reliable sign of a grave outlook, the figures generally accepted as indicating slight grades of renal insufficiency (for example, nonprotein nitrogen 30 to 45, or urea nitrogen of 16 to 27 mg. for each 100 c.c. of blood) cannot be relied upon unreservedly, since, owing chiefly to excess of protein in the diet, they may exceptionally be equaled when the kidneys are normal. Upon the other hand, since the kidneys have a capacity for the excretion of urea far in excess of normal demands they may be able to prevent accumulation of nitrogen even when seriously damaged.

The work of Wakefield, Power, and Keith[1] indicates that in early renal insufficiency the *retention of inorganic sulfates* may be demonstrated in blood serum before there is lowered excretion of phenolsulfonephthalein, or in some instances, before the kidney has lost its ability to concentrate urine to a specific gravity of 1.025 or more in the concentration test.

[1] Wakefield, E. G., Power, M. H., and Keith, N. M.: Inorganic Sulphates in the Serum in Early Renal Insufficiency. Significance of Determinations, Jour. Am. Med. Assn., *97*:913–917 (Sept. 26), 1931.

In chronic passive congestion of the kidneys the blood urea and nonprotein nitrogen undergo little or no change. Their estimation, therefore, taken in conjunction with the phenolsulfonephthalein test, which gives low values in this condition, may be of great service in cardiorenal conditions as a means of separating primary renal disease with secondary cardiovascular change from secondary renal disturbances resulting from passive congestion due to a poorly compensated heart lesion.

The principal substances concerned in "nitrogen retention" are uric acid, urea, and creatinine. It is believed by some, following the work of Myers, Fine and Lough, that estimation of these substances separately gives the best insight into the renal condition. According to their view there is a definite order in which the substances are retained. Thus, damage to the kidneys is first manifested functionally by retention of uric acid; and increase of this substance in the blood may therefore be the earliest sign of kidney disease, especially of chronic interstitial nephritis. Of course, other sources of uric acid accumulation, such as gout, must be excluded. As the disease progresses urea also begins to accumulate; and when the function is markedly depressed all three substances are retained. According to Myers and Lough, the creatinine concentration in the blood is the surest guide to a serious prognosis, any appreciable retention indicating a grave disorder of the renal function. Not all, however, have found the creatinine values so significant. In this connection it is necessary to recognize the work of Behre and Benedict,[1] which indicates that there may be no creatinine in the blood, and that the substance which has been thought to be creatinine is some unknown substance which reacts in a similar manner in the methods now in use. This does not necessarily overthrow the conclusions regarding the value of the determinations.

The **blood picture** in nephritis may for convenience be mentioned here. In chronic interstitial nephritis the blood usually exhibits the changes characteristic of a moderate secondary anemia, which may become fairly marked as the disease progresses. If cardiac decompensation supervenes, the red corpuscles and hemoglobin tend to rise to normal or even above. In chronic diffuse (parenchymatous) nephritis

[1] Behre, J. A. and Benedict, S. R.: Studies in Creatine and Creatinine Metabolism. IV. On the Question of the Occurrence of Creatinine and Creatine in Blood, Jour. Biol. Chem., *52*:11–33 (May), 1922.

the anemia is much more striking, red corpuscles often falling to 2,500,000 and hemoglobin sinking to a corresponding level. Brown[1] considers this of grave prognostic significance, indicating injury to the bone marrow. There may be moderate polymorphonuclear leukocytosis late in the disease.

4. Amyloid Disease of the Kidneys.—Here the pathologic picture is that of a chronic nephritis plus a deposit of amyloid material in the glomerular tufts and interstitial tissue. The urine is abundant, of low specific gravity, and fairly rich in albumin and globulin. Urinary solids are not generally much decreased unless the disease is far advanced. Casts appear in variable numbers. These are chiefly hyaline; but granular, waxy, and occasional fatty casts also occur. The waxy casts are regarded as most typical, although, contrary to an old belief, they are rarely composed of true amyloid material. There may be marked retention of Congo red or vital red as referred to on page 334.

5. Renal Tuberculosis.—In early cases the urine may be practically normal in appearance, but is more frequently pale and somewhat cloudy from the presence of a small amount of pus. Pyuria with no bacterial growth in ordinary cultures is strongly suggestive of tuberculosis. The volume may not be affected, but is apt to be increased, the reaction is acid, and there are traces of albumin and a few renal cells. In advanced cases, or those in which the pelvis of the kidney is involved in the tuberculous process, the urine is usually pale, cloudy, and alkaline, has an offensive odor, and is irritating to the bladder. In such cases albumin and pus are always present, though frequently not abundant. The pus is generally intimately mixed with the urine and does not settle so quickly as does the pus of cystitis. Casts, though present, are seldom abundant, and are obscured by the pus. Traces of blood are common. Tubercle bacilli are nearly always present even when the pus is extremely slight, and their detection is essential for the diagnosis. In most cases they can be found by appropriate staining of the sediment (p. 156). The possibility of confusion with the smegma bacillus must, of course, be kept in mind. In other cases inoculation of guinea-pigs will be necessary. This method of detecting tubercle bacilli, which is described on page 607, is so simple and so trustworthy that it should be resorted to in all doubtful cases.

Temporary or permanent occlusion of the ureter on the diseased

[1] Brown, G. E., and Roth, Grace M.: The Anemia of Chronic Nephritis, Arch. Int. Med., *30*:817–840 (Dec.), 1922.

side, with consequent change in the urinary findings, occurs, according to Braasch, in about 10 per cent of cases.

6. Malignant Tumors of the Kidney.—Hematuria, intermittent or constant, moderate or very marked, is the only urinary sign which occurs with any degree of regularity. It is observed at some time in the course of practically all cases, and in probably three quarters of them it is the first definite symptom.

7. Renal Calculus.—The urine is usually somewhat concentrated, with high color and strongly acid reaction. Small amounts of albumin and a few casts may be present as a result of kidney irritation. Blood is frequently present, especially in the daytime and after severe exercise. Crystals of the substance composing the calculus—

Fig. 86.—Sediment from calculous pyelitis: Numerous pus corpuscles, red blood corpuscles, and caudate and irregular epithelial cells; a combination of hyaline and pus casts and a few uric-acid crystals (Jakob).

uric acid, calcium oxalate, cystine—may often be found in the freshly voided urine (see Sulkowitch test, page 132). The presence of a calculus generally produces pyelitis, and variable amounts of pus then appear, the urine remaining acid in reaction.

8. Pyelitis.—In pyelitis the urine is slightly acid, and contains a small or moderate amount of pus, together with many spindle and caudate epithelial cells. These findings may be intermittent, owing to occasional blocking of the ureter on the diseased side. Pus casts and also other forms may appear when the process extends up into the kidney tubules as is usually the case (Fig. 86). Albumin is always present, and its amount, in proportion to the amount of pus, is decidedly greater than is found in cystitis. This fact is of much value

in differential diagnosis. Even when pus is scanty albumin is rarely under 0.15 per cent, which is the maximum amount found in cystitis with abundant pus.

Bacteriologic studies reveal the colon bacillus in about half of the cases. Staphylococci, *Bacillus proteus vulgaris*, and others have been found.

9. Cystitis.—In acute and subacute cases and in many chronic cases of moderate severity the urine is acid and contains a variable amount of pus, with many epithelial cells from the bladder—chiefly large round and pyriform cells. Red blood corpuscles are often numerous. Albumin is present in small amount—less than 0.15 per cent.

Fig. 87.—Sediment from cystitis (chronic): Numerous pus corpuscles, epithelial cells from the bladder, and bacteria; a few red blood corpuscles and triple phosphate and ammonium biurate crystals (Jakob).

Of the micro-organisms, the colon bacillus is most frequently found in these cases.

In neglected chronic cases and those due to prostatic obstruction the urine is generally alkaline. It is pale and cloudy from the presence of pus, which is abundant and settles readily into a viscid sediment. The sediment usually contains abundant amorphous phosphates and crystals of triple phosphate and ammonium biurate (Fig. 87). Vesical epithelium is common. Numerous bacteria are always present and they may be of many varieties: *Bacillus coli*, *Bacillus proteus vulgaris*, *Staphylococcus aureus* and *albus*, and others.

10. Vesical Calculus, Tumors, and Tuberculosis.—These conditions produce a chronic cystitis, with its characteristic urine. Blood, however, is more frequently present and more abundant than

in ordinary cystitis. With neoplasms, especially, considerable hemorrhages are apt to occur. Particles of the tumor are sometimes passed with the urine. No diagnosis can be made from the presence of isolated tumor cells. In tuberculosis tubercle bacilli can generally be detected.

11. Diabetes Insipidus.—Characteristic of this disease is the continued excretion of very large quantities of pale, watery urine, containing neither albumin nor sugar. The specific gravity varies between 1.001 and 1.005. The daily output of solids, especially urea, is increased.

12. Diabetes Mellitus.—The twenty-four-hour quantity of urine is very large as a rule, usually 4 or 5 liters in severe cases: and in general the volume varies directly with the percentage of sugar. Exceptionally it is above 25 liters. The color is generally pale, while the specific gravity in untreated advanced cases is nearly always high—1.030 to 1.050, very rarely below 1.020. Sometimes in mild or early cases the urine varies little from the normal in quantity, color, and specific gravity, and even very low specific gravity does not exclude diabetes.

The most characteristic sign of the disease is the presence of dextrose in the urine. In the vast majority of instances glycosuria means diabetes. In some extremely mild cases the amount is very small, and can sometimes be detected only in portions of urine secreted several hours after a meal rich in carbohydrates; in ordinary mild cases the urine regularly contains sugar, but can be rendered sugar-free by the withdrawal of carbohydrates from the diet; in severe cases the glycosuria continues even when the carbohydrates are completely withdrawn. The amount of dextrose in severe cases is usually 2 to 4 per cent and may even exceed 8 per cent, while the total elimination may be 600 Gm. in twenty-four hours. Acetone is generally present in moderately advanced cases, especially when the carbohydrate intake is restricted. Diacetic and oxybutyric acids may be present in the more severe cases and, if persistent, usually warrant an unfavorable prognosis. They are responsible for the acidosis which so frequently accompanies this disease and which may reach its culmination in diabetic coma. Accompanying the acidosis there is a corresponding increase in amount of ammonia; and, in general, estimations of ammonium salts will serve as an index of the degree of acidosis in diabetes.

13. Renal Glycosuria.—Recent studies indicate that this condition, sometimes wrongly called renal diabetes, is more common than has been recognized. The essential feature appears to be a lowering

of the renal threshold for sugar.[1] The urine contains sugar, sometimes continuously, sometimes only after meals, while the blood sugar remains within normal limits. Renal function is normal as regards the phenolsulfonephthalein test. The patient's health is affected little or not at all, and the condition may probably be regarded more as an anomaly than as a pathologic entity.

[1] Lewis, D. S., and Mosenthal, H. O.: Renal Diabetes, Bull. Johns Hopkins Hosp., *27*:133–138 (May), 1916. Beard, A. H., and Grave, F.: Renal Glycosuria, Arch. Int. Med., *21*:705–715 (June), 1918. See also Schneiderman, H.: Renal Glycosuria, Jour. Am. Med. Assn., *80*:825–828 (March 24), 1923.

CHAPTER III

THE BLOOD

Preliminary Considerations.—The blood consists of a fluid of complicated and variable composition, the plasma, in which are suspended great numbers of microscopic structures, namely, red corpuscles, white corpuscles, blood platelets, and blood dust.

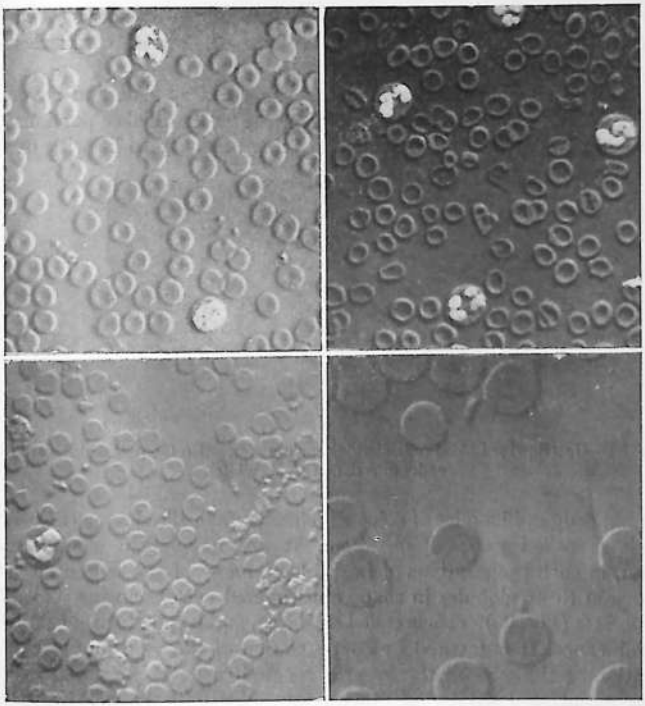


Fig. 88.—Bas relief photomicrographs of blood corpuscles, normal biconcave disks, shallow thin corpuscles in hypochromic anemia. Spherocytes of congenital hemolytic cterus (courtesy R. L. Haden).

Red corpuscles, or **erythrocytes,** appear as biconcave disks (Fig. 88), red when viewed by reflected light or in thick layer, and straw colored when viewed by transmitted light or in thin layer. They give

the blood its red color. They are cells which have been highly differentiated for the purpose of carrying oxygen from the lungs to the tissues. This is accomplished by means of an iron-bearing protein, hemoglobin, which they contain. In the lungs hemoglobin forms a loose combination with oxygen, which it readily gives up when it reaches the tissues. Normal erythrocytes do not contain nuclei. They are formed from preexisting nucleated cells in the bone marrow. Their life, judged from the length of time that transfused corpuscles remain in the circulation in anemia, averages about eighty-three days (Wearn, Warren, and Ames).[1, 2] The total volume of the red corpuscles is slightly less than half that of the blood.

If a small drop of blood be taken upon a clean slide and covered with a clean cover glass, the red corpuscles in the thicker portions of the preparation will often show a striking tendency to lie with over-

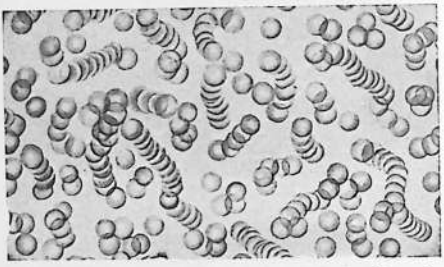


Fig. 89.—Unstained red blood cells, showing rouleaux formation (Stengel and Fox; drawn under direction of C. Y. White).

lapping edges, like piles of coins which have been tilted over (Fig. 89). This is called *rouleaux formation*. It has no clinical significance. Also, in such preparations of fresh blood, many of the red corpuscles are seen to be globular in shape and covered with knob- or spinelike processes (Fig. 90). This is called *crenation*, and has little or no clinical significance. It is favored by concentration of the fluid due to evaporation at the edge of the cover. Crenated corpuscles are often seen in concentrated urine and other body fluids and should always be recognized.

[1] Wearn, J. T., Warren, Sylvia, and Ames, Olivia: The Length of Life of Transfused Erythrocytes in Patients with Primary and Secondary Anemia, Arch. Int. Med., *29:* 527–538 (April), 1922.

[2] Ashby, Winifred: The Present Status of the Question of the Length of Life of the Unagglutinable Transfused Red Blood Corpuscle, Arch. Int. Med., *43:*481–489 (Oct.), 1924.

White corpuscles, or **leukocytes**, are less highly differentiated cells. There are several varieties. They all contain nuclei, and most of them contain granules which vary in size and staining properties. They are formed chiefly in the bone marrow and lymphoid tissues. Their function is not fully understood. It appears to be concerned chiefly with the protection of the body against harmful agencies, in part through phagocytosis, in part through production of antitoxic substances and of ferments which play an important rôle in pathology.

Blood platelets, or *blood plaques*, are colorless or slightly bluish, spheric or ovoid bodies, usually about one third or one half the diameter of an erythrocyte, sometimes even as large as an erythrocyte. They appear to be constricted-off portions of the pseudopodia of certain giant cells of the bone marrow. Their function is not fully known, but is in some way connected with coagulation.

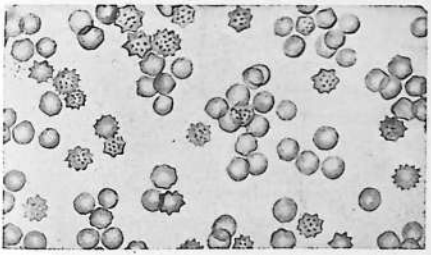


Fig. 90.—Unstained red blood cells, showing crenation (Stengel and Fox; drawn under direction of C. Y. White).

The **blood dust of Müller** (*hemoconia*) consists of fine granules which have brownian motion. The larger granules resemble micrococci. Little is known of them and they are given no consideration in clinical blood examinations. Most of them are probably minute fat globules, others granules from disintegrated leukocytes.

The **total amount** of blood, as shown by the method of Keith, Rowntree, and Geraghty,[1] averages about one twelfth of the body weight. Roughly it may be put at 85 c.c. for each kilogram (2.2 pounds) or about 5 or 6 liters for the average adult.

The **reaction** is faintly alkaline and is maintained throughout life at a remarkably constant level, as is explained in the section on Acidosis (p. 648).

[1] Keith, N. M., Rowntree, L. G., and Geraghty, J. T.: A Method for the Determination of Plasma and Blood Volume, Arch. Int. Med., *16*:547–576 (Oct.), 1915.

The color is due to the presence of hemoglobin in the red corpuscles, the difference between the bright red of arterial blood and the purplish red of venous blood depending upon the relative proportions of oxyhemoglobin and reduced hemoglobin. The depth of color depends upon the amount of hemoglobin. In very severe anemias the blood may be so pale as to be designated as "watery." The formation of carbon-monoxide-hemoglobin in coal-gas poisoning gives the blood a bright cherry-red color, while formation of methemoglobin in poisoning with potassium chlorate and certain other substances gives a chocolate color.

The clear, pale, straw-colored fluid which remains after coagulation (p. 195) and separation of the clot is called **serum.** In the serum are found the numerous substances which the tissues elaborate for protection against bacterial and other harmful agents. In most cases these substances, or "antibodies," are elaborated only when the harmful agent is present in the body, and they are "specific," that is, they are effective only against the one disease which has called them forth. A test for the presence of the antibody is, therefore, a test for the existence of the particular disease. The various tests based upon these principles have within recent years become a very important part of clinical laboratory work. They are discussed in the chapter upon Serodiagnostic Methods.

Clinical study of the blood may be discussed under the following heads: I. Methods of obtaining blood for examination. II. Coagulation. III. Hemoglobin. IV. Enumeration of erythrocytes. V. Color index. VI. Volume index. VII. Enumeration of leukocytes. VIII. Enumeration of blood platelets. IX. Study of stained blood. X. Special blood pathology. XI. Miscellaneous methods. XII. Isohemagglutination groups.

I. METHODS OF OBTAINING BLOOD

For most clinical examinations including cell counts and hemoglobin determinations, blood is best obtained from a vein. However, for making differential counts, and also for the enumeration of cellular elements it may be obtained from the lobe of the ear, the palmar surface of the tip of the finger, or, in the case of infants, the plantar surface of the great toe or of the heel. In the case of the ear, the edge of the lobe, not the side, should be punctured. With bed-ridden patients the finger will be found most convenient, otherwise the ear is preferable, as it is less sensitive. An edematous or congested part should be avoided; also a cold, apparently bloodless one. The site should be well rubbed with alcohol to remove dirt and epithelial débris

and to increase the amount of blood in the part. After allowing sufficient time for the circulation to equalize, the skin is punctured with a blood lancet (Fig. 91) (of which there are several patterns upon the market) or preferably with a short, stout, sharp, three-cornered needle, which is known as a glover's needle. This may be fixed in the cork of a small vial that contains some antiseptic, such as a 5 per cent solution of phenol, or a solution of one of the noncorrosive mercurial compounds, which keeps the needle sterile. It should be rinsed in 50 per cent alcohol before it is used. The puncture is practically painless if properly done with a sharp needle. It is made *with a firm, quick stab*, which, however, must not be so quick nor made from so great a distance that its site and depth are uncertain. The depth may be guarded with the thumb-nail if the lancet is not provided with a guard, but this should not be necessary. The first drop of blood which appears should be wiped away, and the second used for examination. The skin at the site of the puncture must be dry, else the blood will not form a rounded drop as it exudes. The blood should not

Fig. 91.—Daland's blood lancet.

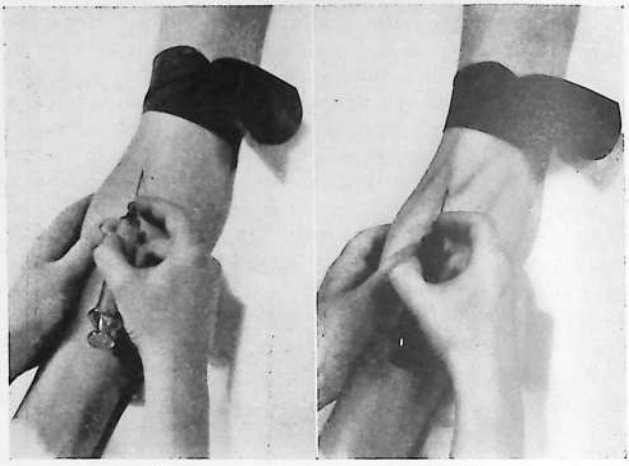
be pressed out, since this dilutes it with serum from the tissues; but moderate pressure some distance above the puncture is allowable.

Method of Obtaining Blood from a Vein.—Cleanse the skin at the bend of the elbow by rubbing well with 70 per cent alcohol, and wipe the skin dry with absorbent cotton.

Bind a rubber bandage firmly around the upper arm. The end is tucked under the last round in such a manner that a slight pull will release the bandage. With a rubber bandage one turn will suffice (Fig. 92). The cuff of the blood pressure apparatus answers admirably. Instead of a bandage it will often be sufficient for an assistant or even the patient to grasp the upper arm firmly.

Have the patient extend his arm fully and open and close the fist a few times to cause the veins to become distended. Even if not seen, they can usually be felt as cords beneath the skin. In fat persons veins which show as blue streaks are usually too superficial and too small. Grasp the forearm with the left hand, draw the skin tense with the thumb, and insert a sterile hypodermic needle attached to a sterile syringe into any vein that is prominent. The needle should be large—about 19 to 21 gauge. It should go through the skin about 3 mm. from the vein with the bevel at its tip upper-

most, thus requiring two movements, one to puncture the skin, one to enter the vein.



A B

Fig. 92.—Obtaining blood from a vein. A, Photograph made with ordinary film, veins palpable but not visible. B, Photograph made with film sensitive to infra-red rays and showing deep veins of arm.

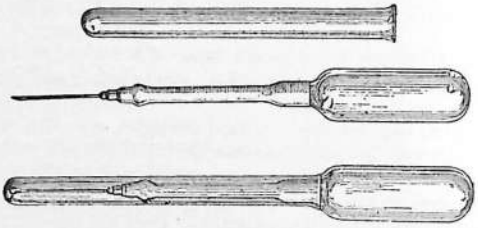


Fig. 93.—Keidel's vacuum tube for collecting blood from a vein, consisting of a sealed ampule, a needle with rubber connection, and a small glass test tube to serve as cap. After the needle has entered the vein the stem of the ampule is crushed within the rubber connection and blood enters because of the vacuum. Similar tubes containing sterile culture media are upon the market. (Courtesy of Hynson, Westcott, and Dunning, Baltimore, Md.)

When sufficient blood is obtained the bandage is first removed and the needle is then withdrawn, this order being followed to avoid formation of a hematoma. It is usually easy to secure 5 to 15 c.c. of blood, or even more,

if required. *If the needle be sharp and smooth* the procedure causes the patient surprisingly little inconvenience, seldom more than does an ordinary hypodermic injection. Dull needles may be sharpened by rubbing the bevel, point forward, on a hard, fine oil stone, and then smoothed with Bon Ami.

There is rarely any difficulty in entering a vein except in children and in adults when the arm is fat and the veins are small. If desired, one of the veins about the ankle can be used. In the case of infants, blood may be secured from the superior longitudinal sinus by puncturing through the posterior angle of the anterior fontanel. A short needle (about $\frac{3}{8}$ inch) of rather large caliber should be used.

Instead of a syringe many other devices for securing blood from a vein may be employed. Two of these are shown in Figures 93 and 94, which indicate their construction in sufficient detail. They possess the advantage that the blood can be drawn directly into any desired reagent or culture medium.

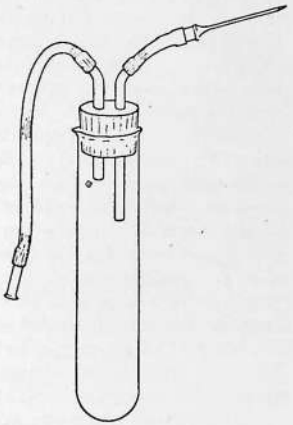


Fig. 94.—Device for drawing blood from a vein, using a large test tube, a centrifuge tube, or a small flask. It may be modified in various ways. The needle may be attached directly to the glass tube which is ground to fit it (Cummer), thus doing away with the rubber connection; or the hub of the needle may be fixed directly in the rubber stopper in place of the glass tube (Woolley). The glass delivery tube may be extended to the bottom of the container if it is desired to collect blood under a layer of liquid paraffin.

II. COAGULATION

Coagulation consists essentially in the transformation of fibrinogen, one of the proteins of the blood plasma, into fibrin by means of a ferment called thrombin. The presence of calcium salts is necessary. The resulting coagulum is made up of a meshwork of fibrin fibrils with entangled corpuscles and platelets. The clear, straw-colored fluid which is left after separation of the coagulum is called *blood serum*.

Because of its great interest in connection with the pathogenesis and differential diagnosis of the primary and secondary hemorrhagic diseases the mechanism of coagulation must be considered more in detail. The process is complicated and not fully understood, and the theories which have been offered to explain it are many and intricate. **Howell's theory** is one of the simplest and serves well as a basis for clinical work. Five "coagulation factors" are assumed to take part. Four of these—fibrinogen, calcium salts, prothrombin, and antiprothrombin—are normal and constant con-

stituents of the blood plasma. Prothrombin is held in some sort of combination with antiprothrombin and is by this means kept inactive so that coagulation within the vessels cannot occur. The fifth coagulation factor, thromboplastin, a coagulation-accelerating substance, is not present in the circulating blood, but resides in the tissue juice outside the blood vessels. When the blood escapes from the vessels it meets the thromboplastin of the tissues, and this at once neutralizes the antiprothrombin and thereby sets free prothrombin. The prothrombin thus released is then activated by calcium salts and becomes thrombin, a ferment-like substance whose function it is to transform fibrinogen of the plasma to fibrin. The fibrin forms a meshwork of threads in which the cellular elements are entangled, and thus constitutes the essential part of the coagulum. After a time the coagulum contracts, pressing out the serum.

Thus the process of coagulation takes place in three stages: (*a*) Neutralization of antiprothrombin and the formation of thrombin from the released prothrombin, these processes being invisible; (*b*) transformation of fibrinogen into fibrin, which is the visible evidence of clotting; (*c*) retraction of the clot. All the substances concerned in clotting except one are normally present in the plasma; that one, thromboplastin, resides outside the vessels in tissue juice. To explain coagulation of blood within the vessels, or of blood removed directly from a large vein without possible addition of tissue juice, it is assumed that enough thromboplastin to neutralize antiprothrombin and thus initiate clotting may be formed from disintegration of blood platelets. The much more rapid clotting of blood from an ordinary puncture is explained by the greater contamination with tissue juice.

Red cells and leukocytes take little or no active part in coagulation, but are passively entangled in the coagulum. Blood platelets, upon the other hand, are intimately connected with the process, and this seems to be their chief function (p. 244).

The Morawitz theory is very similar except that antiprothrombin is not considered to be present. Prothrombin and calcium become thrombin on the addition of thromboplastin. Thrombin and fibrinogen form fibrin (Fig. 95). (See page 326 for a classification of hemorrhagic diseases due to defects in the coagulation mechanism of the blood.)

For certain purposes, notably for bacteriologic and chemical work, it is necessary to prevent coagulation of the blood which is withdrawn. This may be accomplished by receiving it directly in a solution of 1 per cent sodium citrate (or ammonium oxalate) in physiologic salt solution, or into a tube containing a very little finely powdered neutral potassium oxalate. These substances form either soluble or insoluble calcium compounds, which render the calcium unavailable for purposes of clotting.

In clinical work five features of the clotting process are studied in appropriate cases: Coagulation time, character of the clot, prothrombin time, calcium time, and bleeding time. These are discussed in the following paragraphs:

1. Coagulation Time.—Normally, when blood is secured from an ordinary skin puncture, coagulation takes place in two to six minutes after it leaves the vessels, usually about four and a half minutes. The time is influenced by temperature, size of the drop, smoothness and cleanliness of the instruments, and other factors. Clotting may be more rapid after meals. Kugelmass has studied somewhat extensively the effect of diet on coagulation in rats, and also the relation of diet in hemorrhagic diseases. It is much more rapid when the blood is squeezed from a puncture than when it flows freely, owing to admixture with tissue juice. It is never possible to estimate the amount of such admixture when a skin puncture is the source of the blood. *For this reason, if results are to be relied upon, it is imperative that the blood be taken from a vein.* The normal coagula-

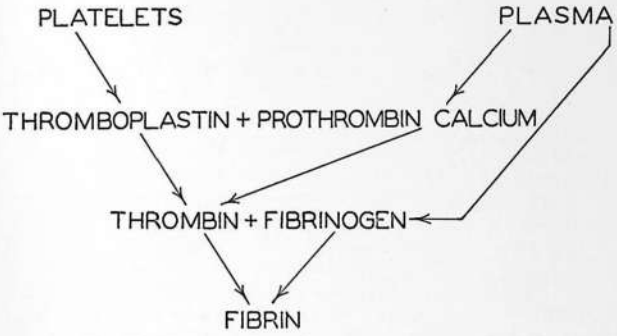


Fig. 95.—Morawitz theory of coagulation (Quick diagram).

tion time is then considerably longer, averaging about twenty minutes by Howell's method, five to ten minutes by Lee and White's; while in some pathologic conditions the discrepancy may be much greater. Lee and White cite an instance in which hemophilic blood with a true coagulation time of fifty minutes coagulated in five minutes when secured from an ear prick.

Shortening of coagulation time, which in some cases may be due to deficiency of antiprothrombin, is not of much clinical significance except in relation to possible thrombosis, as in typhoid. *Prolongation* is important. The greatest prolongation occurs in hemophilia, where it is usually one to several hours. There is less, though still marked, delay in melena neonatorum; in obstructive jaundice; and, less important clinically, in some anemias and leukemias, and in many infectious

diseases, notably pneumonia. Many apparently healthy individuals have coagulation time somewhat beyond the usually accepted normal limit. Aside from its value in the diagnosis of hemorrhagic diseases, estimation of coagulation time is important as a preliminary to operation when there is any reason to expect dangerous capillary oozing, as in tonsillectomies or operations upon jaundiced persons. It should always be supplemented by estimation of the bleeding time (p. 203).

There are many methods of ascertaining the coagulation time, and results by the different methods are not comparable because of difference in their end-points and in the conditions to which the blood is subjected. It is therefore well to adopt a single method for one's routine work and to keep all conditions as nearly uniform as possible. In every case absolute cleanliness of the instruments is imperative. It is always advisable to test the blood of a normal person under exactly the same conditions as a control.

Fig. 96.—Showing difference in shape of blood drops before coagulation (upper drop), and after coagulation (lower drop) (Duke's method).

Methods Which Use Blood from a Skin Puncture.—The puncture should be deep enough to ensure free flow of blood in order to lessen contamination with tissue juice. The first drop should be wiped off and the second used for the test. Time is counted from the first appearance of the drop.

The simplest method is to receive several drops of blood (well rounded drops 4 to 5 mm. in diameter) on a clean slide and to draw a needle through one or another of the drops at one-minute intervals. When shreds of fibrin cling to the needle and are dragged along by it coagulation has taken place. The slide may be kept inverted over a glass of warm water in order to avoid evaporation, which in a dry climate may entirely vitiate results. Duke uses a glass slide to which two glass disks 5 mm. in diameter are cemented. Well-rounded drops of blood—one from the patient, one from a normal person—are received on the disks, and the slide is inverted across the top of a glass or beaker containing water at 40° C. and covered with a towel. Coagulation is judged by the shape of the drops when the slide is held in a vertical position (Fig. 96).

A satisfactory method is to take up the blood in a capillary glass tube about 1.5 mm. in diameter. If clean, the tube fills readily by capillary attraction. Short sections of the tube are then carefully broken off at one-minute intervals after scratching with a file, and the ends are gently separated.

When coagulation has occurred threads of fibrin will be seen to span the gap between the broken ends. Some definite end-point should be adopted, as for example, when the fibrin will span a gap of 5 mm.

Methods Which Use Blood from a Vein.—The time is counted from the first appearance of the blood in the syringe. Any of the methods mentioned above may be used, the drops of blood being transferred from the syringe to the appropriate instrument, but the following are more satisfactory:

Lee and White's Method.—Secure the blood with a small hypodermic syringe, entering the vein as quickly as possible, and avoiding suction. Place 1 c.c. of blood in a test tube 8 mm. in diameter, which has been rinsed with physiologic salt solution just previously. Place the tube in a glass of water at about 75° F., although this is unnecessary if the room temperature lies between 65° and 90° F. Tilt the tube at intervals. Coagulation is assumed to be complete as soon as the tube can be inverted without displacing the clot. The normal coagulation time by this method is five to ten minutes. The greater the diameter of the tube, the slower the clotting.

Howell's method is similar. Fill a hypodermic syringe and its attached needle with a mixture of ether and petrolatum, force it out, and draw air into the syringe a few times. The ether will evaporate, leaving a thin coating of petrolatum. Secure 2 to 4 c.c. of blood with this syringe and place it in a test tube about 21 mm. in diameter. The end-point is judged by tilting and finally inverting the tube as in Lee and White's method. By this method the blood of healthy persons was found to coagulate in ten to thirty minutes, average about twenty minutes.

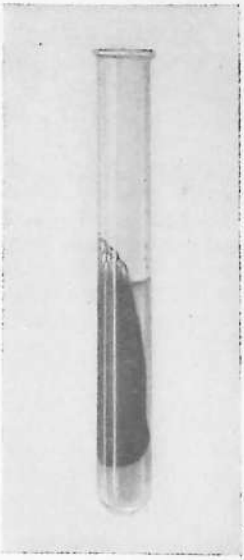


Fig. 97.—Normal clot retraction.

2. Character of the Clot.—Under normal conditions the coagulum begins to retract within a few minutes to one or two hours after it is formed, gradually separating from the wall of the vessel in which it is contained and expressing serum (Fig. 97). The process is completed within eighteen to twenty-four hours.

For the study of retraction 2 or 3 c.c. of blood are taken in a test tube, placed in an incubator at 37° C., and observed at hourly intervals. The time at which retraction occurs is noted.

The phenomenon of retractility is apparently due to presence of

blood platelets, for the degree of retractility closely parallels the number of platelets (p. 243). It is entirely independent of the coagulation time. This is most typically exemplified in purpura haemorrhagica (p. 324). The coagulum is formed within the normal time, but it retracts very little or not at all even after standing several days. In hemophilia, upon the other hand, with normal number of platelets, the blood coagulates very slowly, but the clot when once formed has normal retractile power.

3. Prothrombin Time.—For the differentiation of the various conditions in which the clotting mechanism is disturbed it is necessary to study not only the coagulation time but also to determine quantitatively the various coagulation factors: Prothrombin, fibrinogen, calcium, and so forth. Of these, prothrombin has had great clinical interest. A fair idea of the relative amount or strength of prothrombin was formerly thought to be given by the simple method of Howell detailed below.

The normal "prothrombin time" by this method averages about ten minutes, but may be as much as thirty minutes. It is constantly and very markedly prolonged in hemophilia—a fact which, according to Hurwitz and Lucas, makes the diagnosis of this disease relatively simple. The usual "prothrombin time" of hemophilic blood is five to twenty-five times the normal.

Howell and Cekada[1] showed that prothrombin is normal in quantity and in properties in hemophilia. The platelets are resistant, and because they do not disintegrate there is a lack of thromboplastic material. It is apparent then that the "prothrombin time" test does not indicate relative amounts of prothrombin; nevertheless, the procedure is still of value in the diagnosis of hemophilia.

Howell's Method for "Prothrombin Time."—1. Obtain about 2 c.c. of blood from a vein, using a syringe which has been rinsed out with physiologic salt solution. Avoid suction.

2. At once place the blood in a centrifuge tube which contains 0.25 c.c. of 1 per cent sodium oxalate in physiologic salt solution.

3. Mix by inverting several times and centrifugalize thoroughly.

4. Place 5 drops of the clear plasma in each of four small test tubes.

5. To these tubes add 0.5 per cent solution of calcium chloride in increasing quantities: 2 drops in Tube 1; 3 drops in Tube 2; 4 drops in Tube 3; 5 drops in Tube 4. Mix gently.

6. Coagulation will probably occur in all tubes, but not at the same rate. Its occurrence is recognized by invertibility of the tube as in Howell's coagulation method. *The coagulation time of the tube which clots earliest is*

[1] Howell, W. H., and Cekada, E. B.: The Cause of the Delayed Clotting of Hemophilic Blood, Am. Jour. Physiol., *78*:500–511 (Nov.), 1926.

the "prothrombin time." With each unknown blood a normal is run. The "prothrombin quotient" of Hurwitz and Lucas is found by dividing the time of the unknown by that of the normal control.

Quick[1] suggested a simplified method making use of recalcified plasma. In his test oxalated blood is divided into two equal portions. Centrifuge half for five minutes at a speed not over 1000 revolutions per minute and the other at 3000 revolutions per minute in an angle centrifuge. The clotting time of each specimen is tested after recalcification. In hemophilia the clotting time is markedly prolonged after high speed centrifugation while with slow centrifugation of the oxalated plasma there is an opportunity for the platelets to break up and the clotting time is relatively much shorter, although still somewhat prolonged.

Cheney[2] has utilized the principles of the Howell method for a simple plasma coagulation time test in studying vitamin K deficiency. The constancy of results warrants its use as a simple test provided physical conditions are kept constant. He uses 10 mg. of dry potassium oxalate for each 5 c.c. of blood. Place 0.5 c.c. of two per cent oxalate solution in a centrifuge tube and evaporate to dryness. Centrifuge the oxalated blood for a constant time at a constant speed and pipet off the clear plasma. Place two chemically clean test tubes, $\frac{3}{8}$ inch in diameter, in a rack and add varying amounts of 0.4 per cent calcium chloride as follows: To the first tube, add 0.2 c.c. (0.8 mg. calcium chloride) and to the second tube add 0.1 c.c. (0.4 mg. calcium chloride). Add 0.2 c.c. of the plasma to each of the tubes, shake briskly about ten times and start the stop watch. Note the time required for the formation of a firm clot in each tube. The length of time recorded from the mixing of the plasma and the calcium chloride to the time of clot formation is the plasma coagulation time for each of the two calcium chloride dilutions. The tube that shows the shortest coagulation time will indicate the tube containing the optimal amount of calcium for that sample of plasma. The average normal plasma coagulation time with the optimal amount of calcium is about five or six minutes, while in cases of vitamin K deficiency the time may be greatly prolonged from fifteen minutes to more than an hour. Cheney stated that the test has proved satisfactory in the diagnosis and treatment of jaundice associated with vitamin K deficiency. The speed and time of centrifuging and the temperature of the room should be kept as nearly constant as possible.

Quick's Prothrombin Time Test.—While Howell's method for "prothrombin time" is at times useful, it has been replaced very largely by a test devised by Quick,[3] in which thromboplastin in excess is added to oxalated plasma and then recalcified. The details are as follows:

[1] Quick, A. J.: The Diagnosis of Hemophilia, Amer. Jour. Med. Sci., *201*:469–474 (April), 1941.

[2] Cheney, Garnett: The Plasma Coagulation Time as a Simple Test for Vitamin K Deficiency, Amer. Jour. Med. Sci., *200*:327–337 (Sept.), 1940. Cheney, Garnett: The Normal Plasma Coagulation Time, Amer. Jour. Med. Sci., *203*:325–333 (Mar.), 1942.

[3] Quick, A. J.: The Clinical Application of the Hippuric Acid and the Prothrombin Tests, Amer. Jour. Clin. Path., *10*:222–233 (Mar.), 1940.

Reagents. (*a*) *Thromboplastin.*—Rabbit brain is prepared by completely removing all blood vessels and stripping off the pia. Macerate the brain in a mortar under acetone, replacing the acetone several times. Dry on a suction filter and store the nonadhesive, granular powder in small ampules from which the air is evacuated for three minutes by means of an oil vacuum pump and then chilled. Such a preparation apparently maintains its full activity indefinitely. Thromboplastin material prepared by this method may now be obtained from various biological supply houses. For use, mix 0.3 Gm. of dehydrated rabbit brain thoroughly with 5 c.c. of freshly prepared physiologic salt solution and incubate at 50° C. for ten to fifteen minutes. Remove the coarse particles by slow centrifugation and save the supernatant emulsion for use in the test.

Quick originally prepared thromboplastin material by drying brain tissue without the use of acetone. In many laboratories this older method is still used. The brain tissue is prepared by macerating and spreading it thinly upon large glass plates and drying for twenty-four hours in the incubator at 37° C. Such dried material when scraped from the plates may be stored in ampules or glass bottles and will keep at least two months; however, with this thromboplastin material, the normal prothrombin time will be several seconds longer than with the use of acetone-dehydrated brain tissue.

(*b*) *Sodium Oxalate Solution* (Tenth Molar Solution).—Dissolve 1.34 Gm. of anhydrous sodium oxalate (C. P.) in 100 c.c. of distilled water.

(*c*) *Calcium Chloride Solution* (Fortieth Molar Solution).—Dissolve 1.11 Gm. of anhydrous (C. P.) calcium chloride in 400 c.c. of distilled water.

Method.—1. Place 0.5 c.c. sodium oxalate solution (*b*) in a tube marked to hold exactly 5 c.c. of blood.

2. Withdraw blood by venipuncture and add to the oxalate solution (1) exactly 4.5 c.c. up to the 5 c.c. mark. Mix thoroughly by inverting two or three times.

3. Centrifuge for a few minutes at a moderate speed to obtain clear plasma.

4. Transfer 0.1 c.c. of clear plasma to a small serologic tube. Add 0.1 c.c. of calcium chloride solution (*c*) to the mixture (Fig. 98) and accurately measure with a stop watch or stop clock the number of seconds that elapses between the time that the calcium is added and the formation of a clot, as shown by tilting the tube to a horizontal position.

A "normal control" should be run for comparison. With this method the normal prothrombin time will be from twelve to fourteen seconds. Samples of plasma that require a considerably longer time for coagulation are thought to be definitely lacking in prothrombin.

The test is most useful in the preoperative management of patients with hepatic or gallbladder disease when considered before and after the administration of vitamin K (see page 431).

Smith and his colleagues,[1] using a thromboplastin extract, have recommended a *simple bedside test* using whole blood. Place 0.1 c.c. of thrombo-

[1] Smith, H. P., Ziffren, S. E., Owen, C. A., and Hoffman, G. R.: Clinical and Experimental Studies on Vitamin K, J. A. M. A., *113*:380-383 (July 29), 1939.

plastin extract in a small serologic tube and add freshly drawn blood up to a 1 c.c. mark previously made on the outside of the tube. The tube is inverted to obtain complete mixing of the blood and tilted every few seconds until clotting is observed.

4. Calcium Time.—When the coagulation time is abnormally slow, especially in jaundiced patients, it may be desirable to find whether the delay is due to deficiency of calcium and thus whether clotting can probably be hastened by administration of calcium.

To this end, a few cubic centimeters of blood are obtained from a vein, and 1 c.c. is placed in each of two test tubes 8 to 10 mm. in

Fig. 98.—Quick prothrombin time test: *a*, stop clock; *b*, solution of calcium chloride; *c*, thromboplastin emulsion; *d*, plasma; *e*, M/40 solution of sodium oxalate.

diameter. To one of these is added 3 drops of 1 per cent solution of calcium chloride (or 6 drops of 0.5 per cent solution). Should the blood in the tube containing the calcium coagulate within the normal time, while coagulation in the tube without calcium is delayed, the delay may be assumed to be due, in part at least, to deficiency of calcium in some form in the blood.

5. Bleeding Time.—This is a term used by Duke[1] to indicate

[1] Duke, W. W.: The Relation of Blood Platelets to Hemorrhagic Disease. Description of a Method for Determining the Bleeding Time and Coagulation Time and Report of Three Cases of Hemorrhagic Disease Relieved by Transfusion, J. A. M. A., *14*:1185–1192 (Oct.), 1910.

the time required for a small cut to cease bleeding. It does not necessarily parallel the coagulation time of the blood; for it is largely dependent upon the efficiency of the tissue juice in accelerating clotting, upon the elasticity of the skin, and upon the mechanical and chemical action of the blood platelets. The normal bleeding time is one to three minutes, although it may sometimes be as long as eight minutes. There is slight delay—usually five to ten minutes—in many severe anemias. Duke found great prolongation, ten to ninety minutes or longer, in two classes of conditions: (*a*) Those in which the blood platelets are very greatly reduced, notably purpura haemorrhagica, acute leukemia, and aplastic anemia; and (*b*) those in which the fibrinogen content of the blood is extremely low, as chloroform- and phosphorus-poisoning and certain destructive diseases of the liver

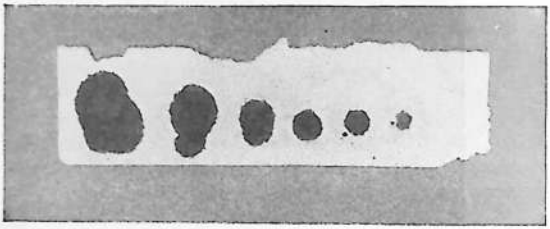


Fig. 99.—A strip of soft filter paper showing a row of blood blots obtained in a test of the bleeding time by Duke's method. Blots are made at half-minute intervals. In this case the bleeding time is normal (photograph natural size).

with a hemorrhagic tendency. Contrary to common belief the bleeding time by this method is usually not prolonged in hemophilia (p. 324).

In surgery the bleeding time may well be studied in connection with the coagulation time as a routine procedure before operation, but it should not take its place.

Duke's Method for Bleeding Time.—1. Make a slight cut in the lobe of the ear. The usual puncture as made for a blood count will answer if fairly deep, although it is generally best to use the point of a scalpel. Within wide limits the size of the cut is not important. It is most satisfactory when it gives a blot about 1 or 2 cm. in diameter at the end of the first half minute.

2. At half-minute intervals blot with a piece of absorbent paper all the blood which has flowed out. This furnishes a series of blots and the rate of decrease in size indicates the rate of decrease of the hemorrhage. The usual bleeding time is one to three minutes, giving two to six blots (Fig. 99). In certain pathologic conditions mentioned above bleeding may continue for

an hour or more. When the time is moderately prolonged the twentieth blot will be about one half the size of the first; when it is enormously prolonged the twentieth blot may be fully as large as the first.

III. HEMOGLOBIN

Hemoglobin is an iron-bearing protein which normally occurs in the circulating blood in two forms: *Oxyhemoglobin*, chiefly in arterial blood; and *reduced* hemoglobin (more correctly called simply *hemoglobin*), chiefly in venous blood. Through the action of acids, alkalies, oxidizing and reducing substances, heat, and other agencies it is readily converted into a series of derivative compounds which can be distinguished by means of the spectroscope.

Most of these derivative compounds are formed only in blood which has left the vessels; a few, however, may be produced in the circulation.

Sulfhemoglobin is found in the circulating blood in most cases of the rare condition known as "enterogenous cyanosis," and in prolonged overuse of coal-tar derivatives. **Methemoglobin** is found in poisoning with potassium chlorate, nitrites, nitrobenzol (as in shoe dyes), and in other substances. Clinically, there is marked cyanosis, and in severe cases the blood has a chocolate-brown color when withdrawn. When the condition is at all marked, sulfhemoglobinemia and methemoglobinemia are easily recognized spectroscopically (p. 356).

Carbon monoxide hemoglobin, formed in carbon monoxide poisoning, gives the blood a brighter red color than is normal.

Acute poisoning has long been well known. Chronic poisoning, due to prolonged exposure to small amounts of carbon monoxide, is less well known, but is assuming increasing importance. The chief sources of the gas are gasoline motors, illuminating gas, gas heaters, and defective stoves and furnaces. Exposure to carbon monoxide is thus one of the hazards of modern civilization. It has even been found in the air of busy streets of large cities in sufficient concentration to cause mild symptoms in persons, such as traffic policemen, who are long exposed to it.

Henderson and Haggard found that healthy persons exposed to various concentrations of the gas for an hour did not experience definite symptoms (headache, dizziness, muscular weakness, nausea) unless the concentration in the blood reaches 26 or 30 per cent of saturation, but it appears that in chronic poisoning, especially in children, serious symptoms may occur with less amounts. The figures reported for clinical cases of poisoning are often misleading, since the carbon monoxide largely or wholly disappears from the blood after the patient has breathed pure air for a few hours, although the symptoms may continue for a long time. CO-hemoglobin has a characteristic spectrum (p. 356), and, when present in sufficient amount (30 per cent or more), is readily identified with the ordinary "pocket" spectroscope, but chemical tests are much more sensitive and more satisfactory. Two

simple qualitative tests are described below. For a relatively simple quantitative method the reader is referred to Sayers and Yant.[1]

Katayama's test is one of the best. It will detect as little as 10 per cent of saturation. Place about 10 c.c. of water in each of two test tubes. To one add 5 drops of the suspected blood, and to the other 5 drops of normal blood to serve as a control. To each tube add 5 drops of fresh orange-colored ammonium sulfide, mix gently, and make faintly acid with acetic acid. Blood containing carbon monoxide hemoglobin develops more or less rose-red color, depending on the concentration; normal blood, a dirty greenish brown.

Hoppe-Seyler's test is less sensitive and less satisfactory, but, since it requires no chemical excepting sodium hydroxide, is useful in emergencies. It may be performed as follows: To 3 c.c. of water in a test tube add 3 to 5 drops of the blood and 1 drop of 5 per cent sodium hydroxide solution, mix gently, and let stand one hour. Normal blood gives a greenish-brown color, carbon monoxide blood more or less pink. It is always necessary to run a control with normal blood.

Normally hemoglobin is confined to the red corpuscles. When it is dissolved out of these cells and appears in the plasma the condition is known as *hemoglobinemia*. This occurs in a great variety of conditions, among which may be mentioned: Severe types of infectious diseases; paroxysmal hemoglobinuria; severe burns and frost bites; and poisoning with potassium chlorate, mushrooms, and some other poisons. When the free hemoglobin reaches a certain concentration in the blood plasma it is excreted in the urine (hemoglobinuria).

To recognize hemoglobinemia, receive a little blood in a small dry test tube and allow it to stand in a cool place for twenty-four hours. The serum, which separates after coagulation, will be colored red or pink instead of pale yellow, as is normally the case.

The normal amount of hemoglobin was formerly given as about 14 Gm. for each 100 c.c. of blood, but recent work has shown this to be too low for adults.[2] The mean hemoglobin concentration of 118 normal English males was determined by two methods of precision and was found to be about 115 per cent in Haldane's scale (14 Gm. per 100 c.c.) when the standard was determined with the Van Slyke apparatus. The mean hemoglobin concentration of 116 women was determined by gas analysis and was found to be 100 per cent in the

[1] Sayers, R. R., and Yant, W. P.: The Tannic Acid Method for Quantitative Determination of Carbon Monoxide in the Blood, U. S. Public Health Reports, Treasury Department, *37*: No. 40, 2433–2439 (Oct. 6), 1922. See also Jour. Am. Med. Assn., *78*: 1745 (June 3), 1922.

[2] Jenkins, C. E., and Don, C. S. D.: The Hemoglobin Concentration of Normal English Males and Females, Jour. Hyg., *33*:36–41 (Jan.), 1933.

Haldane scale. There is no foundation for the belief that the English mean differs materially from the American. An analysis of the figures, which were obtained by these investigators, reveals more interesting results than merely the means for the two sexes. The range for man was 101 to 137.5 per cent (Haldane scale), with a standard deviation of ±6.9 per cent. This discloses that two thirds of the normal individuals had values which varied between 15.2 and 17 Gm. per 100 c.c. of whole blood, and that the other third of the normal individuals, who submitted to this investigation, had values which were higher or lower than those for the other individuals. In fact, one of these individuals had a value of 19.2 Gm. per 100 c.c. of whole blood. In considering the data for women, the range was found to be greater but the values, which were lower, were from 77 to 123 per cent of Haldane's figure of 14 Gm. The standard deviation was ±8.3 per cent. Two thirds of the normal women had values between 12.8 and 15.2 Gm. per 100 c.c. of whole blood; a third of the normal women had values which were higher or lower than these figures; and one woman revealed a value of 17.2 Gm. per 100 c.c. of whole blood. The general average for both sexes during adult life appears to be about 16 Gm. An arbitrary standard of 16.6 Gm. may be taken as 100 per cent. This makes the factor 6 by which the absolute amount may be multiplied to obtain the percentage. The expression, "50 per cent hemoglobin," when used clinically, means that the blood contains 50 per cent of normal. Practically, however, with the various methods of estimation in general use the blood of healthy adults ranges from 80 to 105 per cent or 13.5 to 17.5 Gm. for each 100 c.c.; these figures may, therefore, be taken as representing normal limits. There are, moreover, marked fluctuations with age and sex, which must be taken into account in any careful case study. These are well shown in Fig. 100, which is based upon Williamson's careful spectrophotometric study of the blood of 919 healthy persons in Chicago.

The custom of recording hemoglobin in terms of percentage of an indefinite normal is grossly inaccurate and leads to much confusion. From what has just been said it is clear that no single normal standard can be applied to all ages and both sexes. The situation is complicated by the fact that different hemoglobinometers use different standards. A record, therefore, means little unless one knows what instrument was used and the age and sex of the patient. This confusion could be avoided if records were made in terms of the actual percentage of hemoglobin, that is, in grams for each 100 c.c. of blood. This plan has been adopted by certain large hospitals, and has been proposed to the American Society of Clinical Pathologists for consideration as a standard method. The reading on any type of instrument

can readily be converted into absolute percentage if one knows what amount of hemoglobin was adopted by the makers as normal, provided, of course, that the particular instrument is accurately standardized. This calculation will be given with the description of the various instruments.

Increase of hemoglobin, or *hyperchromemia,* is uncommon, and is usually more apparent than real. It accompanies an increase in number of erythrocytes, and may be noted in change of residence

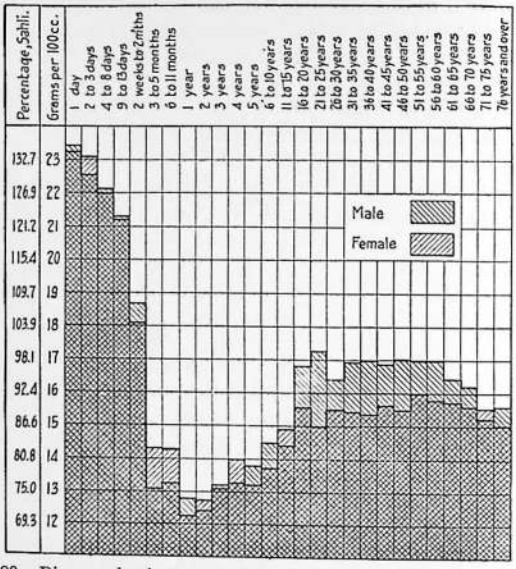


Fig. 100.—Diagram showing average hemoglobin values for both sexes at different ages (after Williamson). Some have obtained slightly lower values for the different ages. The corresponding percentages on the Sahli hemoglobinometer, which assumes 17.3 Gm. for each 100 c.c. blood to be the normal, are also shown.

from a lower to a higher altitude; in poorly compensated heart disease with cyanosis; in concentration of the blood from any cause, as the severe diarrhea of cholera; and in "idiopathic polycythemia" or erythremia.

Decrease of hemoglobin, or *oligochromemia,* is very common and important. It is the distinctive and most striking feature of the anemias (p. 297). In secondary anemia the hemoglobin loss may be slight or very great. In mild cases a slight decrease of hemoglobin is

the only blood change noted. In very severe cases, especially in repeated hemorrhages, malignant disease, and infestation by hookworm and *Diphyllobothrium latum*, hemoglobin may fall to 15 per cent. Hemoglobin is always diminished, and usually very greatly, in chlorosis (average about 40 to 45 per cent), pernicious anemia (average about 20 to 25 per cent), and leukemia (usually about 40 to 50 per cent).

Estimation of hemoglobin is less tedious and, if reasonably accurate, usually more helpful than a red corpuscle count. It offers the simplest and most certain means of detecting the existence and degree of anemia, and of judging the effect of treatment in anemic conditions. Pallor observed clinically does not always denote anemia.

There are many methods, but none is entirely satisfactory. With the different standards for the normal adopted by different makers and the inaccuracies and deterioration of individual instruments records of hemoglobin estimations are often untrustworthy. *The physician should standardize his instrument to give readings in grams per 100 c.c. upon the bloods of healthy adults whose red corpuscles number 5,000,000 for each cubic millimeter.* The methods of estimating hemoglobin which are most widely used are here described:

1. **Direct Matching Methods.**—Methods which are based on the direct matching of the red color of whole fresh blood with some color standard are not satisfactory. (*a*) **The Tallqvist hemoglobin scale** consists of a book of small sheets of absorbent paper and a carefully printed color scale. The percentages of this scale do not have any accurate meaning, and the method is not recommended, except for use at the bedside when other methods are not available.

(*b*) **The Dare hemoglobinometer,** which employs direct matching and undiluted blood, has been used extensively. It has been changed by the present manufacturers in an effort to make it adaptable for the estimation of the grams of hemoglobin per 100 c.c. of whole blood. The instrument is expensive and not very accurate.

2. **Acid Hematin Methods.**—There are several methods that depend on converting hemoglobin into acid hematin with dilute hydrochloric acid, and matching the brownish-yellow color of this solution with a standard in some sort of a colorimeter, or comparator.

(*a*) **The Sahli hemoglobinometer** (Fig. 101) consists of an hermetically sealed comparison tube containing a suspension of acid hematin, a graduated test tube of the same diameter, and a pipet of 20-cu. mm. capacity. The two tubes are held in a black frame with a white ground-glass back.

Place decinormal hydrochloric acid solution in the graduated tube to the mark 10. Obtain a drop of blood and draw it into the pipet to the 20-cu. mm. mark. Wipe off the tip of the pipet, blow its contents into the hydro-

chloric acid solution in the tube, and rinse well. The hemoglobin is changed to acid hematin. Place the two tubes in the compartments of the frame; let stand one minute, and dilute the fluid with water drop by drop, mixing after each addition, until it has exactly the same color as the comparison tube. The graduation corresponding to the surface of the fluid then indicates the percentage of hemoglobin. Mixing may be done by closing the tube with the finger and inverting, but care should be exercised to see that none of the fluid is removed by adhering to the finger. Slightly waxing the finger will aid. Decinormal hydrochloric acid solution is prepared with sufficient accuracy for this purpose by adding 1 c.c. of the concentrated acid to 99 c.c. distilled water. A little chloroform should be added as a preservative.

Under favorable conditions this method is very satisfactory, and is probably accurate to within 5 per cent. Unfortunately, not all instruments upon the market are well standardized, and the comparison tube does not

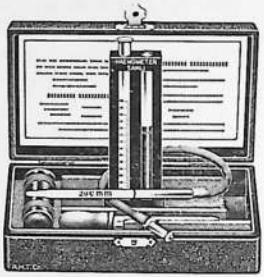


Fig. 101.—Sahli hemoglobinometer.

keep its color well. Sometimes, however, the apparent fading is due to the fact that the hematin is in suspension and settles out when the instrument lies unused for some time. This can be remedied by inverting the tube a number of times. Most tubes contain a glass bead to facilitate mixing. The color tube should be kept from the light as much as possible.

A reading of 100 per cent on the Sahli instrument should, according to the makers, correspond to 17.3 Gm. of hemoglobin in 100 c.c. of blood. The blood of normal adults therefore reads 80 to 90 per cent.

(*b*) **The Sahli-Hellige hemometer** (Fig. 102) is equipped with permanent glass standards and a square comparison tube which allows the value for the hemoglobin to be read as grams per 100 c.c. of whole blood, and also as percentages. The low figure of 14.5 Gm. per 100 c.c. of whole blood corresponds to 100 per cent of hemoglobin.

(*c*) **Sahli-Haden Hemoglobinometer.**—Haden[1] has devised a Sahli type

[1] Haden, R. L.: A New Sahli Type Hemoglobinometer, Jour. Lab. & Clin. Med., *25*: 325–327 (Dec.), 1939.

hemoglobinometer which is probably the most accurate and simple of any of these instruments. A graduated, square glass tube is used for holding the acid hematin. The Ulrich pipet is recommended for making the dilution with tenth normal hydrochloric acid. The calibrations are in grams per 100 c.c. of hemoglobin and are based on allowing the blood to stand thirty minutes before making the comparison with the yellow glass standard in the instrument. This may be done either by daylight or by artificial light. If the result is read by electric light, a blue "Daylite" glass filter is used in comparison with an opal glass filter in the holder on the back of the instrument. There are three windows on the scale and a magnifier is used in focusing on the scale. When the color of the specimen is nearest to a correct match with the central or standard color, the top window will be of lighter shade and the bottom window will be of darker shade. The reading is made by ob-

Fig. 102.—Sahli-Hellige hemometer.

serving the point on the graduated scale to which the dilution has risen. Figure 103 illustrates the parts of this apparatus.

(*d*) **The Haden-Hausser hemoglobinometer** (Fig. 104) uses a yellow glass standard. The blood is diluted 1 to 10 with decinormal hydrochloric acid and should be allowed to stand thirty minutes for the full color to develop. A cell which is formed of a plane glass plate and a glass wedge is filled by capillarity, and comparison is made with the standard through fenestra in the frame which holds the cell and standard. The values are read in grams per 100 c.c., and the scale ranges from 8 to 18 Gm. In cases of anemia, the readings which are made by using type B wedge, which holds twice as much as type A wedge, must be divided by two, thus providing a range of 4 to 9 Gm. per 100 c.c. A larger model, which is illuminated electrically, has also been manufactured.

(*e*) **Newcomer's Standard Disk.**—This consists of a small piece of light brown glass approximately 1 mm. thick, which may be used as a color standard for hemoglobin estimations with any colorimeter of the Duboscq type.

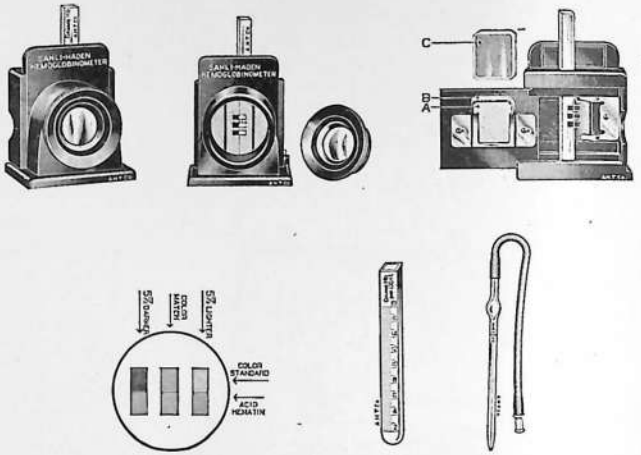


Fig. 103.—Sahli-Haden hemoglobinometer.

Place the disk in one of the light paths of the colorimeter, either above one of the plungers or above or below one of the cups, and fill this cup with water.

In the other cup place exactly 5 c.c. of approximately decinormal hydrochloric acid (1 c.c. concentrated acid, 99 c.c. water). Secure exactly

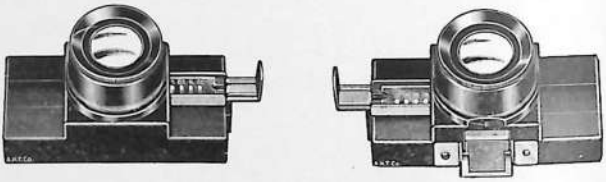


Fig. 104.—Haden-Hausser hemoglobinometer, front and rear views.

20 cu. mm. of blood from a skin puncture (the pipet of the Sahli hemoglobinometer may be used), place the bood at once in the cup with the acid, and mix well. This gives a dilution of 1 in 251. Now adjust the plunger until two fields match and make the reading. Calculate the amount of

hemoglobin in grams for each 100 c.c. of blood by aid of the table on this page, which takes into account two variable factors: (*a*) The time which has elapsed since the blood dilution was made, the acid hematin solution reaching its full depth of color only after some hours; and (*b*) the thickness of the colored glass disk, which varies from 0.95 to 1.05 mm., the exact thickness being engraved upon each.

To translate grams hemoglobin for each 100 c.c. of blood into terms of percentage of normal, as is usually required in clinical work, multiply by 100 and divide by the number of grams hemoglobin which have been adopted as representing the normal (p. 207). For example to express the hemoglobin in percentage according to Sahli's scale multiply by 100 and divide by 17.3.

NEWCOMER'S TABLE FOR USE WITH THE STANDARD DISK

To Obtain Grams Hemoglobin Per 100 c.c. Blood Divide the Colorimetric Reading into the Appropriate Figure.

Minutes since dilution.	Thickness of the colored glass in millimeters.										
	0.95.	0.96.	0.97.	0.98.	0.99.	1.00.	1.01.	1.02.	1.03.	1.04.	1.05.
10	94.4	95.4	96.4	97.4	98.4	99.4	100.4	101.4	102.4	103.4	104.4
15	93.1	94.1	95.0	96.0	97.0	98.0	99.0	100.0	101.0	102.0	103.0
20	92.5	93.5	94.5	95.4	96.4	97.4	98.4	99.4	100.4	101.3	102.3
30	91.8	92.8	93.8	94.8	95.7	96.7	97.7	98.6	99.6	100.5	101.5
40	91.6	92.5	93.5	94.5	95.4	96.4	97.4	98.3	99.3	100.3	101.2
Final	90.6	91.6	92.5	93.5	94.4	95.4	96.4	97.3	98.3	99.2	100.2

With the carefully standardized disks at present supplied this method is accurate. Its use is, however, confined to laboratories equipped with an expensive colorimeter.

(*f*) **Osgood-Haskins Method.**—This method has proved very satisfactory in many laboratories. Use is made of a permanent artificial standard containing 32 Gm. of ferric sulfate and 80 mg. of chromic sulfate, which are made up to 100 c.c. with distilled water. This must be checked, however, with a previously prepared correct standard and preferably should be purchased either from Hynson, Westcott and Dunning, Baltimore, Maryland, or from the Shaw Supply Co., Portland, Oregon. The stock standard should be kept in a pyrex flask which should be closed tightly with a rubber stopper.

Procedure.—Place exactly 1 c.c. of well-mixed oxalated venous blood in a 100 c.c. volumetric flask. Lake with 40 c.c. of distilled water. Add 50 c.c. of a fifth normal solution of hydrochloric acid; mix well; and make up to 100 c.c. Heat a portion of this mixture, which has been placed in a test tube in a water bath at 55° to 60° C., for seven minutes or longer, and cool. Place some of the standard in a colorimeter cup and set this at 15 mm., and compare the unknown with the standard. Make three or four readings and estimate the average reading. Take the temperature of the standard. Reference must be made to a table (furnished with the standard)

and the percentage of hemoglobin is read directly for various colorimetric readings, and for different temperatures of the standard. One hundred per cent represents 13.8 Gm. per 100 c.c., when the standard with a temperature of 15.5° C. is set at 15 mm. and the unknown is read at 10.5 mm. Osgood recommends that the first normal blood that is examined each week be kept as an acid hematin standard in a brown bottle, with the temperature of the standard as originally read marked on the bottle, and that this solution be used as the standard for all subsequent determinations which are made during that week.

3. **Carboxyhemoglobin Method.**—The Palmer[1] method has been very popular in many laboratories for years. Its chief drawback is that hemoglobin must be converted into carbon monoxide hemoglobin by passing illuminating gas, or carbon monoxide gas which is obtained from some other source, through blood diluted with 0.4 per cent solution of ammonia; this is also its chief advantage as the standard which is prepared in this manner is very stable. The sample of blood that is used should have an oxygen capacity of 18.5 per cent, as determined by the Van Slyke method, which represents 14 Gm. of hemoglobin per 100 c.c. This is 100 per cent according to Haldane's scale, although Palmer pointed out that the normal average for males is more nearly 16.6 Gm. per 100 c.c. or 22 per cent oxygen capacity. A 20 per cent solution of blood in 0.4 per cent solution of ammonia, which is prepared by bubbling carbon monoxide through it for ten minutes, will make a stock standard which may be kept in the icebox for many months if it is sealed with paraffin.

Procedure.—(1) Take 5 c.c. of stock solution, and dilute with 95 c.c. of 0.4 per cent solution of ammonia to make a 1 per cent standard. This may be kept for two or three weeks in a dark glass bottle.

(2) Make a 1 per cent solution of the patient's blood in 0.4 per cent solution of ammonia.

(3) Bubble carbon monoxide through the solution for ten minutes. *This should be done under a hood.*

(4) Place the standard 1 per cent solution in the left-hand cup of the colorimeter; set at 10 mm.; place the unknown in the right-hand cup; and then raise or lower the right-hand cup until the colors match.

The *calculation* is very simple:

$$\frac{10 \text{ (reading of standard)}}{\text{R (reading of unknown)}} \times 100 = \text{per cent hemoglobin.}$$

It is evident that this percentage can also be readily converted to read in grams per 100 c.c. of blood, which is a preferable manner of reporting.

4. **Van Slyke's Oxygen-capacity Method.**—This is an indirect method, which estimates the amount of hemoglobin from the amount of oxygen it will absorb and utilizes the Van Slyke apparatus described for CO_2 estima-

[1] Palmer, W. W.: The Colorimetric Determination of Hemoglobin, Jour. Biol. Chem., *33*:119–126 (Jan.), 1918.

tions (p. 397). It serves as an accurate standard method for standardizing the various hemoglobinometers, but is too complicated for clinical work. The technic will be found in Van Slyke's papers.[1]

5. **Iron-content Determinations.**—Two methods of determining the iron content of the blood have proved satisfactory, the Kennedy method, and the newer Wong method; Osterberg's combination of these two methods is described in the chapter on clinical chemistry, page 414. The value for the iron content may be transposed into terms of grams of hemoglobin per 100 c.c. of blood by dividing the amount of iron in mg. per 100 c.c. by 3.35, since hemoglobin contains 0.0335 per cent of iron.

6. **The spectrophotometric method** is very accurate and is often used for research; it requires expensive apparatus and is not suited for clinical use.

7. **Photo-electric-cell Methods.**—These methods are excellent substitutes for colorimetric or spectrophotometric methods. The first use of the

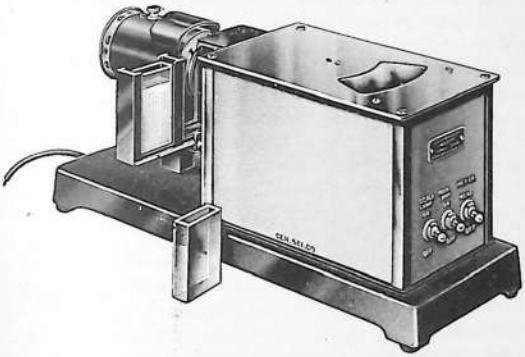


Fig. 105.—Cenco-Sheard-Sanford photelometer.

photo-electric colorimeter for the determination of hemoglobin values was reported by Sheard and Sanford.[2] This method was further improved by the development of the photelometer.

The Sheard-Sanford photelometer[3] (Fig. 105) has proved to be very

[1] Van Slyke, D. D.: Gasometric Determination of the Oxygen and Hemoglobin of Blood, Jour. Biol. Chem., *33*:127–132 (Jan.), 1918. Van Slyke, D. D. and Stadie, W. C.: The Determination of the Gases of the Blood, Jour. Biol. Chem., *49*:1–42 (Nov.), 1921.

[2] Sheard, Charles and Sanford, A. H.: A Photoelectric Hemoglobinometer. Clinical Applications of the Principles of the Photo-electric Photometry to the Measurement of Hemoglobin, Jour. Lab. and Clin. Med., *14*:558–574 (Mar.), 1929. Sanford, A. H. and Sheard, Charles: The Determination of Hemoglobin with the Photoelectrometer, Jour. Lab. and Clin. Med., *15*:483–489 (Feb.), 1930.

[3] Sanford, A. H., Sheard, Charles, and Osterberg, A E.: The Photelometer and Its Use in the Clinical Laboratory. Am. Jour. Clin. Path., *3*:405–420 (Nov.). 1933.

The "Cenco-Sheard-Sanford Photelometer" is manufactured by the Central Scientific Company, 1700 Irving Park Boulevard, Chicago, Illinois.

satisfactory, especially in laboratories in which a number of routine determinations are made daily. Its only disadvantage is in the cost of the equipment, which is somewhat more than that of a colorimeter. However, the instrument may be used as a colorimeter. or a nephelometer, in fact, there are innumerable uses for it other than as a hemoglobinometer. The advantages of this method are that it is accurate, calibrated with the Van Slyke oxygen-capacity method; the readings are interpreted directly in grams per 100 c.c.; it is a very rapid method, as the determinations, which may be made immediately after diluting the blood, or several hours later, take only a few seconds. There is no attempt to match colors, therefore, subjective errors are avoided. The only *reagent* used is a 0.1 per cent solution of sodium carbonate. The *materials* needed in addition to the photelometer itself are pipets, with which 0.1 c.c. of blood may be measured accurately, such as the Kahn pipet, or the Folin microsugar pipet, and a pipet with which to measure accurately 20 c.c. of diluent into a 50 c.c. flask, or centrifuge tube. The *procedure* is to dilute 0.1 c.c. of blood in 20 c.c. of 0.1 per cent solution of sodium carbonate, thus making a 1 to 200 solution of oxyhemoglobin. This is most conveniently and most accurately done, in my experience, by making the dilutions from a sample of blood immediately after it is obtained by venipuncture, for some other purpose. The photelometer has a green glass filter in front of the photronic type of photo-electric cell. This filter transmits light in its maximal intensity at that portion of the spectrum where the maximal absorption occurs in one of the oxyhemoglobin bands. The light intensity through a standard spectroscopic cell, which is filled with 0.1 per cent sodium carbonate, is first adjusted with an iris diaphragm so that the reading on the meter is 100. The specimen of diluted blood is placed in a similar spectroscopic cell, is then moved into the path of light in the carrier, and the reading is made on the meter. This lower reading really represents the decrease in current from the photronic cell which is the result of the light absorption of oxyhemoglobin in the green portion of the spectrum (see Fig. 184, p. 356). This *reading is translated directly* into a value for grams of hemoglobin per 100 c.c. of blood, by referring to a chart which is prepared individually for each instrument by the manufacturer, based on oxygen-capacity determinations. Karr and Clark[1] in a comparative study of various hemoglobin methods, stated that the method of reading hemoglobin as oxyhemoglobin in the photelometer is consistently more accurate in its results than are determinations of oxygen capacity as generally made. They recommended strongly that this method be used for routine work because of its simplicity and accuracy.

IV. ENUMERATION OF ERYTHROCYTES

In health there are about 5,000,000 red corpuscles per cubic millimeter of blood. The number is generally a little less—about

[1] Karr, W. G., and Clark, J. H.: Comparison of Various Hemoglobin Methods as Performed in Hospital and Physicians' Laboratories, Amer. Jour. Clin. Path. (Tech. Suppl. V) *11*:127–147 (Sept.), 1941.

4,500,000—in women. At birth the number averages about 7,000,000 and falls gradually to reach the adult figure at about the fifteenth year. This should be compared with the hemoglobin curve shown in Fig. 94. Hawk finds the normal for athletes in training to be 5,500,000.

Increase of red corpuscles, or *polycythemia,* is unimportant. There is a decided increase following change of residence from a lower to a higher altitude, reaching a maximum after several days' sojourn. The increase, however, is not permanent. In a few months the erythrocytes return to nearly their original number. At the University of Colorado (altitude 5400 feet) the average for healthy medical students is about 5,800,000. Several views have been offered in explanation of this effect of altitude: (*a*) Concentration of the blood, owing to increased evaporation from the skin; (*b*) altered distribution of corpuscles, the reserve cells in the splanchnic vessels being thrown into the peripheral circulation; (*c*) accumulation of red cells in the capillaries of the skin; (*d*) new formation of corpuscles or delayed destruction, this giving a compensatory increase of aeration surface. The work of Schneider at Colorado Springs and of Liebesny of Vienna with the skin microscope supports the second and third of these views, although the fourth must be accepted to explain the moderate permanent increase.

Pathologically, polycythemia is uncommon. It may occur in: (*a*) Concentration of the blood from severe watery diarrhea; (*b*) chronic heart disease, especially the congenital variety, with poor compensation and cyanosis; (*c*) acute poisoning by phosphorus, cantharides, or carbon monoxide; and (*d*) polycythemia vera or erythremia (p. 316), which is considered to be an independent disease, and is characterized by a dark red cast of countenance, erythrocyte counts of 7,000,000 to 12,000,000, hemoglobin 120 to 150 per cent, and a normal number of leukocytes. An erythrocyte count of 15,900,000 has been recorded (Morris). In these cases the cells are abnormally small; otherwise the cell volume would exceed that of the whole blood.

Decrease of red corpuscles, or *oligocythemia.* Red corpuscles and hemoglobin are commonly decreased together, although usually not to the same extent.

Oligocythemia occurs in all but the mildest symptomatic anemias. The blood count varies from near the normal in moderate cases down to 1,500,000 in very severe cases. There is always a decrease of red cells in chlorosis, but it is often slight, and is relatively less than the decrease of hemoglobin. Leukemia gives a decided oligocythemia, the average count being about 3,000,000.

Method of Counting.—Although simple in principle, accurate

counting of blood corpuscles involves a technic which is acquired only after considerable practice. Exact and fairly rapid work is demanded. Before beginning, one should familiarize himself with the instrument and its ruling, and should read the directions carefully, giving especial attention to sources of error. It is likewise an advantage to practice sucking the diluting fluid into the pipet and stopping it at a predetermined height. After learning the technic, students should make numerous recounts of the same areas of the counting chamber, and also several different counts from the same dilution of blood, in order that they may appreciate the discrepancies that easily occur in this procedure.

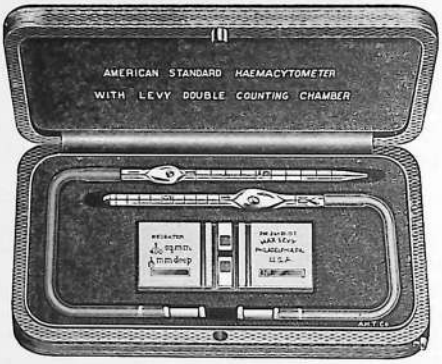


Fig. 106.—Hemacytometer, consisting of a counting chamber, a cover glass, and two pipets for diluting the blood.

When purchasing a hemacytometer it is well, despite the additional cost, to secure one which has been tested by the United States Bureau of Standards. The Bureau of Standards mark is placed upon each piece of such instruments. In the case of the cover glass it is placed on the side which is always to be used uppermost.

The **hemacytometer** consists of two pipets for diluting the blood and a counting chamber (Fig. 106). The rubber tubes which come with the pipets are often too short and too flexible and should be replaced. For this purpose nothing is as good as a rubber catheter.

The *Bürker* type counting chamber (Fig. 107), now most generally used, consists of a heavy glass slide, upon the middle third of which are fixed

three narrow parallel platforms extending across the slide. The middle platform or "floor piece" is exactly 0.1 mm. lower than the two others. Upon it is ruled a square millimeter subdivided into 400 small squares. In older counting chambers of this type each fifth row of small squares is subdivided by an extra line for convenience in keeping track of the areas to be counted. Surrounding this are eight other square millimeters which are subdivided differently by Neubauer, Zappert, and others. Only the central square of 400 small squares is used in counting red corpuscles.

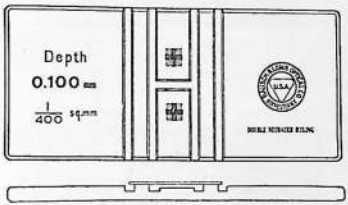


Fig. 107.—A new "open" counting chamber of excellent type. It is made in one piece and the surfaces of the platforms are highly polished, making it possible to obtain Newton's bands as a criterion of proper application of the cover glass. The lower figure shows the chamber in cross section with cover glass in place.

In the "improved Neubauer ruling" now used on Levy and Levy-Hausser counting chambers, the extra line mentioned above as subdividing each fifth row of small squares is omitted, and the 400 small squares are divided into twenty-five groups of sixteen squares each by a "split" boundary line (Fig. 108).

Fig. 108.—Group of 16 small squares of "improved Neubauer ruling," showing split boundary lines.

In the "double counting chamber" the middle platform is divided into two by a transverse groove and a ruling is scored upon each.

The Spencer Lens Company offers a "bright line" hemacytometer in which the ruling is made on a metallic coating on the glass. The lines are very visible, and the makers claim that the nature of the surface makes for a more even distribution of cells (Fig. 109).

Hausser's counting chamber (Fig. 110) is a new form consisting of a bakelite slide in which is placed a small removable counting chamber which is made entirely in one piece and thus avoids the troublesome loosening of

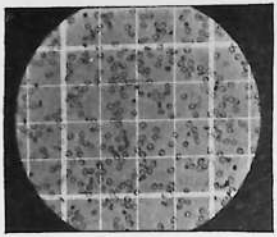


Fig. 109.—Spencer "bright line" counting chamber. The new construction makes the lines appear white against a dark background.

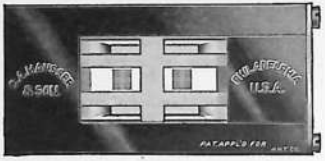


Fig. 110.—Hausser's counting chamber in bakelite holder. The cover glass and clamps are not shown.

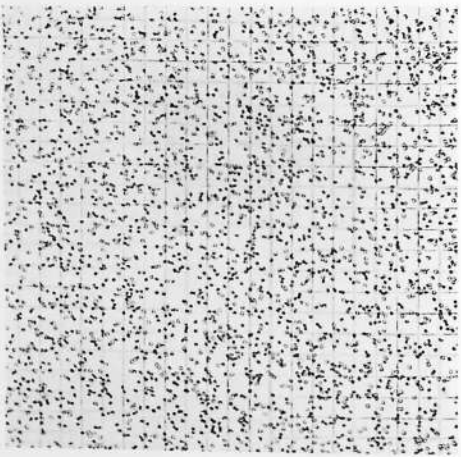


Fig. 111.—Central square millimeter of the ruling of the blood-counting chamber. It is subdivided into 400 small squares.

parts which have been cemented in place with balsam. Counting chambers with various rulings, of both single and double form, and of different depths for counting blood platelets, blood corpuscles, and the cells of spinal fluid, can be had and are interchangeable in the bakelite holder. All are of the "open" type. The instrument is equipped with cover glass clips which, however, are not shown in the illustration.

A thick cover glass, ground perfectly plane, accompanies the counting chamber. Ordinary cover glasses are of uneven surface, and should not be used with this instrument. For use with objectives of short working distance, heavy cover glasses can be obtained with a flat-bottomed excavation or "well" in the center. This combines the advantage of a thin cover with the rigidity of a thick one.

It is evident that, when the cover glass is in place upon the platform of either type of counting chamber (Fig. 107) there is a space exactly 0.1 mm. thick between it and the ruled platform or disk; and that, therefore, each square millimeter of the ruling forms the base of a space holding exactly 0.1 cubic millimeter.

A new style of counting chamber is illustrated in figure 112. This chamber is ruled so that there are four chambers, making it possible to make duplicate counts of both red and white corpuscles without the necessity of cleaning and refilling the chamber or refocusing the microscope. The two rulings for counting red corpuscles are the improved Neubauer ruling and the two new rulings for counting white corpuscles are the Fuchs-Rosenthal ruling, originally designed for counting cells in spinal fluid, but very convenient, because of the large area, for making white corpuscle counts (see Fig. 307, p. 606).

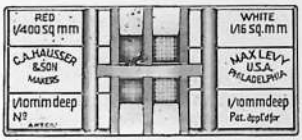


Fig. 112.—Levy counting chamber, quadruple, with two Neubauer rulings and two Fuchs-Rosenthal rulings.

Diluting Fluids.—The most widely used are Hayem's and Toisson's. Both of these have high specific gravities, so that, when well mixed, the corpuscles do not separate quickly. Toisson's fluid is perhaps the better for beginners, because it is colored and can easily be seen as it is drawn into the pipet. It stains the nuclei of leukocytes blue, but this is no real advantage. It must be filtered frequently because of the ready growth of fungi in it. Hayem's fluid is to be preferred for routine work. For convenience in filling pipets the fluids should be kept in small wide-mouthed bottles.

Hayem's Fluid.

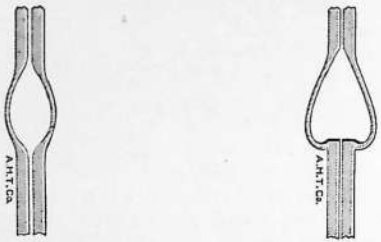
Mercuric chloride	0.5
Sodium sulfate	5.0
Sodium chloride	1.0
Distilled water	200.0

Toisson's Fluid.

Sodium chloride	1.0
Sodium sulfate	8.0
Glycerin	30.0
Distilled water	160.0

Methyl-violet, 5 B to give a strong purple color.

Filling the Pipet.—To count the red corpuscles use the pipet with 101 engraved above the bulb. It must be clean and dry. Puncture the skin, wipe off the first drop of blood, and fill the pipet from the second, sucking the blood to the mark 0.5 or 1, according to the dilution desired. While doing this, hold the pipet horizontally at nearly right angles to the line of vision, so that the exact height of the column may be easily seen. The side of the tip should rest against the skin, but the end must be free. Air bubbles will enter with the blood if the drop is too small or if the tip is not kept immersed. Should the blood go slightly beyond the mark, draw it back by touching the tip of the pipet to a moistened towel. Quickly wipe off the blood adhering to the tip, plunge it into the diluting fluid, and suck the fluid up to the mark 101, slightly rotating the pipet meanwhile. At this stage it is best to hold the pipet nearly vertically in order to avoid inclusion of a large air bubble in the bulb. This dilutes the blood 1 : 200 or 1 : 100, according to the amount of blood taken. Except in cases of severe anemia, a dilution



A B

Fig. 113.—Cross section of bulb and part of stem of diluting pipets: A, Original Thoma pipet; B, Trenner automatic pipet. In the latter, blood fills the stem by capillarity and automatically stops when it reaches the bulb.

of 1 : 200 is preferable. Close the ends of the pipet with the fingers, and shake vigorously until the blood and diluting fluid are well mixed, keeping the pipet horizontal meanwhile. It should not be shaken in the direction of the long axis. One to two minutes' shaking is usually sufficient.

To fill the Trenner automatic pipet (Fig. 113, *B*) draw the blood by suction into the capillary portion until it is nearly full. Discontinue the suction and, if the pipet be held nearly horizontally, the blood will continue to rise to the end of the capillary and will automatically stop there. The diluting fluid then is immediately drawn in by suction as described above for the Thoma pipet.

When it is not convenient to count the corpuscles at once, place a heavy rubber band around the pipet so as to close the ends, inserting a small piece of rubber cloth or other tough, nonabsorbent material, if necessary, to prevent the tip from punching through the rubber. It may be kept thus for twenty-four hours or longer. Still better closure devices can be purchased at slight cost. One of these is shown in Fig. 114.

Charging the Counting Chamber.—When ready to make the count, clean the counting chamber and cover glass. Adjust the cover glass and clamp it in place if the counting chamber is supplied with clamps. If the cover is properly adjusted and the slide be viewed obliquely, faint concentric lines of the prismatic colors—Newton's bands—can be seen between the cover and the platforms upon which it rests. They indicate that the two surfaces are in close apposition. If they do not appear at once, slight pressure upon the cover may bring them out. Failure to obtain them is usually due to dirty slide or cover—both must be perfectly clean and *free from dust.* Some counting chambers have mat surfaces which make it impossible to bring out Newton's bands.

When the cover is properly adjusted, mix the fluid in the pipet thoroughly by shaking for about a minute. Now quickly blow 2 or 3 drops

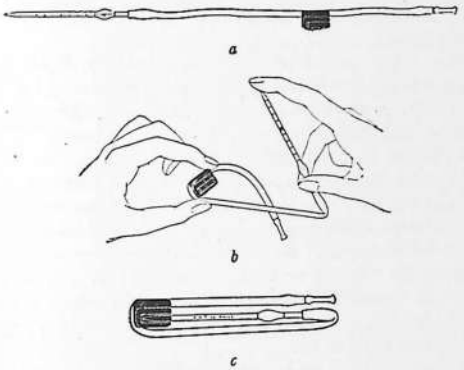


Fig. 114.—New closure device for diluting pipets, showing manner of application.

from the pipet, wipe off its tip, and holding the pipet in an inclined position, touch its tip to the angle between the edge of the cover glass and one of the projecting ends of the floor piece. The fluid will run under the cover by capillary attraction. Care must be exercised to use just enough fluid to fill the space beneath the cover. This is especially important when the instrument lacks cover-glass clamps, since an excess of fluid will tend to raise the cover appreciably and thus increase the cell count. In a properly filled counting chamber the fluid nearly or quite fills the space beneath the cover, none has run over into the moat, and there are no bubbles. If these conditions are not met the work must be done over again.

Counting.—Allow the corpuscles to settle for a few minutes, and then examine with a low power to see that they are evenly distributed. If they are not *evenly distributed over the whole disk*, the counting chamber must be cleaned and a new drop placed in it.

Inexperienced workers often have difficulty in locating the ruled area. On this account the Levy and Levy-Hausser counting chambers now have a deeply ruled line extending from the end of the platform to the ruled area. This prominent "finding line" is quickly located with either high or low objectives.

Probably the most satisfactory objective for counting is the 8 mm. or the 4 mm. with long working distance. To understand the principle of counting it is necessary to remember that the square millimeter (400 small squares) represents a capacity of 0.1 cu. mm. Find the number of corpuscles in the square millimeter, multiply by 10 to find the number in 1 cu. mm. of the diluted blood, and finally, by the dilution, to find the number in 1 cu. mm. of undiluted blood. Instead of actually counting all the corpuscles it is customary to count those in only a limited number of small squares, and from this to calculate the number in the square millimeter. Nearly every worker has his own method of doing this. The essential thing is to adopt a method and adhere to it.

In practice a convenient procedure is as follows: *With a dilution of 1 : 200, count the cells in 80 small squares, and to the sum add 4 ciphers; with dilution of 1 : 100, count 40 small squares and add 4 ciphers.* Thus, if with 1 : 200 dilution, 450 corpuscles were counted in 80 squares, the total count would be 4,500,000 for each cubic millimeter. This method is sufficiently accurate for most clinical purposes, provided the corpuscles are evenly distributed and 2 drops from the pipet be counted. It is convenient to count five blocks of sixteen small squares (Fig. 115), one such block in each corner of the square millimeter, and one block near the center.

When using the "double counting chamber," it is convenient to count the corpuscles in twenty small squares at the top and those in twenty small squares at the bottom in each of the ruled portions, making eighty squares in all and add four ciphers.

In order to avoid confusion in counting cells which lie upon the borderlines the following rule is generally adopted: *Corpuscles which touch the upper and left sides should be counted as if within the squares, those touching the lower and right sides, as outside; or vice versa.*

Sources of Error.—The most common sources of error in making a blood count are:

(*a*) Inaccurate dilution, usually from faulty technic, occasionally from inaccurately graduated pipets. Only an instrument of standard make can be relied upon, and it is best to purchase one which has been tested by the United States Bureau of Standards.

(*b*) Too slow manipulation, allowing a little of the blood to coagulate and remain in the capillary portion of the pipet.

(*c*) Inaccuracy in depth of counting chamber usually due to imperfect application of the cover glass, but sometimes to faulty manufacture or to softening of the cement by alcohol or heat. A cemented slide should not be cleaned with alcohol or left to lie in the warm sunshine.

(*d*) Uneven distribution of the corpuscles. This results when the blood

has partially coagulated, when it is not thoroughly mixed with the diluting fluid, when the cover glass is moved after the counting chamber is filled.

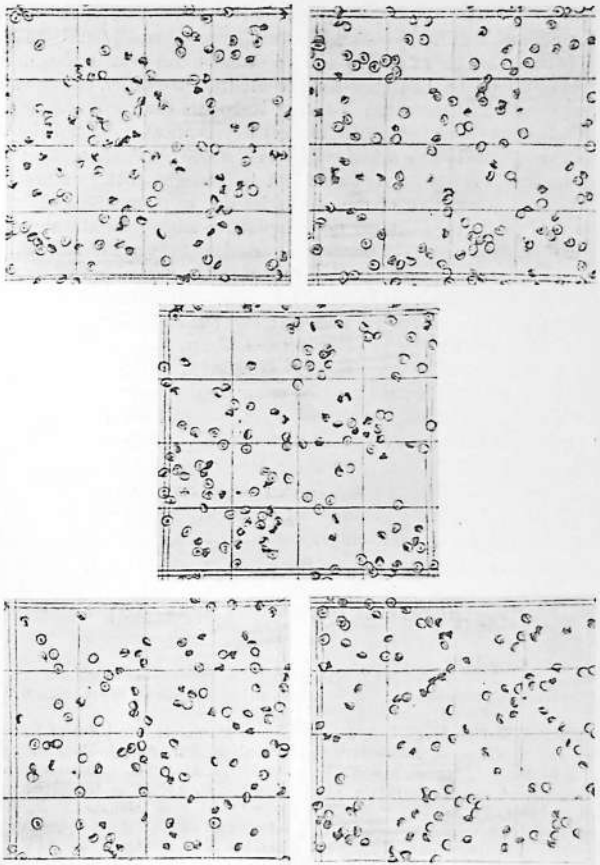


Fig. 115.—Five fields, sixteen squares each (× 430).

or when the suspension overflows into the moat. The last may sometimes reduce the count as much as 1,000,000.

(*e*) The presence of yeasts, which may be mistaken for corpuscles, in the diluting fluid.

The "Normal" Error of the Count.—Even if all usual precautions are taken to avoid the errors listed above, the estimate of the erythrocyte count per cubic millimeter of blood in the body is subject to error, as are all instrumental measures. In the hemacytometer method in particular one is dealing with estimating the count in a very large volume by taking a *sample* which is relatively very small, and it is a common observation that different samples of an identical batch will differ one from the other. If one uses the method of estimate recommended, that is, counts the corpuscles in eighty squares, it is obvious that he will not get the identical count that he would if he used one particular set of eighty of the 400 available squares rather than another (Fig. 111). It should not be thought that this necessarily indicates maldistribution of the corpuscles due to poor mixing. Each corpuscle that enters the chamber takes a position among the 400 squares "at random," and as a result there will be a variation of the number of corpuscles which fall into each square. The theoretical mathematical law of this variation, which was first stated by Abbe[1] was more elaborately and independently presented by "Student"[2] and later was reinvestigated experimentally together with an analysis of other sources of error of the blood count by Berkson, Magath and Hurn.[3] The law is called the "Poisson distribution" after the French mathematician who enunciated the general law of which the distribution of blood corpuscles in the hemacytometer is a particular case. According to this law, the variation among the different squares in the *chamber* is given by S. D. $= \sqrt{m}$ where m is the mean number of corpuscles per unit area and S. D. is the standard deviation* of the counts in these areas. For instance, if the mean count per eighty squares is 500, as will be the case for a count of 5 million per cubic millimeter, the S. D. of counts of different sets of eighty squares in the chamber will be $\sqrt{500}$ or 22.4. Expressed relatively as a per cent, this is $\frac{22.4}{500} \times 100 = 4.5$ per cent. Between

[1] Abbe, E.: Ueber Blutkörper Zählung. Jenaische Sitzber. 1879: xcviii-cv.

[2] "Student," On the Error of Counting with a Hemocytometer, Biometrika, *6*:351–360, 1907.

[3] Berkson, Joseph, Magath, T. B., and Hurn, Margaret: The Error of Estimate of the Blood Cell Count as Made with the Hemocytometer. Am. J. Phys. *128*:309–323 (Jan.) 1940. Berkson, Joseph, Magath, T. B., and Hurn, Margaret: Laboratory Standards in Relation to Chance Fluctuations of the Erythrocyte Count as Estimated with the Hemocytometer. J. Am. Stat. Assn. *30*:414–426 (June) 1935. Magath, T. B., Berkson, Joseph, and Hurn, Margaret: The Error of Determination of the Erythroctye Count. Am. J. Clin. Path. *6*:568–579 (Nov) 1936. Berkson, Joseph: Some Difficulties of Interpretation Encountered in the Application of the Chisquare Test. J. Am. Stat. Assn. *33*:526–536 (Sept.) 1938.

* The standard deviation is a measure of variability given by the formula $\sqrt{\frac{\Sigma (x - m)^2}{n}}$ where x is the observation whose variation is measured, m is the mean or average, n is the number of observations, and Σ stands for summation.

±1 S. D. are included two-thirds of the repeated counts, and between ±2 S. D. are included 95 per cent of repeated counts. Since ±2 S. D. is generally accepted as significant limits, the error of a count of 5 million per cubic millimeter made in the hemacytometer chamber *owing merely to the variation in the field of observation is* $\pm 2 \times 4.5 = \pm 9$ *per cent.* This "error of the field" measured as S. D. $= \sqrt{m}$ is the minimal error to which a single estimate of the count can be considered subject so long as the hemacytometer is used, but the error due to the variation within the field of observation is not the only one to which the count as usually made is subject. Separate fillings of different standard chambers with the same blood will result in different total counts per measured unit volume in the different chambers, owing to variations in calibration, variations in the filling technic, variations in pressure of the cover-slip, and so forth. This may be referred to as the "error of the chamber." Similarly, separate fillings of different standard pipets with the same blood will result in different total counts per measured unit volume in the different pipets. This may be referred to as the "error of the pipet." Berkson, Magath and Hurn have determined the S. D. of the chamber error as 4.6 per cent of the mean count and the S. D of the pipet error as 4.7 per cent of the mean count. For the total error, they gave for a count of 5 million $\sqrt{4.1^2 + 4.6^2 + 4.7^2} = 7.7$* or about 8 per cent. *Since twice the S. D. is the usually accepted limits of significance, the error of a single estimate of the erythrocyte count was given by them as* ±*16 per cent.* A large number of repeated counts of this kind will of course vary and 95 per cent of them will be within the limits of ±16 per cent; 68 per cent, or about two-thirds, will be within ±8 per cent. In Fig. 116 is shown the distribution of repeated estimates for a total count of 5 million per cubic millimeter.

Cleaning the Instrument.—The instrument should be cleaned immediately after using, and the counting chamber and cover must be cleaned again just before use.

Transfer the rubber tube to the small end of the pipet and draw through it, successively, water, alcohol, ether, and air; or water, acetone, and air. This can be done with the mouth, but it is much better to use a rubber bulb or suction filter pump. When the mouth is used, the moisture of the breath will condense upon the interior of the pipet unless the fluids be shaken, and not blown, out. If blood has coagulated in the pipet—which happens when the work is done too slowly—dislodge the clot with a horsehair (which can be obtained from the hair-cloth interlining of a coat), never with a wire, and clean with strong sulfuric acid, or let the pipet stand overnight in a test tube of the acid. Even if the pipet does not become

* The total error is given as the square-root of the sum of the squares of the constituent errors. The 4.1 per cent for the error of the field is a slight modification found by Berkson, Magath and Hurn of what is given by the Poisson distribution ($\sqrt{500}/500 \times 100 = 4.5$ per cent).

clogged, it should be occasionally cleaned in this way. When the etched graduations on the pipets become dim, they can be renewed by rubbing with a wax pencil.

Wash the counting chamber and the cover with water and dry them with clean soft linen. Alcohol may be used to clean the latter, but never the former, unless it be of the new noncement type, although a handkerchief

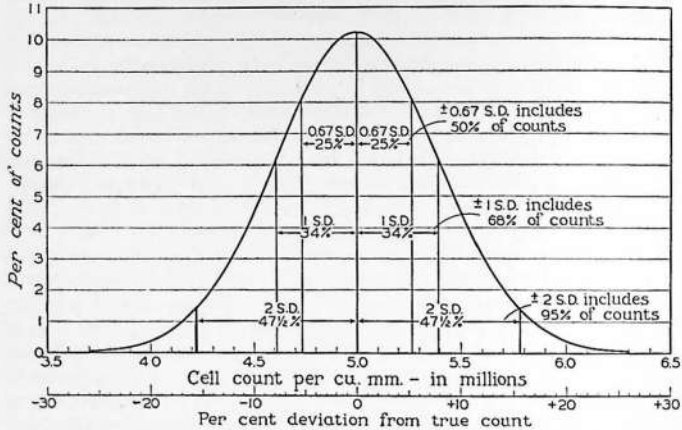


Fig. 116.—Distribution of repeated estimates of the erythrocyte count made by enumerating the erythrocytes in eighty squares of the hemacytometer chamber, for an individual with 5,000,000 erythrocytes per cubic millimeter of blood. The ordinate gives the per cent of the counts which will fall in each 100,000 interval. For instance, between 5,000,000 and 5,100,000 there will be 10.2 per cent of the counts. The standard deviation (S.D.) of the counts is 390,000 and from this may be calculated the frequency of counts falling in any interval. Between ±0.67 S.D. (the probable error) will fall 50 per cent of the counts; hence one may expect 50 per cent to fall between 4,740,000 and 5,260,000. Between ±1 S.D. will fall 68 per cent of the counts; hence one may expect 68 per cent to fall between 4,610,000 and 5,390,000. Between ±2 S.D. will fall 95 per cent or almost all of the counts; hence one may expect 95 per cent to fall between 4,220,000 and 5,780,000.

slightly moistened with alcohol may be used to wipe off the surface of the ruled disk and the platform.

V. COLOR INDEX

This is an expression which indicates the amount of hemoglobin in each red corpuscle compared with the normal amount. For example, a color index of 1 indicates that each corpuscle contains the normal amount of hemoglobin; of 0.5, that each contains one half the normal.

The color index is very significant in chlorosis and pernicious anemia. In the former it is usually much decreased; in the latter, generally much increased. In hemorrhagic anemia it is low and may be very low, suggesting chlorosis. In symptomatic anemia it is moderately diminished.

To obtain the color index, divide the *percentage* of hemoglobin by the *percentage* of corpuscles. For this purpose the normal number of corpuscles (100 per cent) is assumed to be 5,000,000 for each cu. mm. The percentage of corpuscles may be found by multiplying the first two figures of the red corpuscle count by 2. This simple method holds good for all counts of 1,000,000 or more. Thus, a count of 2,500,000 is 50 per cent of the normal. If, then, the hemoglobin has been estimated at 40 per cent divide 40 (the percentage of hemoglobin) by 50 (the percentage of corpuscles). This gives $\frac{4}{5}$, or 0.8, as the color index.

From what has already been said regarding the variations in hemoglobin instruments, and of the impossibility of fixing a normal standard for either red cells or hemoglobin which is applicable to all ages and in all localities, it would appear that color-index calculations, as above described, have little value. They do, however, yield information of great value in many cases of anemia, provided that the hemoglobinometer used has been standardized to give an average reading of 100 per cent upon the bloods of healthy adults with red corpuscle counts of 5,000,000 in each cubic millimeter (p. 207). In childhood, from about the sixth month to the fifteenth year, the color index is normally low, since the hemoglobin values fall disproportionately during this period of life. It is probable that the normal corpuscles are saturated with hemoglobin, and therefore that a high color index must be dependent on increased size of the corpuscles. A low index, on the other hand, may be due either to small size of the red cells or to lowered concentration of hemoglobin in their substance.

VI. VOLUME INDEX

The term "volume index" was introduced by Capps to express the average size of the red cells of an individual compared with their normal size. It is the quotient obtained by dividing the *volume* of red corpuscles, expressed in percentage of the normal, by the *number* of red corpuscles, also expressed in percentage of the normal. This means of expressing the average size of the red cells gives accurate information more readily than does measuring them under the microscope.

The volume index more or less closely parallels the color index, but is more dependable and more significant. A volume index above 1 is practically constant in pernicious anemia and is now regarded as one of the most important signs of the disease. The following are averages of the examinations reported by Larrabee:

	Red corpuscles for each cubic millimeter.	Hemoglobin per cent by Sahli instrument.	Color index.	Volume index.
Normal males	5,267,250	103.0	0.98	1.007
Normal females	4,968,667	106.0	1.06	1.001
Primary pernicious anemia	1,712,166	50.0	1.47	1.270
Secondary anemia	3,737,160	61.0	0.81	0.790
Chlorosis	3,205,000	34.5	0.55	0.695

Method.—The Wintrobe hematocrit tube (Fig. 117) is very often used for determining corpuscular volume. Prepare a solution containing 0.8 Gm. of potassium oxalate and 1.2 Gm. of ammonium oxalate in 100 c.c. of distilled water. Place exactly 0.5 c.c. of this mixture in each test tube and evaporate off the water and thoroughly dry before use. This ammonium and potassium mixture does not shrink the erythrocytes, so no correction of the final reading of cell solution is necessary. Place 5 c.c. of venous blood in a test tube containing 10 mg. of the oxalate mixture. Transfer with a capillary pipet the oxalated blood to the hematocrit tube which is filled exactly to the 10 cm. mark, cap to prevent evaporation and centrifuge for twenty minutes at 3000 r. p. m. Make a reading and continue the centrifugation for ten minutes longer to insure complete packing. The reading on the tube multiplied by ten indicates directly the percentage of packed erythrocytes per 100 c.c. of blood. The sedimentation rate may be determined in the same tube before centrifugation by the Wintrobe and Landsberg method (see p. 330).

Fig. 117.—Wintrobe hematocrit tube.

A similar method which requires a still larger quantity of blood has been described by Haden and is now much used. A graduated 15-c.c. centrifuge tube is filled exactly to the 2 c.c. mark with 1.1 per cent[1, 2] sodium oxalate solution; and 10 c.c. of blood is added exactly to the 12 c.c. mark. The tube is inverted to oxalate the blood. The cells are packed to the maximum amount, normal blood packing to 4.6 c.c. or 4.8 c.c. The value for normal blood should be determined with each centrifuge. The

[1] The strength of oxalate solution which originally was recommended by Haden was 1.6 per cent. Later, he advised a 1.4 per cent oxalate solution. However, 1.1 per cent oxalate solution is more nearly isotonic, and should be used. The results will be slightly different with different strength oxalate solutions.

[2] Magath, T. B., and Hurn, Margaret: Concern ing Anticoagulants, Am. Jour. Clin. Path., 5:548-557 (Nov.), 1935.

subsequent calculations are similar to those described in the preceding paragraph.

Sanford and Magath[1] described a new centrifuge tube for volume index determinations with a slight modification of the Haden method (Fig. 118). This tube is made of heavy pyrex glass and will not break in the centrifuge. It is 5 inches long and will fit in the 15 c.c. centrifuge tube shell. The capacity of the tube is only 6 c.c., so that the graduations in 0.1 c.c. are well separated and the level of the packed cells is easily seen. The walls are parallel throughout the entire graduated portion, thus making the reading of volumes at all levels equally easy. Half the original Haden quantities are used, and the cells from normal blood will pack to the 2.4 c.c. mark. The tube is also very useful in determinations of total blood volume (p. 332).

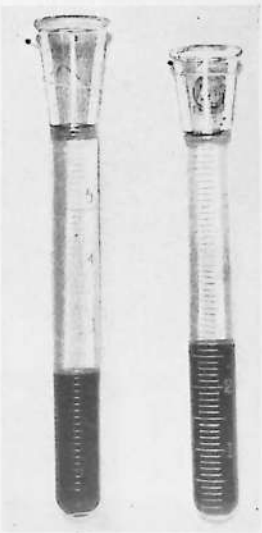


Fig. 118.—Sanford-Magath volume index tube.

VII. DIAMETERS OF ERYTHROCYTES

Many years ago, Price-Jones[2] described a method for measuring the mean diameters of erythrocytes. Blood films are made and stained in the usual manner. Some method of projecting them on to a sheet of paper on the table with a magnification of 1000 diameters is necessary. Outline in pencil the individual cells, measuring 2 diameters to within 0.5 mm. with a millimeter rule. The mean of the two measurements is taken as the diameter of the cell. Not less than 200 erythrocytes should be measured in this manner, and preferably a greater number, that is, up to 500. The measurements are grouped by 0.25 microns and the mean diameter of the entire number is taken as the mean for the sample. Price-Jones' original studies gave 7.4 microns as the mean diameter of normal erythrocytes. This is considered now to be slightly low for a normal mean.

Although Price-Jones' curves have been made and studied by many hematologists ever since, this method is laborious and has been replaced by

[1] Sanford, A. H., and Magath, T. B.: A New Centrifuge Tube for Volume Index Determinations (Modified Haden Method), Jour. Lab. and Clin. Med., *15*:172–173 (Nov.), 1929.

[2] Price-Jones, Cecil: The Variation in the Sizes of Red Blood Cells. Brit. Med. Jour. *2*:1418–1419 (Nov. 5) 1910.

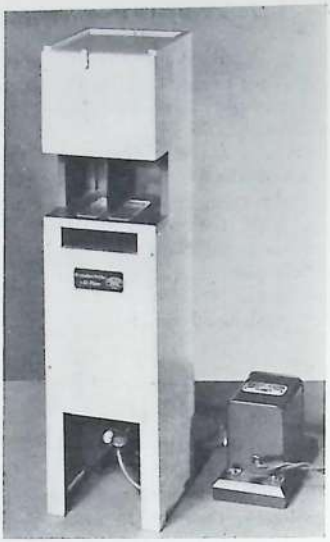


Fig. 119.—Pijper apparatus for measuring erythrocytes.

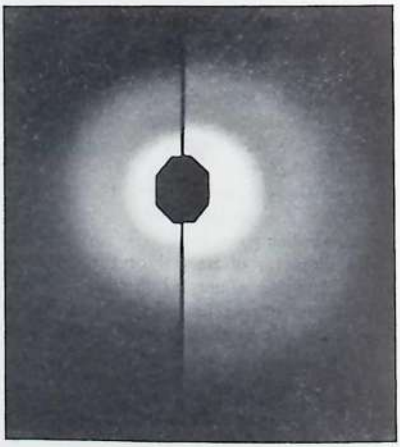


Fig. 120.—Corona of normal sized corpuscles (*right*); Corona of macrocytosis (*left*).

simpler technics. Pijper[1] described and improved a diffraction method for measuring erythrocytes (Fig. 119). With this method an ordinary blood film

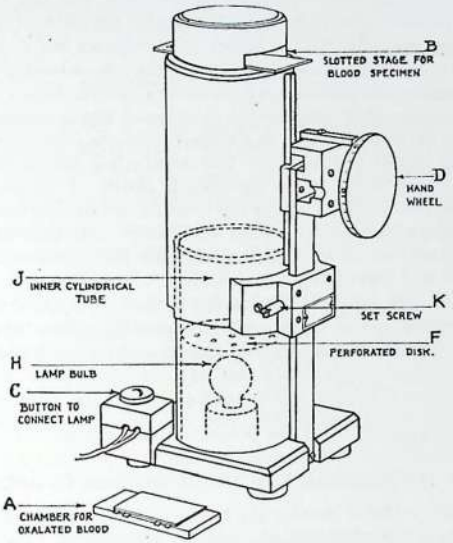


Fig. 121.—Diagrammatic view of Haden-Hausser erythrocytometer.

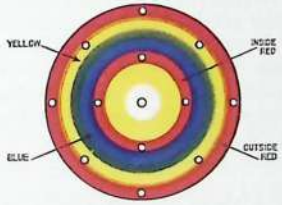


Fig. 122.—Field of view in the Haden-Hausser erythrocytometer (A. H. Thomas Co.).

is used as a diffraction grating. This method uses the phenomenon of diffraction first applied by Young a century ago for the measuring of wool fibers.

[1] Pijper, Adrianus: The Diagnosis of Addison's (Pernicious) Anemia by a New Optical Method. Lancet. *2*:367–368 (Aug. 23) 1924. Pijper, Adrianus: An Improved Diffraction Method of Diagnosing and Following the Course of Pernicious and Other Anemias. Brit. Med. Jour. *1*:635–638 (Apr. 6) 1929.

A beam of parallel rays of white light produced by a series of lenses is passed through an evenly made blood film. As the rays strike the periphery of each erythrocyte they are bent in toward the corpuscle and also split into the component colors so as to form concentric rings of the colors of the spectrum. These images are collected by lenses and projected on a ground glass screen. The apparatus is so constructed that the corona formed by a normal blood film makes half the images projected on a ground glass. The film made from blood composed of erythrocytes of unknown size is placed so that the corona made by this slide used as a diffraction grating forms the other half of the image on the ground glass. The examination should be made in a darkened room in order to see the coronas clearly. A comparison of the two coronas is very simple. If the erythrocytes are smaller than normal in the blood being examined, the corona will be larger than that made with the normal erythrocytes. If there is macrocytosis, the corona will be smaller than normal (see Fig. 120).

Using this same principle, Haden[1] has devised a very useful instrument for the measurement of erythrocyte diameters by diffraction. With this instrument, the light is moved back and forth and the calibrations are made on the large wheel which moves the light until the corona is adjusted to a certain position in relation to four small points of light. The mean diameter of the erythrocytes is read directly from the calibrations on the wheel (see Figs. 121 and 122).

VIII. CORPUSCULAR SIZE AND HEMOGLOBIN CONTENT

Wintrobe's study[2] of the size and hemoglobin content of the erythrocyte produced three valuable indices useful in hematology, especially in the classification of the anemias according to normochromic, or hypochromic normocytosis, macrocytosis, or microcytosis as shown in Fig. 160, page 301. The mean corpuscular volume, the mean corpuscular hemoglobin by weight, and the mean corpuscular hemoglobin concentration in percentage may all be easily calculated if the erythrocytes have been accurately enumerated, the hemoglobin determined in grams for each 100 c.c. of blood, and the volume of cells determined by some hematocrit method.

The *mean corpuscular volume* (C.V.) = volume of packed cells (in 1000 c.c. blood) ÷ R.B.C. (in millions per cu. mm.). The mean corpuscular volume is recorded in cubic microns (cu. μ). The normal range is from 80 to 94 cubic microns.

The *mean corpuscular hemoglobin* (C.H.) = hemoglobin in grams per 1000 c.c. of blood ÷ R.B.C. (in millions per cu. mm.). This gives the average weight of hemoglobin in each erythrocyte in micromicrograms ($\gamma\gamma$), or millionth part of a microgram (gram $\times$ 10^{-12}). The normal range is from 27 to 32 micromicrograms.

[1] Haden, R. L.: A New Instrument for the Diffractometric Measurement of the Diameter of Red Blood Cells. Jour. Lab. and Clin. Med *25*:399–403 (Jan.), 1940.

[2] Wintrobe, M. M.: The Size and Hemoglobin Content of the Erythrocyte, Jour. Lab. and Clin. Med., *17*:899–912 (June), 1932.

The *mean corpuscular hemoglobin concentration* (C.C.) = hemoglobin in grams per 100 c.c. ÷ volume of packed cells, in c.c. per 100 c.c. × 100. This gives the average concentration of hemoglobin in the erythrocytes expressed in per cent (%). This normally is from 32 to 38 per cent.

IX. ENUMERATION OF LEUKOCYTES

The normal number of leukocytes varies from 5000 to 10,000 per cubic millimeter of blood. The number is larger in robust individuals than in poorly nourished ones, and, if disease be excluded, may be taken as a rough index of the individual's nutrition. It rises appreciably in the afternoon regardless of the intake of food. Sabin finds the highest count on any day to be approximately twice the lowest count on the same day (the highest usually between 1 and 5 P. M.), and she also finds an hourly rhythm in the count. These variations depend on the neutrophils chiefly. Since it is well to have a definite standard, 7500 is generally adopted as the normal for the adult. With children the number is somewhat greater, averaging about 10,000 to 12,000 in infants and somewhat below 10,000 in older children. Leukocyte counts have much less diagnostic value in young children than in adults, since the blood of children reacts much more markedly and often in an atypical manner.

Decrease in Number of Leukocytes

Counts of 3500 to 4000 per cubic millimeter are not uncommon in persons who are poorly nourished, although not actually sick. The infectious diseases in which leukocytosis is absent (p. 238) often cause a slight and sometimes a marked decrease in number of leukocytes, owing probably to toxic inhibition of bone-marrow activity. Leukocytes occasionally fall below 1000 for each cubic millimeter, and if agranulocytosis (p. 322), or lack of polymorphonuclears, exists, the prognosis will be poor. Chlorosis may produce leukopenia, as also pernicious anemia, which usually gives it in contrast to the secondary anemias, which are frequently accompanied by leukocytosis. Leukocyte counts are, therefore, of some aid in the differential diagnosis of these conditions.

In contrast to the normal digestive leukocytosis (p. 237), Widal has called attention to a digestive leukopenia, along with accelerated coagulation of the blood and a fall in blood pressure, in persons suffering from hepatic disease. This so-called "hemoclastic crisis" is induced by 200 c.c. of milk given upon an empty stomach, the maximal response occurring in about forty-five to fifty minutes. At one time

it promised to be useful as a test of hepatic insufficiency, but is now little regarded excepting as an interesting phenomenon.

Increase in Number of Leukocytes

Increase in number of leukocytes is common and of great importance. It may be considered under two heads:

A. Increase of leukocytes due to chemotaxis and stimulation of the blood-making organs, or *leukocytosis*. The increase affects one or more of the normal varieties.

B. Increase of leukocytes due to *leukemia*. Normal varieties are increased, but the characteristic feature is the appearance of great numbers of abnormal cells.

The former may be classed as a *transient*, the latter as a *permanent*, increase.

A. Leukocytosis

This term is variously used. By some it is applied to any increase in number of leukocytes; by others it is restricted to increase of the polymorphonuclear neutrophilic variety. As has been indicated, it is here taken to mean a transient increase in number of leukocytes, that is, one caused by chemotaxis and stimulation of the blood-producing structures, in contrast to the permanent increase caused by leukemia.

By *chemotaxis* is meant that property of certain agents by which they attract or repel living cells—positive chemotaxis and negative chemotaxis respectively. An excellent illustration is the accumulation of leukocytes at the site of inflammation, owing to the positively chemotactic influence of bacteria and their products. A great many agents possess the power of attracting leukocytes into the general circulation. Among these are many bacteria and certain organic and inorganic poisons.

Chemotaxis alone will not explain the continuance of leukocytosis for more than a short time. It is probable that substances which are positively chemotactic also stimulate the blood-producing organs to increased formation of leukocytes, and in at least one form of leukocytosis such stimulation apparently plays the chief part.

As will be seen later, there are several varieties of leukocytes in normal blood, and most chemotactic agents attract only one variety, and either repel or do not influence the others. It practically never happens that all are increased in the same proportion. The most satisfactory classification of leukocytoses is, therefore, based upon the type of leukocyte chiefly affected.

Theoretically, there should be a subdivision for each variety of leukocyte, for example, neutrophilic leukocytosis, lymphocytic leukocytosis, eosinophilic leukocytosis, monocytosis, and so forth. Practically, however, only two of these, polymorphonuclear leukocytosis and lymphocytic leukocytosis, need be considered under the head of Leukocytosis. Increase in number of the other leukocytes will be considered when the individual cells are described (pp. 267–285). They are present in the blood in such small numbers normally that even a marked increase scarcely affects the total leukocyte count; and, besides, substances which attract them into the circulation frequently repel the polymorphonuclears, so that the total number of leukocytes may actually be decreased.

The polymorphonuclear neutrophils are capable of active ameboid motion, and are by far the most numerous of the leukocytes. Lymphocytes are about one third as numerous and have little independent motion. As one would, therefore, expect, marked differences exist between the two types of leukocytosis; neutrophilic leukocytosis is more or less acute, coming on quickly and often reaching high degree; whereas lymphocytic leukocytosis is more chronic, comes on more slowly, and is seldom so marked.

1. Polymorphonuclear Neutrophilic Leukocytosis.—Neutrophilic leukocytosis may be either physiologic or pathologic. A count of 20,000 would be considered a marked leukocytosis; of 30,000, high; above 50,000, extremely high.

(1) **Physiologic Neutrophilic Leukocytosis.**—This is never very marked, the count seldom exceeding 12,000 or 14,000 in each cubic millimeter. It may occur: (*a*) In the newborn, as high as 18,000 in the first few days of life; (*b*) in pregnancy during the ninth month, most marked in primiparae; (*c*) during labor, averaging about 18,000 in primiparae, much less in multiparae, and subsiding during the first few days of the puerperium; (*d*) during digestion, and (*e*) after cold baths. There is moderate leukocytosis in the moribund state, occasionally reaching 20,000; this is commonly classed as physiologic, but is probably due mainly to terminal infection.

The increase in these conditions is not limited to the neutrophils. Lymphocytes are likewise increased in varying degrees, most markedly in the newborn.

In view of the normal rise in the afternoon and of the leukocytosis of digestion, which usually increases the leukocytes by about 30 per cent, the hour at which a leukocyte count is made should always be recorded. Digestive leukocytosis is most marked three to five hours after a hearty meal rich in protein, especially when such a meal fol-

lows a long fast. It is absent in pregnancy and when leukocytosis from any other cause exists. It is usually absent in cancer of the stomach, a fact which may be of some help in the diagnosis of this condition, but repeated examinations and careful technic are essential. The absence of digestive leukocytosis in liver disease (Widal's test of liver function) has been mentioned on page 235.

(2) **Pathologic Neutrophilic Leukocytosis.**—In general, the response of the leukocytes to chemotaxis is a conservative process. It has been compared to the gathering of soldiers to destroy an invader. This is accomplished partly by means of phagocytosis—actual ingestion of the enemy—and partly by means of chemical substances which the leukocytes produce.

In those diseases in which leukocytosis is the rule the degree of leukocytosis depends upon two factors: The *severity of the infection* and the *resistance of the individual.* A well-marked leukocytosis usually indicates good resistance. A mild degree means that the body is not reacting well, or else that the infection is too slight to call forth much resistance. Leukocytosis may be absent altogether when the infection is extremely mild, or when it is so severe as to overwhelm the organism before it can react. When leukocytosis is marked, a sudden fall in the count may be the first warning of a fatal issue. These facts are especially true of pneumonia, diphtheria, and abdominal inflammations, in which conditions the degree of leukocytosis is of considerable prognostic value.

The classification here given follows Cabot in the main:

(*a*) *Infectious and Inflammatory Diseases.*—The majority of infectious diseases produce leukocytosis. The degree varies with the site and nature of the disease, the virulence of the infection, the resistance of the individual, and the presence of complications, hence no definite figures can be given for any particular disease. However, the counts which are to be expected in typical cases of the more important infectious diseases which produce leukocytosis are somewhat as follows: Pneumonia, 20,000 to 30,000, a few counts over 100,000 having been recorded; scarlet fever, 20,000 to 30,000; diphtheria, 15,000 to 25,000; erysipelas, 20,000 to 25,000; Asiatic cholera, 25,000 to 30,000; meningococcus meningitis, 20,000 to 30,000; tuberculous meningitis, 10,000 to 20,000; acute articular rheumatism, 10,000 to 15,000.

The most important infectious diseases *which do not cause leukocytosis* are influenza; measles; German measles; malaria; mumps; tuberculosis, except when invading the meninges or when complicated by mixed infection; and typhoid and paratyphoid fevers, in which leukocytosis indicates an inflammatory complication. In influenza

uncomplicated cases generally show low normal leukocyte counts, averaging 5000 to 6000, with a tendency to subnormal counts on the first day or two and with the lymphocytes at a high normal level or slightly increased. In an occasional case the count may be 12,000 to 15,000. With the onset of pneumonia a definite leukocytosis usually occurs.

All inflammatory and suppurative processes cause leukocytosis, except when slight or well walled off. This is particularly marked in infections with staphylococci, streptococci, and pneumococci. Appendicitis has been studied with especial care in this connection, and the conclusions now generally accepted probably hold good for most acute intra-abdominal inflammations. A marked leukocytosis (20,000 or more) nearly always indicates abscess, peritonitis, or gangrene, even though the clinical signs be slight. Absence of or mild leukocytosis indicates a mild process, or else an overwhelmingly severe one; and operation may safely be postponed unless the abdominal signs are very marked. On the other hand, no matter how low the count, an increasing leukocytosis—counts being made hourly—indicates a spreading process and demands operation, regardless of other symptoms.

Leukocyte counts alone are often disappointing in infectious and inflammatory conditions, but are of much greater value when considered in connection with the percentage of neutrophils (p. 270). *Every total leukocyte count should be accompanied by a differential count.*

(*b*) *Malignant Disease.*—Leukocytosis occurs in about one half of the cases of malignant disease. In many instances it is probably independent of any secondary infection, since it occurs in both ulcerative and nonulcerative cases. It seems to be more common in sarcoma than in carcinoma. Very large counts are rarely noted.

(*c*) *Postoperative.*—The number of leukocytes rises moderately after operations irrespective of infection, reaching a maximum in about six hours. The degree depends upon the severity of the operation, amount of tissue damaged, loss of blood, and possibly upon the anesthetic used.

(*d*) *Posthemorrhagic.*—Moderate leukocytosis follows hemorrhage and disappears in two to four days. This is especially true of hemorrhage into the serous cavities which nearly always increases the leukocyte count 150 to 300 per cent within ten hours. In cases of ruptured tubal pregnancy with hemorrhage into the peritoneal cavity the count usually reaches 18,000 to 30,000.

(*e*) *Toxic.*—This is a rather obscure class, which includes gout, chronic nephritis, acute yellow atrophy of the liver, ptomaine poison-

ing, prolonged chloroform narcosis, and quinine poisoning. Leukocytosis may or may not occur in these conditions, and is not important.

(*f*) *Drugs.*—This also is an unimportant class. Most tonics and stomachics and many other drugs produce a slight leukocytosis. A moderate leukocytosis may also occur as a result of prolonged anesthesia.

2. Lymphocytic Leukocytosis.—This is characterized by an increase in the total leukocyte count, accompanied by an increase in the percentage of lymphocytes. The word "lymphocytosis" is often used in the same sense. It is better, however, to use the latter as referring to any increase in the absolute number of lymphocytes, without regard to the total count, since an absolute increase in number of lymphocytes is frequently accompanied by a normal or subnormal leukocyte count, owing to loss of neutrophils.

Lymphocytic leukocytosis is probably due more to stimulation of blood-making organs than to chemotaxis. It is less common, and is rarely so marked as a neutrophilic leukocytosis. When marked, the blood cannot be distinguished from that of lymphatic leukemia.

A marked or high lymphocytic leukocytosis occurs in pertussis. It is said to appear early in the catarrhal stage and to reach its maximum at the height of the paroxysmal stage, after which it gradually subsides. In thirty well-marked cases studied by Schneider the average leukocyte count was 19,000 in the first week, rising to about 27,000 in the third. His lowest counts in the first week were 12,600, and in the third 16,800. Leukocyte counts would, therefore, seem to have great value in diagnosis, but in our experience they have often been disappointing, since in many mild cases the count does not rise above what may be regarded as a high normal for children before the characteristic whoop begins.

There is slight or moderate lymphocytic leukocytosis in other diseases of childhood, as rickets, scurvy, and especially hereditary syphilis. In the last mentioned disease the blood picture may at times approach that of pertussis. It must be borne in mind in this connection that lymphocytes are normally more abundant in the blood of children than in that of adults.

Within the past few years a number of cases of "acute infectious mononucleosis" with glandular enlargement and fever have been reported (p. 321).

B. Leukemia

This is an idiopathic disease of the blood-making organs which is accompanied by an enormous increase in number of leukocytes. The leukocyte count sometimes reaches 1,000,000 per cubic millimeter,

and leukemia is always to be suspected when it exceeds 50,000. Lower counts do not, however, exclude it. The subject is more fully discussed later (p. 317).

Method of Counting Leukocytes

The leukocytes are counted with the hemacytometer already described (p. 218). Numerous modifications of the original ruling have been introduced, notably the Türk, the Zappert-Ewing, and the Neubauer (Fig. 123), which give a ruled area of 9 sq. mm., the center having the ruling which is used for counting the red corpuscles. Of these, the Neubauer may be especially commended. Some of them were originally devised for counting the leukocytes in the same dilution with the red corpuscles. The two kinds of cell are easily

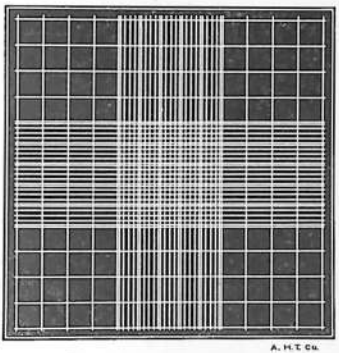


Fig. 123.—Entire area of improved Neubauer ruling.

distinguished, especially when Toisson's diluting fluid is used. The red cells are counted in the central portion in the usual manner, after which all the leukocytes in the whole area of 9 sq. mm. are counted, and the number in a cubic millimeter of undiluted blood is then calculated.

Although less convenient, it is more accurate to count the leukocytes separately, with less dilution of the blood, as described in the following paragraphs.

Technic.—A larger drop of blood is required than for counting the erythrocytes, and more care in filling the pipet, since the bore is considerably larger than that of the "red" pipet. If desired a "throttle" may be used in the rubber tubing on the pipet. Amstutz suggested the use of a 25-gauge hypodermic needle as a mouthpiece for this purpose.

Use the pipet with 11 engraved above the bulb.[1] Suck the blood to the mark 0.5 or 1, and the diluting fluid to the mark 11. This gives a dilution of 1 : 20 or 1 : 10, respectively. The dilution of 1 : 20 is easier to make. If the blood should accidentally go much above the 0.5 mark, draw it to 0.6 and multiply the final count by 5/6. Mix well by shaking in all directions except in the long axis of the pipet; blow out 2 or 3 drops, place a drop in the counting chamber, and charge the counting chamber as already described (p. 223).

Examine with a low power to see that the cells are evenly distributed. Count with the 16-mm. objective and a high-power eyepiece, or with the long-focus 4-mm. and a low-power eyepiece. An 8-mm. objective also will be found very satisfactory for this purpose. As one gains experience one will rely more upon the lower powers. A square millimeter may be included in the field of the 16-mm. objective with a medium ocular.

Count all the leukocytes in several square millimeters and find the average per square millimeter. Multiply this by 10 to find the number in 1 cu. mm. of diluted blood, and by the dilution to find the number in a cubic millimeter of undiluted blood. In every case at least 200 leukocytes must be counted as a basis for calculation, and it is much better to count 500. With Neubauer and similar rulings and a dilution of 1 in 20, a convenient plan is the following: With a low-power objective count the leukocytes in the square millimeters at each of the four corners of the ruled area and multiply by 50, or divide the count by two, and add two ciphers. This should be repeated upon a second and a third slide and the average taken.

In routine work Todd's modification of the "circle" method is very satisfactory. It requires a 4-mm. objective, and is, therefore, especially desirable for beginners, who are usually unable accurately to identify leukocytes with a lower power. The student is frequently confused by particles of dirt, remains of red cells, and yeast cells which are prone to grow in the diluting fluid. Draw out the sliding tube of the microscope until the field of vision is such as shown in Fig. 124. One side of the field is tangent to one of the ruled lines, A, while the opposite side just cuts the corners, B and C, of the seventh squares in the rows above and below the diameter line. When once adjusted, a scratch is made upon the draw tube, so that for future counts the tube has only to be drawn out to the mark. The area of this microscopic field is 0.1 sq. mm. With a dilution of 1 : 20, count the leukocytes in 20 such fields upon different parts of the disk without regard to the ruled lines, and to their sum add two ciphers. With dilution of 1 : 10, count 10 such fields, and add two ciphers. Thus, with 1 : 10 dilution, if 150 leukocytes were counted in 10 fields, the leukocyte count would be 15,000 for each cubic millimeter. To compensate for possible unevenness of distribution, it is best to count a row of fields horizontally and a row vertically across the disk. This method is applicable to any

[1] In some cases of leukemia with very high count it may be necessary to use the "red" pipet with dilution of 1 : 100.

degree of dilution of the blood, and is simple to remember: *One always counts a number of fields equal to the number of times the blood has been diluted, and adds two ciphers.* The count should be repeated upon several slides and the average taken.

It is sometimes impossible to obtain the proper size of field with the objectives and eyepieces at hand. In such case place a cardboard or stiff paper disk with a circular opening upon the diaphragm of the eyepiece, and adjust the size of the field by drawing out the tube. The circular opening can be cut with a sharp cork borer.

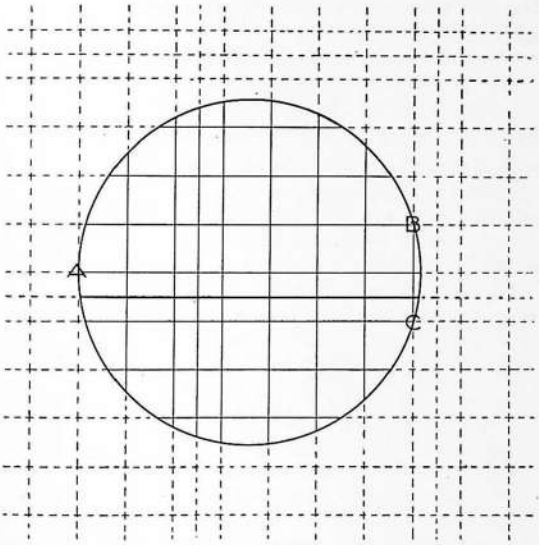


Fig. 124.—Size of field required in counting leukocytes as described in the text.

Diluting Fluids.—The diluting fluid should dissolve the red corpuscles so that they will not obscure the leukocytes. The simplest fluid is a 1 per cent solution of acetic acid. More satisfactory is the following: Glacial acetic acid, 1 c.c.; 1 per cent aqueous solution of gentian violet, 1 c.c.; distilled water, 100 c.c. These solutions must be filtered frequently to remove yeasts and molds.

X. ENUMERATION OF BLOOD PLATELETS

The normal number of blood platelets, when counted by the direct method described below, ranges from 250,000 to 350,000 for

each cubic millimeter of blood. They are intimately connected with the process of coagulation and this appears to be their chief function. The precise rôle which they play is not, however, entirely clear. They are apparently the chief source of the prothrombin of the blood plasma; they probably furnish at least part of the thromboplastic substance of the tissues; and, after coagulation has taken place, they undoubtedly serve to bring about retraction of the clot, since the rapidity and degree of retraction vary directly with the number of platelets present. In general, whenever from any cause the platelets fall below 60,000 for each cubic millimeter of blood, a hemorrhagic tendency becomes evident.

Physiologic variations in number of platelets are marked. Thus, the number increases as one ascends to a higher altitude, and is higher in winter than in summer. There are unexplained variations from day to day; hence a single abnormal count should not be taken to indicate a pathologic condition. In disease, variations are often extremely great. An increase may be due to increased number or increased activity of the bone marrow giant cells (megakaryocytes) which are the mother cells of the platelets. A decrease is more important. It may be referable to injury or destruction of the megakaryocytes, or to destruction of platelets in the circulation. There is no means of recognizing increased destruction of platelets as there is in the case of red corpuscles. The normal life of the platelets is estimated (Lee and Minot) at about four days. Even when platelets are present in normal numbers they may be functionally defective.

Upon the clinical side the following facts seem to be established:

(*a*) In acute infectious diseases the number is usually normal or subnormal. In diphtheria, especially, the count may fall to a very low level with development of a tendency to hemorrhage. As a rule, the platelets increase during convalescence.

(*b*) In secondary anemia, especially posthemorrhagic, platelets are generally increased, although sometimes decreased. In pernicious anemia they are nearly always greatly diminished, and an increase would question the diagnosis of this condition.

(*c*) They are decreased in chronic lymphatic leukemia; variable but usually greatly increased in chronic myelogenous leukemia, much decreased in acute leukemia of either form.

(*d*) They are somewhat increased in tuberculosis.

(*e*) Platelet counts are of great value in differentiating the hemorrhagic diseases. Both hemophilia and purpura haemorrhagica are caused by disturbances of the platelets, but may be sharply differentiated. In hemophilia platelets are normal in number, but defective

in function, bringing about coagulation very slowly. The clot, though delayed, is well formed and has normal retractile power. In purpura haemorrhagica, upon the other hand, platelets are functionally normal, but greatly reduced in numbers, ranging from about 40,000 to 75,000 for each cubic millimeter in mild cases, and down to 15,000 or below in severe cases. Coagulation time is about normal, but the platelets are too few to cause normal retraction of the clot.

Blood platelets are difficult to count, owing to the rapidity with which they disintegrate and to their strong tendency to adhere to any foreign body and to each other. The unavoidable error is greater than in counting red corpuscles or leukocytes, but is negligible in practice, because only very great variations in the count have clinical significance. It is often possible to recognize a great loss of platelets from a careful inspection of stained films, provided these are made evenly and very quickly in order to avoid clumping.

Method of Counting Platelets.—Many methods have been proposed. Of these, the direct method, which employs the hemacytometer already described, is the most practicable for clinical purposes, although the counts are regularly lower than are yielded by some of the indirect methods.

Rapid work is necessary in order to prevent clumping of the platelets. The diluting fluid is drawn to near the 1 mark in the "red" pipet, blood from a freely bleeding puncture is drawn exactly to the 0.5 mark, and finally the diluting fluid is quickly drawn to the 101 mark. This gives a blood dilution of 1 in 200. The blood and diluting fluid are immediately mixed by shaking for about two minutes; the counting chamber is filled at once, and ten minutes are allowed for the corpuscles to settle before counting is begun. The count is made with the high dry objective and a high ocular (10×) in the manner described for red corpuscles. A control count of the platelets should always be made with the blood from a normal person, at the same time, and using the same diluting fluid and exactly the same technic.

Diluting Fluid of Wright and Kinnicutt.[1]—Platelets appear as rounded, lilac-colored bodies; red corpuscles are decolorized, appearing only as shadows; leukocytes are stained.

Aqueous solution brilliant cresyl blue (1 : 300)	2 parts
Aqueous solution potassium cyanide (1 : 1400)	3 "

These two solutions are kept in separate bottles and mixed and filtered just before using. The cresyl blue solution is permanent, but molds have a tendency to grow in it. The cyanide solution deteriorates after about ten days.

[1] Wright, J. H., and Kinnicutt, Roger: A New Method of Counting the Blood Platelets for Clinical Purposes, and Some of the Results Obtained with It, Jour. Am. Med. Assn., *56*:1457–1459 (May 20), 1911.

Diluting Fluid of Rees and Ecker.[1]—This solution preserves the red corpuscles, which may be counted in the same specimen.

Sodium citrate, 3.8 per cent aqueous solution	100.0 c.c.
Formaldehyde, 40 per cent solution	0.2 "
Brilliant cresyl blue	0.1 Gm.

Diluting Fluid of Guy and Leake.[2]—This fluid has been found especially satisfactory. It does not destroy the red corpuscles.

Distilled water	94.0 c.c.
Formalin, 40 per cent solution	6.0 "
Sodium oxalate	1.6 Gm.
Crystal violet	0.01 "

The fluid is warmed, filtered, and preserved in stoppered bottles. It keeps well.

Van Allen[3] **"Thrombocytocrit."**—This method has proved a satisfactory substitute for counting methods. It requires a special spherical sedimentation chamber of 20 c.c. capacity, and a specially constructed centrifuge tube (Fig. 125). The capillary portion of this tube has a capacity of 0.03 c.c., and is graduated in thousandths of a milliliter. In use it is necessary to close this tube with a spring clip which must be purchased with the apparatus.

Method.—Draw exactly 6 c.c. of 1.3 per cent solution of sodium oxalate into a Record syringe. Exclude the air bubble, and draw exactly 4 c.c. of blood from the patient's vein into the syringe containing the anticoagulant. Deposit this in the sedimentation chamber, and add exactly 10 c.c. of the oxalate solution. Thoroughly mix the contents with the syringe, and set the chamber aside for three and a half hours. By means of the syringe and needle, carefully remove exactly 5 c.c. of the supernatant fluid; this represents 1 c.c. of the original blood. Include a slight trace of the corpuscular sediment to give the fluid a slightly reddish tinge. Place this in the thrombocytocrit, apply the sealing clips, and centrifugalize at 3500 revolutions per minute for one and a half hours. The capillary portion of the tube will be partly filled with sediment which will be sharply defined in two layers. The red tinged lower layer represents the corpuscles, while the platelets lie above in an ivory white layer. The volume of this layer can be read in thousandths of a cubic centimeter. By shifting the decimal point two places to the right, the result will indicate the percentage of platelets by volume in whole blood. The normal range for human beings is 0.35 to 0.67 per cent with this method. Laboratory animals have a higher range.

[1] Rees, H. M., and Ecker, E. E.: An Improved Method for Counting Blood Platelets Jour. Am. Med. Assn., *80*:621–622 (March 3), 1923.

[2] Leake, C. D., and Guy, E. F.: A Diluting Fluid for Platelet Counting, Jour. Am. Med. Assn., *84*:890–891 (March 21), 1925.

[3] Van Allen, C. M.: Volumetric Measurement of Blood Platelets, Jour. Lab. and Clin. Med., *12*:282–285, 1926.

Plasma Platelet Count.—Nygaard[1] has developed a simple "plasma platelet count" method. This method utilizes a modification of the Van Allen method, and also a principle which was described by Thomsen.[2] The only *reagent* required is a 1.1 per cent solution of sodium oxalate.[3] (This may contain a little brilliant cresyl blue for better identification of the platelets, but this is not necessary.) The oxalate solution should be sterilized in the autoclave, and kept in a sterile container. Use a 10 c.c. all-glass syringe which is accurate at the 9 c.c. mark, and at the 10 c.c. mark. As a sedimentation chamber, use the Van Allen chamber or any convenient tube of suitable size.

Method.—1. Draw exactly 9 c.c. of oxalate solution into the syringe.

2. By venipuncture, draw exactly 1 c.c. of the patient's blood into the oxalate solution in the syringe. That is, the blood is withdrawn so that the mixture measures exactly 10 c.c. in the syringe.

3. Expel the contents of the syringe into the sedimentation chamber, and draw exactly 10 c.c. of oxalate solution into the syringe; this is added to the mixture in the sedimentation chamber.

4. Mix oxalate solution and blood thoroughly by closing the mouth of the chamber and gently inverting the chamber once or twice. The dilution of blood is 1 to 20.

5. After sedimentation of the erythrocytes has occurred, and at any time up to four to six hours, transfer, with a capillary pipet, a portion of the oxalated plasma to a counting chamber.

6. Count with the 4-mm. objective all of the platelets in 80 small squares in five groups of 16 small squares in different parts of the hemacytometer. It is convenient to count one group in each corner of the central cross-ruled area, and one group near the center.

Fig. 125.—Van Allen thrombocytocrit.

Add three zeros to the total count for the number of platelets per cubic millimeter of blood. With this method the average normal platelet count is about 320,000 per cubic millimeter.

Still another method which combines some of the features of the methods described has been found useful by Walker and Sweeney.[4] Draw 1.1 per cent sodium oxalate into a white blood cell pipet to the 1 mark and expel

[1] Nygaard, K. K.: A Direct Method of Counting Platelets in Oxalated Plasma, Proc. Staff Meetings of Mayo Clinic, *8*:365–370 (June 14), 1933.

[2] Thomsen, Oluf: A Method for Direct Count of the Blood Plates in the Blood, Acta Med. Scand., *53*:507–516, 1921.

[3] Nygaard used 1.6 per cent solution of sodium oxalate, but a 1.1 per cent solution is recommended as an anticoagulant as it is more nearly isotonic, and is used in blood volume determinations.

[4] Walker, T. F. and Sweeney, P. A.: A Method of Counting Blood Platelets. J. Lab. and Clin. Med. *25*:103–105 (Oct.) 1939.

by blowing. This wets that portion of the pipet and prevents the platelets from adhering to the glass. Draw blood to the 0.5 mark, then fill the pipet to the 11 mark with 1.1 per cent sodium oxalate solution. Mix by shaking thoroughly. Place a heavy, rubber band around the pipet to close the ends and centrifuge for half a minute at 1600 revolutions per minute to drive the erythrocytes into the stem of the pipet. Gently expel the erythrocytes from the stem of the pipet and place a drop of clear, supernatant fluid in the counting chamber. Allow the platelets to settle for ten minutes and then count all the platelets in 80 small squares. It is important to focus carefully over each square so as to count all the platelets at different levels in the chambers. Add three zeros to the total count for the number of platelets per cubic millimeter of blood.

XI. STUDY OF STAINED BLOOD

A. Making and Staining Blood Films

1. Spreading the Film.—*Properly spread films are essential to accurate work.* They more than compensate for the time spent in learning to make them. There are certain requisites for success with any method: (*a*) The slides and covers must be perfectly clean; new slides should be soaked in a 10 per cent solution of acetic acid, and then rinsed in clean distilled water, and dried; old slides are cleaned by thorough washing with soap and water, rubbing with alcohol and drying on a clean towel; (*b*) the drop of blood must not be too large; (*c*) the work must be done quickly, before coagulation begins.

The blood is obtained from the finger tip or the lobe of the ear, as for a blood count; only a very small drop is required, usually about twice the size of a pinhead. The size of the drop largely determines the thickness of the film. The proper thickness will depend upon the purpose for which the film is made. For the structure of blood cells and the malarial parasite it should be so thin that, throughout the greater part of the film, the red corpuscles lie in a single layer, close together but not overlapping. For routine differential counting of leukocytes a film in which the red cells are piled up somewhat is best because the leukocytes are more evenly distributed, and because the number of leukocytes in a given area is greatly increased and the tedium of counting is correspondingly lessened. The film must not, upon the other hand, be so thick that identification of the various leukocytes becomes difficult. In some cases of severe anemia it is very difficult to make good films owing to the large proportion of plasma, which leads to slow drying, with consequent distortion of the red cells and the appearance of artifacts. To overcome this the films should be made very thin and dried quickly over a low flame.

Nearly all ordinary slides are slightly curved. In order that they may lie firmly upon the microscope stage without rocking, the blood film should be spread upon the convex side, which is recognized by laying the slide flat upon the table and twirling it rapidly by snapping the end with a finger. The side upon which it twirls the better is the convex side.

Ehrlich's Two-cover-glass Method.—This method is widely recommended, but considerable practice is required to get good results. Touch a large cover glass to the top of a small drop of blood, and place it, blood side down, upon another large cover glass. If the drop be not too large, and the covers be perfectly clean, the blood will spread out in a very thin

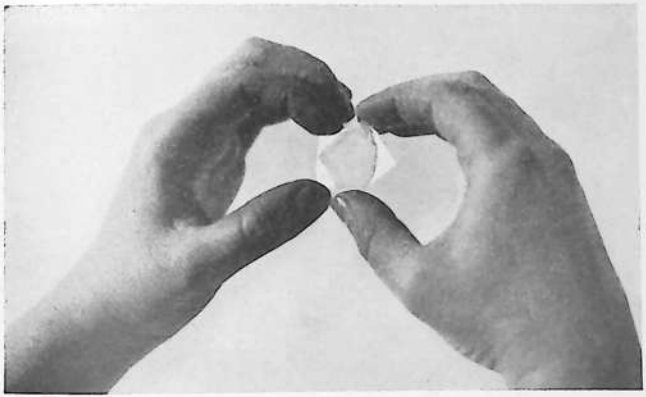


Fig. 126.—Spreading the film; two-cover-glass method.

layer. Just as it stops spreading, before it begins to coagulate, pull the covers quickly but firmly apart on a plane parallel to their surfaces (Fig. 126).

This method is especially to be recommended for very accurate differential counts, since all the leukocytes in the drop will be found on the two covers, and thus the error due to unequal distribution can be excluded by counting all the leukocytes. One of the covers is usually much better spread than the other.

Slide-and-cover Method.—Beacom used a slide and cover in a manner similar to that described above for two cover glasses. A small drop of blood from a puncture is taken upon a clean slide about $\frac{1}{2}$ inch from the end, and a clean thin cover glass is quickly applied. As soon as the blood has ceased spreading the tips of two fingers are placed on the cover, and, with *the lightest possible pressure*, the cover is slid quickly along the slide, living a

thin film of blood behind it. The method is much easier to learn than is the two-cover-glass method, gives more uniform films, and apparently gives more uniform distribution of leukocytes. All of the blood remains on the slide, none, or practically none, on the cover; hence the method is especially useful for very accurate differential counts, since every leukocyte in the drop of blood can be classified. Slow work, allowing partial coagulation, results in rupture of many of the leukocytes, as is also the case with the two-cover-glass method.

Two-slide Method.—Take a small drop of blood upon a clean slide about $\frac{3}{4}$ inch from the end, using care that the slide does not touch the skin. Place the end of a second slide against the surface of the first at an angle of 30 to 40 degrees, and draw it up against the drop of blood, which will immediately run across the end, filling the angle between the two slides. Now push the "spreader slide" back along the other in the manner indicated in Fig. 127. The blood will follow. The thickness of the film can be

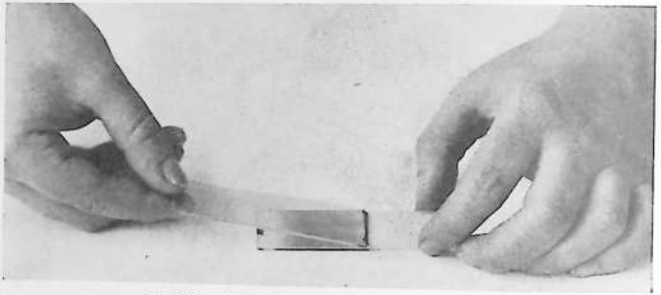


Fig. 127.—Spreading the film; two-slide method.

regulated by changing the angle, by varying the pressure, and by using a smaller or larger drop of blood.

It is very easy by this method to make large, thin, even films which are especially useful for studying the red corpuscles and the malarial parasite. Their use for differential leukocyte counting is discussed on page 266.

The films may be allowed to dry in the air, or may be dried by gently warming high above a flame (where one can comfortably hold the hand). F. W. Lacy suggested an interesting novelty. After the film is made and dried, half of it is covered with a card and the other half is placed near the mouth and breathed on a number of times. The red corpuscles in this part are nearly or quite destroyed, leaving the leukocytes, which stand out distinctly when stained. The part of the film which has been protected from the breath stains in the usual way.

When slides are used the label can be written with a soft lead pencil directly on the blood film, as was suggested by von Ezdorf.

2. Fixing the Film.—In general, films must be "fixed" before they are stained. Fixation may be accomplished by chemicals or by heat. *Those stains which are dissolved in methyl alcohol combine fixation with the staining process.*

Chemical Fixation.—Soak the film one to two minutes in pure methyl alcohol or absolute ethyl alcohol, or fifteen minutes or longer in equal parts of absolute alcohol and ether. One minute in 1 per cent solution of mercuric chloride or in 1 per cent formalin in alcohol is preferred by some, especially for the carbolthionin stain. The film must be well washed in water after mercuric chloride fixation. Chemical fixation may precede hematoxylin-eosin and other simple stains.

Heat Fixation.—This may precede any of the methods which do not combine fixation with the staining process. The best method is to place the film in an oven, raise the temperature to 150° C., and allow to cool slowly. Without an oven, the proper degree of fixation is difficult to attain.

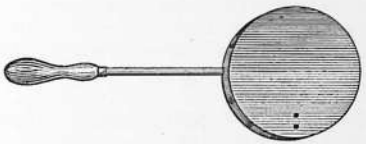


Fig. 128.—Kowarsky's plate for fixing blood (Klopstock and Kowarsky).

Kowarsky has devised a small plate of two layers of copper (Fig. 128), upon which the films are placed together with a crystal of urea. The plate is heated over a flame until the urea melts, and is then set aside to cool. Some prefer to use slides and to place the crystal of urea directly upon the slide. This is said to give the proper degree of fixation, but in Todd's experience the films have always been underheated. He obtained better results by use of tartaric acid crystals (melting point 168°–170° C.). The plate, upon which have been placed the cover glasses, film side down, and a crystal of the acid, is heated over a low flame until the crystal has completely melted. It should be held sufficiently high above the flame that the heating will require five to seven minutes. The covers are then removed. Freshly made films of normal blood should be allowed to remain upon the plate for a minute or two after heating has ceased. Fresh films require more heat than old ones, and normal blood more than the blood of pernicious anemia and leukemia.

Blood films can be satisfactorily fixed for most purposes by covering with absolute alcohol, quickly dashing off the excess, and igniting the remainder.

3. Staining the Film.—The aniline dyes, which are extensively used in blood work, are of two general classes: Basic dyes, of which methylene blue is the type, and acid dyes, of which eosin is the type. Nuclei and certain other structures in the blood are stained by the basic dyes, and are hence called *basophilic*. Certain structures take up only acid dyes, and are called *acidophilic*, or *oxyphilic*, or *eosinophilic*. Certain other structures are stained by combinations of the two, and are called *neutrophilic*. Recognition of these staining properties marked the beginning of modern hematology.

(1) **Hematoxylin and Eosin.**—This method is most useful in studying eosinophilic cells and the structure of nuclei, hematoxylin being, in fact, one of our best nuclear stains. It may therefore be recommended for the Arneth count (p. 273). Red corpuscles are pink or red, all nuclei blue, eosinophilic granules bright red; neutrophilic granules and platelets are not stained. Neither polychromatophilia nor basophilic granular degeneration of the red cells is demonstrated.

1. Fix by heat or chemicals.
2. Stain with any standard hematoxylin solution until nuclei are well colored, usually three to five minutes.
3. Wash well with water.
4. Apply a weak aqueous or alcoholic solution of eosin (about 0.5 per cent) for a minute or two.
5. Wash well in water, dry, and examine. If the eosin stains too deeply, longer washing in water will usually remove some of the excess. The hematoxylin will not wash out, but is rather intensified by washing in water.

The procedure may be simplified by mixing the hematoxylin and eosin. Such a mixture was much used before modern staining methods were introduced. Almost any of the standard hematoxylin solutions may be employed; to this is added enough of a saturated aqueous solution of eosin to color the reds properly while the hematoxylin is staining the nuclei. The combined stain keeps well. The fixed smear is immersed in the staining fluid for the required time, usually five to fifteen minutes, and is then rinsed, dried, and mounted.

(2) **Polychrome Methlylene-blue-eosin Stains.**—These stains, outgrowths of the original time-consuming Romanowsky method, have largely displaced other blood stains for clinical purposes. They may be recommended for all routine work. They stain differentially every normal and abnormal structure in the blood. Most of them are dissolved in methyl alcohol and combine the fixing with the staining process. Numerous methods of preparing and applying these stains

have been devised, among the best known being Giemsa's, Wright's, Hastings', and Leishman's.

Wright's Stain.—This is one of the best and is the most widely used in this country. The practitioner will find it convenient to purchase the stain ready prepared, but, since much of the solution offered for sale is unsatisfactory, it is best to purchase the powder and dissolve it in methyl alcohol as needed. Most microscopic supply houses carry it in stock. Wright's most recent directions for its preparation and use are as follows:

Preparation.—To a 0.5 per cent aqueous solution of sodium bicarbonate add methylene blue (B. X. or "medicinally pure") in the proportion of 1 Gm. of the dye to each 100 c.c. of the solution. Heat the mixture in a steam sterilizer at 100° C. for one full hour, counting the time after the sterilizer has become thoroughly heated. The mixture is to be contained in a flask, or flasks, of such size and shape that it forms a layer not more than 6 cm. deep. After heating, allow the mixture to cool, placing the flask in cold water, if desired, and then filter it to remove the precipitate which has formed in it. It should, when cold, have a deep purplish-red color when viewed in a thin layer by transmitted yellowish artificial light. It does not show this color while it is warm.

To each 100 c.c. of the filtered mixture add 500 c.c. of a 0.1 per cent aqueous solution of "yellowish water-soluble" eosin and mix thoroughly. Collect the abundant precipitate, which immediately appears, on a filter. Owing to lack of uniformity in the dyes now obtainable it may be necessary to add more or less of the eosin to obtain a satisfactory precipitate. When the precipitate appears it may be recognized by placing a drop of the fluid upon filter paper. Dry the precipitate thoroughly, dissolve it in methylic alcohol (Merck's "reagent") in the proportion of 0.1 Gm. to 60 c.c. of the alcohol. In order to facilitate solution the precipitate is to be rubbed up with the alcohol in a porcelain dish or mortar with a spatula or pestle. This alcoholic solution of the precipitate is the staining fluid. We frequently find that freshly made solutions stain the red cells blue owing to slight alkalinity; such solutions usually work properly after a few months.

Application.—1. Without previous fixation cover the film with a noted quantity of the staining fluid by means of a medicine dropper. There must be plenty of stain in order to avoid too great evaporation and consequent precipitation. When slides are used, the stain may be confined to the desired area by two heavy wax-pencil marks.

2. After one minute add to the staining fluid on the film the same quantity of distilled water by means of a second medicine dropper. This may be done by counting drops. Instead of distilled water it is much better to use the buffer solution of *p*H 6.4 described below. Blow gently on the diluted stain to make an even distribution. The quantity of the fluid on the preparation must not be so large that some of it runs off. Allow the

mixture to remain for four minutes. A longer period of staining may produce a precipitate. Eosinophilic granules are best brought out by a short period of staining.

3. Wash the preparation in water for thirty seconds, or until the thinner portions of the film become yellow or pink in color. The preparation should be flooded with water while the stain is still upon it. If the stain is poured off before rinsing, the scum tends to settle upon the blood film, where it clings in spite of subsequent washing.

4. Dry, best by waving high above a flame, and mount in balsam. When the films are on slides examine directly with the oil-immersion objective.

McJunkin[1] has shown that much of the trouble in staining with polychrome stains is done away with if a buffer solution is used instead of distilled water in the second step of the procedure. A solution with a *p*H of 6.4 is made by dissolving 6.63 Gm. of monobasic potassium phosphate, and 3.20 Gm. of dibasic sodium phosphate in 1 liter of distilled water. The phosphates should be recrystallized, and the sodium phosphate should be exposed to the air for two weeks to lose its water of crystallization.

When properly applied Wright's stain gives the following picture (Plate I and Plate V): Erythrocytes, yellow or pink; nuclei, various shades of purple; neutrophilic granules, reddish lilac, sometimes pink; eosinophilic granules, bright red; basophilic granules of leukocytes and degenerated red corpuscles, very dark bluish purple; blood platelets, dark lilac; bacteria, blue. The cytoplasm of lymphocytes is generally robin's-egg blue; that of the monocytes has a faint bluish tinge. Malarial parasites stain characteristically: The cytoplasm, sky blue; the chromatin, purplish red. These colors are not invariable: Two films stained from the same bottle sometimes differ greatly. *In general, a preparation is satisfactory when both nuclei and neutrophilic granules are distinct, regardless of their color, and when the film is free from precipitated dye.* In addition, it is desirable, but not essential, that the red corpuscles show a clear pink or yellowish pink; they should not be blue. The colors are prone to fade if the preparation is mounted in a poor quality of balsam or exposed much to the light.

Failure to get satisfactory results with the polychrome methylene-blue-eosin stains, when they are properly used, may be due to imperfect polychroming of the powder; but is most frequently a question of incorrect reaction of the staining fluid. When the solution is too acid the red corpuscles stain bright red and the nuclei of the leukocytes are pale sky blue or even colorless. When it is too alkaline the

[1] McJunkin, F. A.: A Benzidine-polychrome Stain for Blood, Jour. Am. Med. Assn., *74*:17–19 (Jan. 3), 1920.

red corpuscles stain deep slate blue and there is little differentiation of colors. The reaction of the solution is determined partly by that of the powder, when, as in the case with Wright's stain, its reaction is not accurately adjusted; but it depends to a still greater degree upon the methyl alcohol, which is prone to develop formic acid as a result of oxidation upon standing. A given powder may afford perfect results when dissolved in methyl alcohol from a freshly opened bottle, and give poor results when dissolved in the same lot of alcohol after it has stood for some months exposed to the air. Deterioration of old solutions is largely due to the same cause. It is possible to correct imperfectly acting fluids with traces of potassium hydrate or acetic acid, as suggested by Peebles and Harlow, but this is not very satisfactory. We find it better to keep on hand all fluids which have become too acid for use and to mix them as required with the fresh solutions, which are generally too alkaline when made with alcohol from a newly opened bottle. However, such solutions will correct themselves in time. Pathologic bloods will sometimes not stain well with solutions which are correct for normal blood.

Other Useful Blood Stains.—While Wright's stain suffices for most clinical work and is to be recommended if only one blood stain is to be used, certain others demand brief mention.

1. **Giemsa's Stain.**—This widely used stain is probably the best modification of the Romanowsky stain for blood parasites and other protozoa, and is also very satisfactory as a routine blood stain. It consists of:

Azur II-eosin	3.0 Gm.
Azur II	0.8 "
Glycerin (Merck, C. P.)	250.0 c.c.
Methyl alcohol (Kahlbaum I or Merck's reagent)	250.0 "

The solution is troublesome to make and is best purchased ready prepared. Blood films are fixed in methyl alcohol, and are then immersed for twenty minutes or longer in a freshly prepared mixture of 1 c.c. of stain and 10 c.c. distilled water. In order to prevent precipitates falling upon them, the slides or covers should be placed upon edge in the stain. Satisfactory results may also usually be obtained by placing about 30 drops of distilled water upon the fixed film, adding 3 drops of Giemsa's stain, mixing, and allowing it to act for fifteen or twenty minutes.

The use of this stain for *Treponema pallidum* is described later (p. 642).

2. **Pappenheim's Panoptic Method.**—In order to combine the advantages of the several stains Pappenheim recommended the following procedure: Stain for one minute with the May-Grünwald stain; add an equal quantity of water; after one minute pour off the fluid and stain fifteen minutes with the diluted Giemsa solution. The May-Grünwald stain is the

same as Jenner's. Wright's stain, diluted with an equal quantity of water, may be substituted for the Giemsa solution, but the time of staining should then not exceed five minutes.

Rosenthal[1] has used a slightly different technic with excellent results in studying the leukocytes in pneumonia (see p. 277). Make a control smear of normal blood on a clear space on the same slide, at an angle so as to distinguish it from the patient's blood. Use a buffer solution which is nearly neutral (*p*H 6.8 to 7) and which has been prepared from distilled water; the *p*H of this solution should be determined by adding carefully 0.5 to 3 c.c. of fifteenth molar solution of dibasic sodium phosphate (Na_2HPO_4) to 1000 c.c. of distilled water until the solution is of the desired hydrogen ion concentration. Cover the slide for one minute with Jenner's stain, and then dilute the stain with buffer solution. Stain for three minutes. Wash the slide with the buffer solution. Add 1 drop of Giemsa's stain to 1 c.c. of buffer solution and stain the smear with this for ten minutes. Wash, and allow the slide to dry in the air.

3. **Jenner's Stain.**—Jenner's eosinate of methylene blue, dissolved in methyl alcohol, brings out leukocytic granules well, and is, therefore, especially useful for differential counting. It stains nuclei poorly, and is much inferior to Wright's stain for the malarial parasite since it does not give the so-called "Romanowsky staining."

It may be purchased in solution, in the form of tablets, or as a powder, 0.5 Gm. of which is to be dissolved in 100 c.c. neutral absolute methyl alcohol. The unfixed blood film is covered with the staining solution, and after three to five minutes is rinsed with water, dried in the air, and mounted.

4. **Carbolthionin** (p. 830) is especially useful for the study of basophilic granular degeneration of the red cells. Nuclei, malarial parasites, and basophilic granules are brought out sharply. Polychromatophilia is also evident. Fixation may be by alcohol formalin (p. 251) or saturated solution of mercuric chloride.

5. **Pappenheim's pyronine-methyl green** (p. 832) can be used as a blood stain, and is very satisfactory for study of the red cells and of the lymphocytes and for demonstration of Döhle's inclusion bodies (p. 276). All nuclei are blue to reddish purple; basophilic granules, cytoplasm of lymphocytes, and inclusion bodies red. Polychromatophilia is well demonstrated, the affected cells taking more or less of the red color. Heat fixation is probably best.

B. Study of Stained Films

It has been said with much truth that an intelligent study of the stained film, together with an estimation of hemoglobin, will yield 90 per cent of all the diagnostic information obtainable from a blood examination. The stained films furnish the best means of

[1] Rosenthal, Nathan, and Sutro, C. J.: The Blood Picture in Pneumonia with Special Reference to Pathologic Changes in the Neutrophils, Am. Jour. Clin. Path., *3*:181–197 (May), 1933.

studying the morphology of the blood and blood parasites, and, to the experienced, they give a fair idea of the amount of hemoglobin and the number of red and white corpuscles. An oil-immersion objective is required.

1. Erythrocytes.—Normally, the red corpuscles are acidophilic. The colors which they take with different stains have been described. When not crowded together, they appear as circular, homogeneous disks, of nearly uniform size, averaging 7.8 μ in diameter (Fig. 129). In any normal blood, however, there may be individual cells as small as 5.5 μ and as large as 9.5 μ. The center of each is somewhat paler than the periphery. Red cells are apt to be crenated when the film

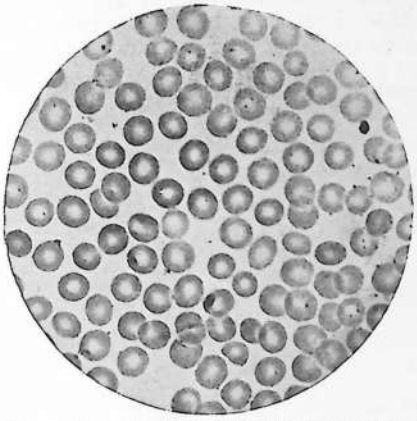


Fig. 129.—Red corpuscles of normal blood. Wright's stain (photograph, × 750).

has dried too slowly. Four or five cases are on record in which the majority of the red corpuscles were markedly elongated or elliptical in healthy individuals, probably an hereditary anomaly.

Pathologically, red corpuscles vary in hemoglobin content, size and shape, staining properties, and structure.

(1) **Hemoglobin Content.**—The depth of staining furnishes a rough guide to the amount of hemoglobin in the corpuscles, that is, to the color index. When hemoglobin is diminished the central pale area becomes larger and paler. This condition is known as *hypochromia*. In pernicious anemia, upon the other hand, as a result of the high color index, many of the red corpuscles may stain deeply and lack the pale center entirely.

(2) **Variations in Size and Shape** (Plate V, Fig. 1).—The cells may be abnormally small (called *microcytes*, 5 μ or less in diameter); abnormally large (*macrocytes*, 10–12 μ), or very large (*megalocytes*, 12–25 μ). Abnormal variation in size is called *anisocytosis*.

Variation in shape is often very marked. Oval, pyriform, caudate, saddle-shaped, and club-shaped corpuscles are common (Fig. 130). They are called *poikilocytes*, and their presence is spoken of as poikilocytosis. *Elliptic erythrocytes** have been reported in predominant numbers in healthy individuals as a congenital hereditary anomaly.

Red corpuscles which vary from the normal in size and shape are present in most anemias, and in the severer grades are often very numerous. Irregularities are particularly conspicuous in leukemia and pernicious anemia, where, in some instances, a normal erythrocyte is the exception. In pernicious anemia there is a decided tendency to

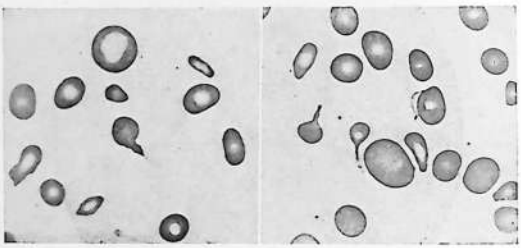


Fig. 130.—Red corpuscles showing variations in size and shape, from a case of pernicious anemia (photographs, × 750).

large-size and oval forms, and megalocytes are rarely found in any other condition. So constant is this in typical cases that a mere glance at a few fields often justifies the report "looks like pernicious anemia."

(3) **Variations in Staining Properties** (Plate V, Fig. 2).—These include polychromatophilia, basophilic granular degeneration, and malarial stippling. With exception of polychromatophilia, they are probably degenerative changes.

(*a*) *Polychromatophilia.*—The affected corpuscles do not show normal affinity for acid stains and take the basic stain to greater or less degree. Wright's stain gives such cells a faint bluish tinge when the condition is mild, and a rather deep blue when severe. Sometimes only part of a cell is affected. A few polychromatophilic corpuscles can

* For a discussion of this rare anomaly and references to the literature, see editorial.[1]

[1] Editorial: Elliptic Erythrocytes in Man. Jour. Am. Med. Assn. *119*:952–953 (July 18) 1942.

Description of Plate V

Abnormal red-corpuscles. All drawn from actual specimens and all stained with Wright's stain except where noted. × 1000 (1 mm. = 1 μ.)

Fig. 1.—Variations in size, shape, and hemoglobin content; from cases of pernicious anemia and chlorosis.

Fig. 2.—Polychromatophilia and basophilic granular degeneration; from cases of lead-poisoning and pernicious anemia.

Fig. 3.—Normoblasts, reticulated red cells, and one microblast. The top row represents stages in the development of the normoblast. The two reticulated red cells are stained with brilliant cresyl blue.

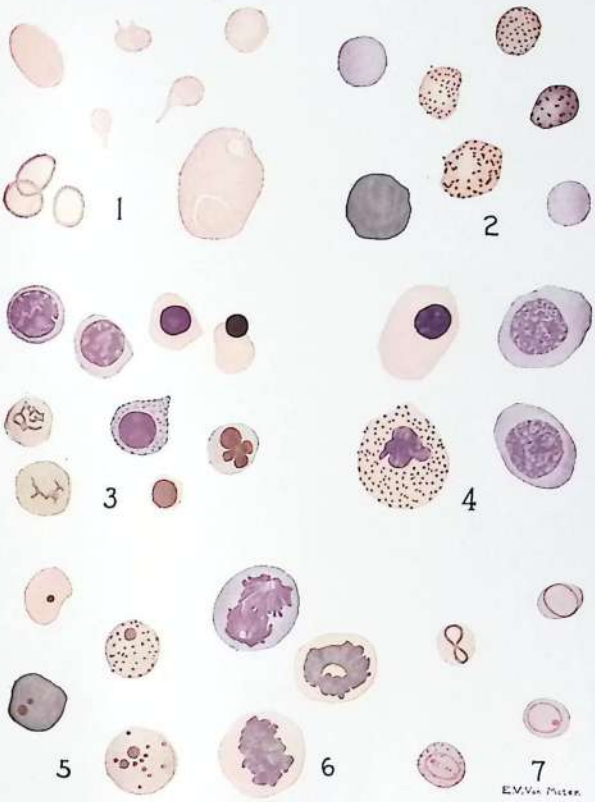
Fig. 4.—Megaloblasts from cases of pernicious anemia. Two show polychromatophilia and fairly typical nuclei, two have condensed nuclei, and one of these has basophilic cytoplasmic granules.

Fig. 5.—Nuclear particles or "Howell-Jolly bodies." One cell also shows basophilic granular degeneration.

Fig. 6.—Mitotic figures, two from myelogenous leukemia, one with polychromatophilic cytoplasm, from von Jaksch's anemia. The last was stained with Leishman's stain.

Fig. 7.—Cabot's ring bodies, from a case of von Jaksch's anemia. Two cells also contain nuclear particles and one shows basophilic granular degeneration. Leishman's stain.

PLATE V



be found in marked symptomatic anemias. They occur most abundantly in malaria, leukemia, and pernicious anemia.

Polychromatophilia has been variously interpreted. It is thought by many to be evidence of youth in a cell, and hence to indicate an attempt at blood regeneration. There are probably several forms referable to different causes.

(*b*) *Basophilic Granular Degeneration* (*Degeneration of Grawitz, Basophilic Stippling*).—This is characterized by the presence, within the corpuscle, of irregular basophilic granules, which vary in size from scarcely visible points to granules nearly as large as those of basophilic leukocytes (Fig. 131). The number present in a red cell commonly varies in inverse ratio to their size. They stain deep blue with carbol thionin or Wright's stain. The cell containing them may stain normally in other respects, or it may exhibit polychromatophilia. Polychromatophilic cells generally contain the smaller granules, which may be so fine that the cell appears dusted with them.

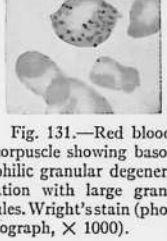


Fig. 131.—Red blood corpuscle showing basophilic granular degeneration with large granules. Wright's stain (photograph, × 1000).

Numerous cells showing this degeneration are commonly found in chronic lead poisoning, of which they were at one time thought to be pathognomonic. They can probably be found in every case with clinical symptoms and in some severe cases are present in nearly every microscopic field. Except in this disease the degeneration indicates a serious blood condition. It occurs in well-marked cases of pernicious anemia and leukemia, and, much less commonly, in very severe symptomatic anemias.

(*c*) *Malarial Stippling*.—This term has been applied to the finely granular appearance often seen in red corpuscles which harbor tertian malarial parasites (Plates XIII and XIV). It was formerly classed with the degeneration just described, but is undoubtedly distinct. Not all stains will show it. With Wright's stain it can be brought out by staining longer and washing less than for the ordinary blood stain. The minute granules, "Schüffner's granules," stain purplish red. They are sometimes so numerous as almost to hide the parasite.

(4) **Variations in Structure.**—The most important is the presence of a nucleus (Plate V, Figs. 3–7). Nucleated red corpuscles, or *erythroblasts*, are classed according to their size: *Microblasts*, 5 μ or less in diameter; *normoblasts*, 5 to 10 μ; and *megaloblasts*, above 11 μ.

Microblasts and **normoblasts** contain one, rarely two, small, round, sharply defined nuclei whose structure is decidedly different from that

of the nuclei of leukocytes. As a rule they are the most deeply stained nuclei to be seen in the blood film, being approached in this respect only by the smaller lymphocytes. Young normoblasts are large, often exceeding 11 μ. Their nuclei are relatively large and their chromatin is arranged in a more or less reticular manner with rather clean-cut open spaces. Not infrequently the openings are arranged at the

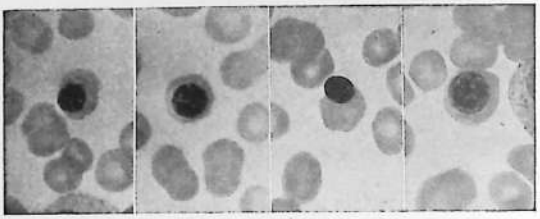


Fig. 132.—Normoblasts from cases of secondary anemia and leukemia. The next to the last is oldest; the last is youngest of the series (photographs, × 1000).

periphery and, with the chromatin bars between them, suggest a wheel with broad spokes. Mitoses are not uncommon in leukemia and pernicious anemia. The older normoblasts are smaller. Their nuclei, also, are smaller and more dense, some being entirely homogeneous and very deeply stained (pyknotic nuclei). These last are apt to be located eccentrically, and sometimes appear as if in process of extrusion

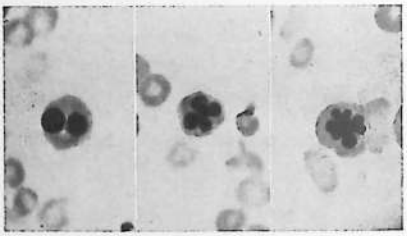


Fig. 133.—Normoblasts with irregular and fragmented nuclei. Wright's stain (photographs, × 1000).

from the cell. These characteristics are shown in Fig. 132. It is important to distinguish the younger from the older cells. As a result of degenerative changes the nuclei may be irregular in shape (Fig. 133), clover-leaf forms being common; or they may be completely broken up into fragments—the so-called **nuclear particles** or Howell-Jolly bodies (Fig. 134)—of which all but one or two may have disappeared

from the cell. These particles are smooth, round remnants of the parachromatin of the nucleus and take the azure stain.

The **megaloblast** (Plate I) is probably a distinct cell, not merely a larger size of the normoblast. In the typical megaloblast the nucleus is characteristic. This is large, oval, and rather palely staining, and

Fig. 134.—Nuclear particles or Howell-Jolly bodies in red corpuscles. From a case o pernicious anemia. Wright's stain (photographs, × 1000).

it has a more delicate chromatin network with larger and more numerous openings than has the nucleus of the normoblast (Plate V and Fig. 135). Sometimes it appears as if made up of coarse granules. As the megaloblast grows older the nucleus becomes smaller (Fig. 136) and more solidly stained and ultimately shows evidences of

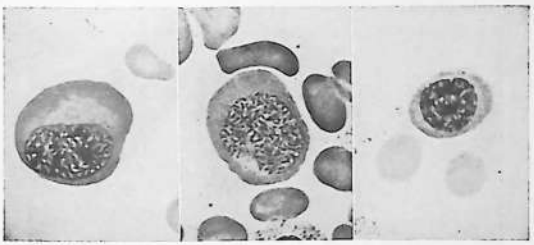


Fig. 135.—Megaloblasts showing typical nuclei; from cases of pernicious anemia. Wright's stain (photographs, × 1000).

degeneration (pyknosis, karyorrhexis). At the same time the entire cell becomes smaller and the cytoplasm shows less tendency to polychromatophilia.

The recognition of megaloblasts is important, but is not always easy unless the nucleus is typical. Mitosis (Fig. 137) may occur, as in the nuclei of normoblasts. Many hematologists now believe that

the most characteristic feature is the evidence of disproportionate maturation in the nucleus in the cytoplasm of the megaloblast, the nucleus developing much more slowly than the cytoplasm.

Young nucleated red cells, especially megaloblasts, are prone to exhibit polychromatophilia. In some cells the cytoplasm is so blue and shows so little of its characteristic smooth texture that it is

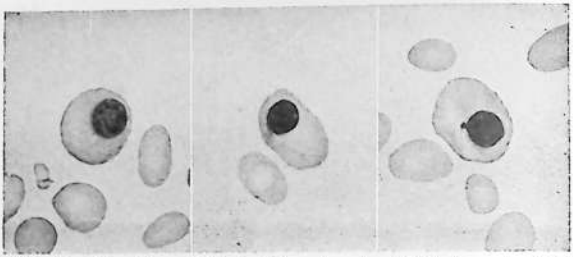


Fig. 136.—Small (aged) megaloblasts with condensed nuclei (photographs, × 1000).

difficult to recognize the cell as an erythrocyte except by the character of the nucleus. Such cells are often mistaken for lymphocytes or for Türk's irritation leukocytes, an error which careful observation of the nucleus even with the low power would usually prevent.

Cabot's ring bodies are ring- or figure-of-8-shaped structures (Fig. 138) which have been observed rarely in certain of the red cells

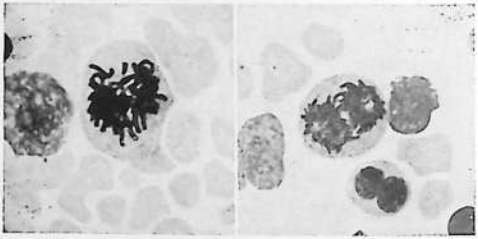


Fig. 137.—Two megaloblasts in the process of mitosis. Mitotic figures are often seen in the blood especially in leukemia, but are rarely so definite as these (photographs, × 1000).

in pernicious anemia, lead poisoning, and lymphatic leukemia. They stain red or reddish purple with Wright's stain and have been thought to be the remains of a nuclear membrane. However, Schleicher[1] stated that Cabot ring bodies are neither nuclear remnants nor are they

[1] Schleicher, E. M.: The Origin and Nature of the Cabot Ring Bodies of Erythrocytes. Jour. Lab. and Clin. Med. *27*:983–1000 (May), 1942.

identical with the nuclear membrane, but are laboratory creations—denatured and aggregated colloid protein in cellular degeneration induced by hemolytic agents.

Significance of Nucleated Red Corpuscles.—Normally, erythroblasts are present only in the blood of the fetus and of very young infants. In the healthy adult they are confined to the bone marrow and they appear in the circulating blood only in disease, where their presence denotes an excessive demand made upon the blood-forming organs to regenerate lost or destroyed red corpuscles. In response to this demand immature and imperfectly formed cells are thrown into the circulation. Their number, therefore, is usually regarded as an indication of the extent to which the bone marrow reacts rather than of the severity of the disease. A contributing factor may be a "lowered bone-marrow threshold" which allows immature cells to pass into the circulation more readily at some times than at others. Some times

Fig. 138.—Cabot's ring bodies in red blood corpuscles from a case of von Jaksch's anemia of infancy. The cell at the right contains a ring, a nuclear particle, and basophilic granules. Leishman's stain (photographs, × 1000).

great numbers of nucleated red corpuscles appear rather suddenly. This is called a **blood crisis,** and the name is rather arbitrarily applied whenever there are present more than five nucleated red cells for every hundred leukocytes.

In general normoblasts appear when blood regeneration takes place in a normal manner, although with excessive activity. They are found in severe symptomatic anemia, leukemia, and pernicious anemia. They are most abundant in myelogenous leukemia. While always present in pernicious anemia, they are often difficult to find. Microblasts have much the same significance as normoblasts, but are less common. Nuclear particles, or Howell-Jolly bodies, are most common in pernicious anemia and have been noted in greatest numbers after splenectomy.

The presence of megaloblasts indicates a change in the type of blood regeneration. This is seen most characteristically in pernicious

anemia and the finding of megaloblasts is therefore extremely important in the diagnosis of this disease, although less significance is attached to them than formerly. They are probably present in every case, although often intermittently or in so small numbers as to require a long search; and in well-marked cases they generally exceed normoblasts in number—a ratio which is very rare in other diseases in which they have been found, such as myelogenous leukemia, or malignant growths in the bone marrow.

Isaacs[1] has studied a group of erythrocytes, which do not comprise more than 1 per cent of the total number of erythrocytes, which show, as their only differential structure, a refractive granule, and which have some of the properties of immature erythrocytes. This granule, one to a cell,

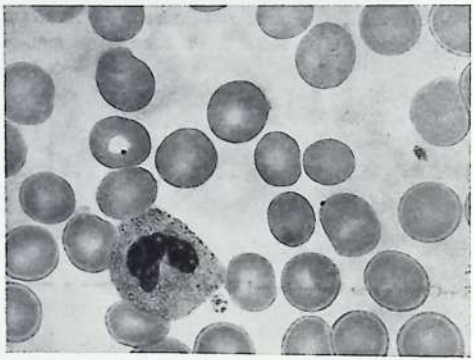


Fig. 139.—Isaacs' refractile granule.

does not stain with ordinary stains, but appears to be a clear black dot when the cell is in focus, and a brilliant refractive disk when the cell is just out of focus (Fig. 139). Isaacs claims that cells which contain these granules are more resistant to hemolytic and agglutinating agents, and to temperatures of 55° to 60° C. He also claims that, when an increased production of erythrocytes is stimulated, these cells are the first to reach the peripheral circulation after the rate of production of mature cells is insufficient.

2. The Leukocytes.—An estimation of the number or percentage of each variety of leukocyte in the blood is called a differential count. It probably yields more helpful information than any other single procedure in blood examinations.

[1] Isaacs, Raphael: The Refractive Granule Red Blood Corpuscle. Its Behavior and Significance, Anat. Rec., *29*:299–313 (Feb.), 1925.

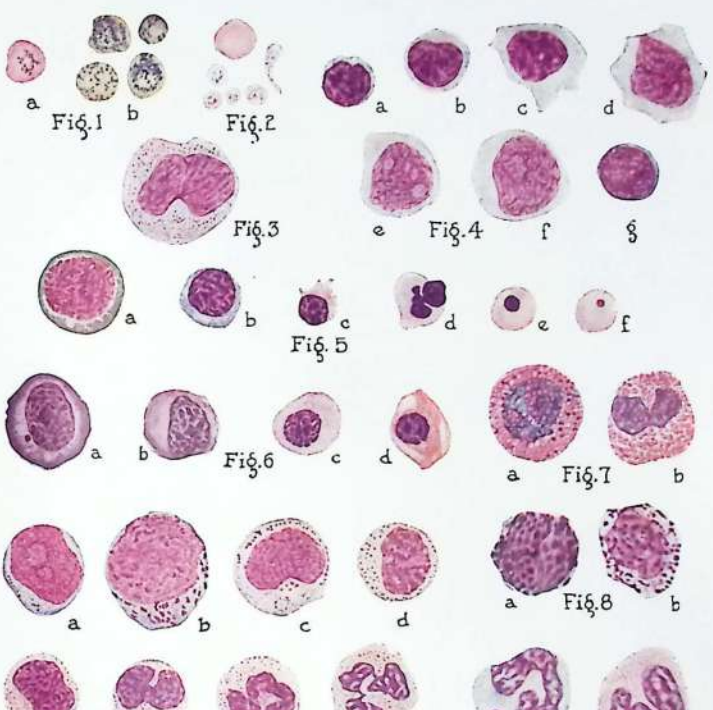


Fig. 1.—Reticulocytes from case of hereditary hemolytic jaundice. Supravital staining with brilliant cresyl blue; *a*, counter-stained with Wright's stain; *b*, without counter-stain.

Fig. 2.—Erythrocyte and blood platelets from normal blood.

Fig. 3.—Monocyte, normal blood.

Fig. 4.—Lymphocytes; *a-d*, from normal blood; *e-g*, from acute lymphatic leukemia; *a*, microlymphocyte; *b* and *c*, mesolymphocytes; *d*, macrolymphocyte; *e*, *f* and *g*, immature lymphocytes.

Fig. 5.—Normoblasts; *d*, karyorrhexis; *f*, Jolly body.

Fig. 6.—Megaloblasts from pernicious anemia.

Fig. 7.—Eosinophil leukocytes; *a*, myelocyte with some dark granules; *b*, mature cell from normal blood.

Fig. 8.—Basophil leukocytes (mast leukocytes); *a*, myelocyte; *b*, mature cell.

Fig. 9.—Development of neutrophil leukocytes from the stem cell (myeloblast); *a-g*, from myelogenous leukemia; *h*, normal blood; *a*, myeloblast with a few fine azure granules; *b*, leukoblast with coarse azure granules; *c*, promyelocyte; *d*, young myelocyte; *e*, myelocyte; *f*, metamyelocyte; *g*, band form; *h*, mature neutrophil.

Fig. 10.—Atypical cells from subacute myelogenous leukemia; *a*, Rieder cell; *b*, young neutrophil developed from a cell similar to *a*. (From Bell, "Textbook of Pathology," Lea and Febiger.)

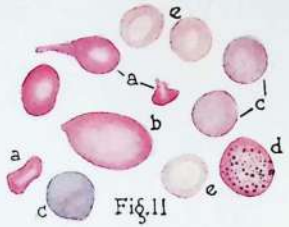


Fig. 11.

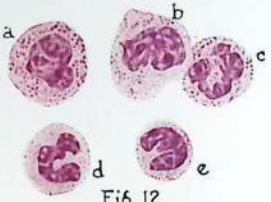


Fig. 12.

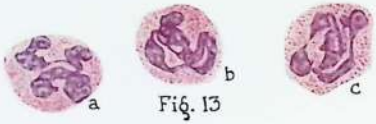


Fig. 13.

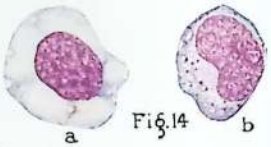


Fig. 14.

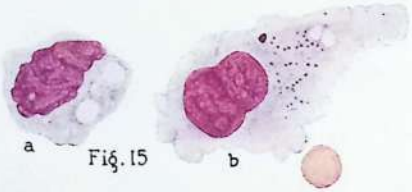


Fig. 15.

Fig. 16.

Parasites

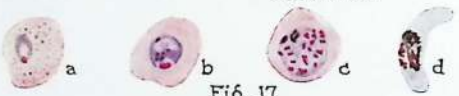


Fig. 17.

Fig. 18.

Fig. 11.—Abnormal erythrocytes; *a*, poikilocytes; *b*, macrocyte; *c*, polychromatophilic cells; *d*, basophilic stippling; *e*, anochromasia.

Fig. 12.—Neutrophils from case of pneumonia; *a*, *b* and *c*, toxic forms; *d* and *e*, cells with simplified nuclei illustrating "shift to the left."

Fig. 13.—Characteristic neutrophils from pernicious anemia.

Fig. 14.—Atypical "leukocytoid" lymphocytes from acute infectious mononucleosis.

Fig. 15.—Reticulo-endothelial cells from blood smear, subacute bacterial endocarditis.

Fig. 16.—Atypical stem cells from acute myelogenous leukemia.

Fig. 17.—Malarial parasites from human blood. *a*. Tertian malaria. Small ring-shaped merozoite. Cytoplasm, blue; chromatin, red. The erythrocyte shows basophilic stippling. *b*. Tertian malaria. Large ameboid form with pigment. *c*. Tertian malaria. Stage immediately preceding segmentation. *d*. Aestivo-autumnal malaria. Gamete, or crescent.

Fig. 18.—Trypanosome from the blood of a horse. (From Bell, "Textbook of Pathology," Lea and Febiger.)

The **differential count** is best made upon a film stained with Jenner's, Wright's, or a similar stain. Wright's stain is probably most widely used, but differentiates the leukocytes somewhat less satisfactorily than Jenner's. The blood film need not be quite so thin as is required for study of the red cells, but it must be thin enough to enable one to identify the leukocytes without difficulty. One should first glance over the preparation to find what the general tinting of the cells may be. Two films stained side by side will often show marked differences in the color reactions of the cells.

To make the differential count, go carefully over the film with an oil-immersion lens, using a mechanical stage if available. Experienced workers often use the lower powers (even the 16-mm., as recommended by Simon) in routine work; but the film must then be mounted, or at least wet with water or oil, since these lenses cannot be used satisfactorily upon dry, unmounted films. Classify each leukocyte seen, and calculate what percentage each variety is of the whole number classified. For accuracy, 500 to 1000 leukocytes must be classified; for approximate results, 300 are sufficient, but it is imperative to count cells in all parts of the smear, since the different varieties may be unevenly distributed. Track of the count may be kept by placing a mark for each leukocyte in its appropriate column, ruled upon paper. Some workers divide a slide-box into compartments with slides, one for each variety of leukocyte, and drop a coffee-bean into the appropriate compartment when a cell is classified. When a convenient number of coffee-beans is used (any multiple of 100) the percentage calculation is simple. Leukocytes which cannot be classified should be placed together in an "unidentified group." In some pathologic conditions, notably leukemia, there may be many of these unidentified cells.

The actual number of each variety in a cubic millimeter of blood is easily calculated from these percentages and the total leukocyte count, and should form part of the record if this is to be complete. An increase in actual number is an *absolute increase;* an increase in percentage only, a *relative increase.* It is evident that an absolute increase of any variety may be accompanied by a relative decrease.

One should make it a rule, when making a differential count, always to attempt to estimate the total leukocyte count from the number of leukocytes seen in a field with the low-power objective. After some practice this can be done with a considerable degree of accuracy.

The number of nucleated red corpuscles seen while making the count is generally noted in the record.

It is an unfortunate fact that differential leukocyte counts as ordinarily made on films on slides are often extremely unreliable, owing to irregular distribution of leukocytes, which may be very marked in thin films. For this reason many workers totally condemn the use of slides for differential counting. Undoubtedly, good cover-glass films, made as described on page 249, allow a really accurate differential count, but only when every leukocyte on both covers is classified, which is impracticable in routine work, since it requires classification of 8000 to 30,000 leukocytes. The distribution of leukocytes on the covers is not, however, more uniform than in properly prepared films on slides. In class work it is preferable to use slides, and to require that the films be moderately thick, but not thick enough to render the leukocytes difficult of recognition. A definite number of leukocytes (100 or 200) is then classified in each of three areas extending across the film, one at the beginning, one in the middle, and one at the end, and *reaching to the very edges of the film*. When the count is made in this way the percentage of polymorphonuclear neutrophils (which is taken as the criterion because of the unlikelihood of errors in classification) usually agrees within 2 points with the true percentage as ascertained by classifying every leukocyte on two cover-glass films made from the same puncture.

Proposals have been made from time to time to carry out the differential count in the counting chamber along with the total count by use of diluting fluids which color the leukocytes differentially. None of these has found general favor because of the impossibility of securing good differentiation of all types. The method is most useful when one is interested in the percentage of only one type, as, for example, the eosinophil in suspected trichinosis. As a special diluting fluid for this purpose Stitt uses 1.5 c.c. of neutralized formalin in 98.5 c.c. of 0.5 per cent glycerol. Just before use this fluid is colored with Giemsa's stain by adding 1 drop of the stain for each 1 c.c. of the fluid. Formalin may be neutralized by adding a few drops of phenolphthalein indicator and then adding very dilute sodium hydroxide until a pink color just appears.

Regarding the nomenclature and classification of some of the leukocytes, particularly those found in pathologic conditions, there is much confusion. In the following pages we shall try to describe these cells with sufficient clearness to facilitate their recognition, and shall, as far as possible, avoid disputed ground, particularly the tangled web of the conflicting theories of histogenesis. The student should thoroughly familiarize himself with the five types of leukocytes found in normal blood and with at least three—myelocytes, myeloblasts, lymphoblasts—of those which appear in disease.

The following leukocytic percentages represent about the average for normal persons in this country:

	Per cent
Lymphocytes	25–33
Monocytes	2– 6
Polymorphonuclear neutrophils	60–70
Eosinophils	1– 4
Basophils	0.25–0.5

Recent studies indicate that variations among healthy individuals may be greater than has been supposed and that climatic factors or altitude may exert a decided influence. One should, therefore, hesitate to base diagnostic conclusions upon slight variations in the differential count unless one has previously determined the normal for the individual.

(1) **Normal Varieties.**—(*a*) **Lymphocytes** are small mononuclear cells without specific cytoplasmic granules (Frontispiece). They are about the size of a red corpuscle or slightly larger (6–10 μ), although their diameter is influenced to a great degree by the thickness of the film, being greatest in very thin films where the cells are much flattened. The typical lymphocyte is a cell with a single, sharply defined nucleus containing heavy blocks of chromatin, staining blue with Wright's stain, while the parachromatin stains pink, and the cytoplasm robin's-egg blue. The characteristic feature of the lymphocyte nucleus is that there is a gradual transition between the chromatin and the parachromatin, so that it is practically impossible to tell where chromatin or parachromatin begins. The nucleus is generally round, but is sometimes indented at one side. Larger lymphocytes, 12 to 15 μ in diameter, with paler nuclei and more abundant cytoplasm, are frequently found, especially in the blood of children, and are difficult to distinguish from monocytes. It is believed that the larger forms are young lymphocytes which become smaller as they grow older. Some workers record the large and small lymphocytes separately. There is no clear line of distinction, but if it seems desirable to separate them, the terms "immature" and "mature" may appropriately be used. In the cytoplasm of many of the larger lymphocytes the Romanowsky stains show a variable number, usually 5 to 10, of rounded, discrete, reddish-purple (azurophilic) granules. They are larger than the granules of neutrophilic leukocytes and are regarded by some as specific for the lymphocyte.

Lymphocytes are formed in the lymphoid tissues, including that of the bone marrow. They constitute about 25 to 33 per cent of all leukocytes, or 1200 to 3000 in each cubic millimeter of blood. They are more abundant in the blood of children, averaging about 60 per

cent in the first year of life and decreasing to about 36 per cent in the tenth, the immature cells being especially abundant.

The percentage of lymphocytes is usually moderately increased in those conditions which give leukopenia, especially chlorosis, pernicious anemia, and many debilitated conditions. There is a decided absolute and relative increase at the expense of the polymorphonuclears at high altitudes, although the extent of this is somewhat uncertain. A similar increase is noted in residents of the tropics and in persons who are much exposed to the sun in temperate zones, as in heliotherapy, where the percentage of lymphocytes seems to increase in proportion to the tanning of the skin. A marked increase, accompanied by an increase in the total leukocyte count, is seen in

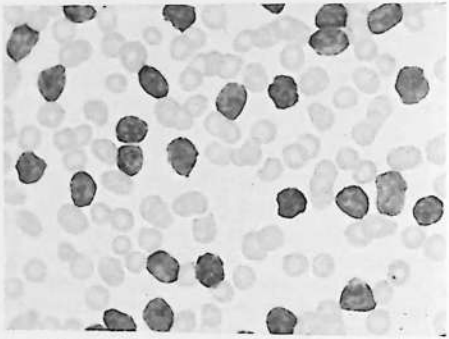


Fig. 140.—Lymphocytosis (photograph, × 500).

pertussis and lymphatic leukemia (Fig. 140). In the former lymphocytes average about 60 per cent. In the latter they sometimes exceed 98 per cent. Exophthalmic goiter commonly gives a marked relative lymphocytosis, while simple goiter does not affect the lymphocytes. In pulmonary tuberculosis a high percentage of lymphocytes or, especially, a progressive increase is a favorable prognostic sign, while a progressive decline should be looked upon with apprehension. Downing and Allison found a marked increase of lymphocytes in tuberculous patients after induction of artificial pneumothorax.

There is at present a tendency toward greater conservatism in ascribing diagnostic significance to lymphocytosis of moderate degree, that is, of less than 40 per cent, unless the normal for the individual has been previously established. Lymphocytic percentages as low as

15 or as high as 45 are occasionally met with in apparently healthy individuals.

(*b*) **Monocytes (Endothelial Leukocytes, Endotheliocytes)** (Frontispiece and Fig. 141).—Under this head we include the two types which have long been known as large mononuclear and transitional leukocytes. They are merely different forms or ages of the same cell.

The monocyte is the largest cell of normal blood, being generally about two to three times the diameter of a red corpuscle (14–20 μ), although smaller individuals are sometimes encountered. It contains a single nucleus, which is lobulated, deeply indented, or horseshoe-shaped, or, less often, round or oval, and which is commonly located eccentrically.

The zone of protoplasm surrounding the nucleus is relatively wide. With Wright's stain the characteristic feature of the nucleus is

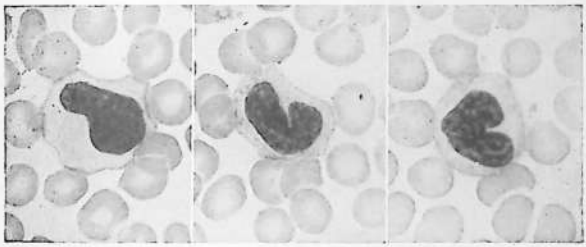


Fig. 141.—Three monocytes from normal blood (photographs, × 1000).

for the chromatin to be in strands. There is also a relatively sharp distinction between the chromatin and the parachromatin which results in a less densely stained nucleus than that seen in the lymphocyte, while the cytoplasm is slate colored or "muddy" in appearance, and sometimes appears dusted, uniformly or in patches, with fine reddish granules which are very much less distinct than the granules of neutrophilic leukocytes. The size of the cell, the width of the zone of cytoplasm, and the depth of color of the nucleus are the points to be considered in distinguishing between those monocytes which have a round nucleus and lymphocytes, but it must be borne in mind that the thickness of the film has a marked influence upon the apparent size of all leukocytes. They appear larger and paler when flattened out in very thin films.

Comparatively little is known regarding the origin of the monocytes, and it is possible that more than one cell is included. There

have been many reports by Sabin, Doan,[1] and their colleagues of their studies in hematology with supravital stains. They differentiate, by reactions to vital neutral red, monocytes and the wandering phagocytic cells which are known as "clasmatocytes." Altogether they constitute about 2 to 6 per cent of the total number of leukocytes; 100 to 600 for each cubic millimeter of blood. Only a few pathologic conditions raise this figure to any marked degree. A distinct increase, to 15 per cent or even higher, is a feature of the blood in typhoid fever and may be of some value in differential diagnosis. It is also quite constant in malaria, where sometimes many of the cells contain engulfed pigment (Plate XIV). In chronic tetrachlorethane poisoning Minot and Smith have demonstrated a marked and progressive increase of this type of cell (12–40 per cent). Other conditions in which an increase is usual are endocarditis lenta, possibly due to proliferation of capillary endothelium (Wollenberg), chronic amebic dysentery, Rocky Mountain spotted fever, trypanosomiasis, and kala-azar.

(*c*) **Polymorphonuclear Neutrophilic Leukocytes** (Frontispiece).—There is usually no difficulty in recognizing these cells. Their average diameter (about 12 μ) is decidedly less than that of the monocytes. The nucleus stains rather deeply, and is very irregular, often assuming shapes comparable to letters of the alphabet, E, Z, S, and so forth (Fig. 142). Frequently there appear to be several separate nuclei, hence the widely used name, "polynuclear leukocyte." Upon careful inspection, however, delicate nuclear bands connecting the parts can usually be seen. The cytoplasm is relatively abundant, and contains great numbers of fine neutrophilic granules (Fig. 150, A). With Wright's stain the chromatin of the nucleus is purple and the cytoplasmic granules are lilac, while in the well-stained preparation the cytoplasm itself is light pink or acidophilic.

Polymorphonuclear neutrophilic leukocytes are formed in the bone marrow from neutrophilic myelocytes. Ordinarily they constitute 60 to 70 per cent of all the leukocytes: 3000 to 7000 for each cubic millimeter of blood. An occasional normal adult may give a count as low as 40 per cent or as high as 80 per cent. In children the average runs from about 35 per cent in the first year to 50 per cent in the tenth. Any marked increase in their number practically always produces an increase in the total leukocyte count, and has already been discussed under Neutrophilic Leukocytosis (p. 237). The leukocytes of pus, *pus corpuscles*, belong almost wholly to this variety.

[1] For a good bibliography of the work of Sabin, Doan, and others, see Doan, C. A.: The Clinical Implications of Experimental Hematology, Medicine, *10*:323–371 (Sept.), 1931.

In infectious and inflammatory conditions, notably in pneumonia and appendicitis, a comparison of the percentage of neutrophilic cells with the total leukocyte count yields more information than a consideration of either alone. In a general way, as was first pointed out by Sondern,[1] the percentage represents the severity of the infection or, more correctly, the *degree of toxic absorption;* while the total count indicates the patient's *power of resistance.* With moderate infection and good resisting powers the leukocyte count and the percentage of neutrophils are increased proportionately. When the neutrophilic percentage is increased to a notably greater extent than is the total

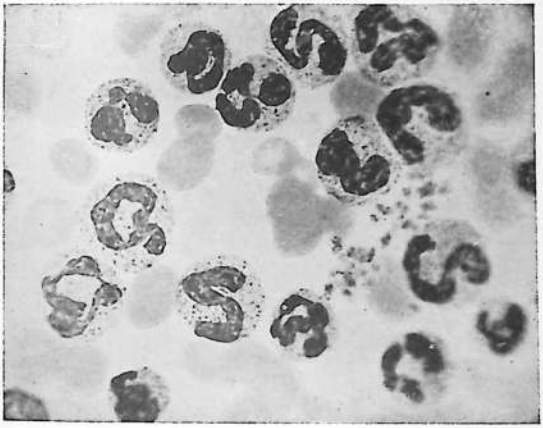


Fig. 142.—Marked polymorphonuclear neutrophilic leukocytosis (photograph, × 1000) (courtesy of Dr. W. P. Harlow).

number of leukocytes, no matter how low the count, either very poor resistance or a very severe infection may be inferred.

Gibson has suggested the use of a chart to express this relationship graphically (Fig. 143). Its arrangement is purely arbitrary, but it will be found very helpful in interpreting counts. An ascending line from left to right indicates an unfavorable prognosis in proportion as the line approaches the vertical. All fatal cases show a rising line. A descending or horizontal line suggests a very favorable prognosis. Because the chart is somewhat

[1] Sondern, F. E.: The Present Status of Blood Examination in Surgical Diagnosis, Med. Rec., *67*:452–455 (March 25), 1905. The Value of the Differential Leukocyte Count in Diagnosis, Am. Jour. Med. Sci., *132*:889–891 (Dec.), 1906.

cumbersome in hospital records, when repeated entries must be made, Wilson[1] has devised a formula which expresses the same idea by a numerical index. His formula is as follows: IR = (T − 10) − (P − 70); IR being the "index of resistance," T, the total leukocyte count expressed in thousands, and P, the polymorphonuclear neutrophilic percentage. If, for example, the total count is 12,000 and the neutrophilic percentage is 90, then the formula would give an index of resistance of − 18: IR = (12 − 10)

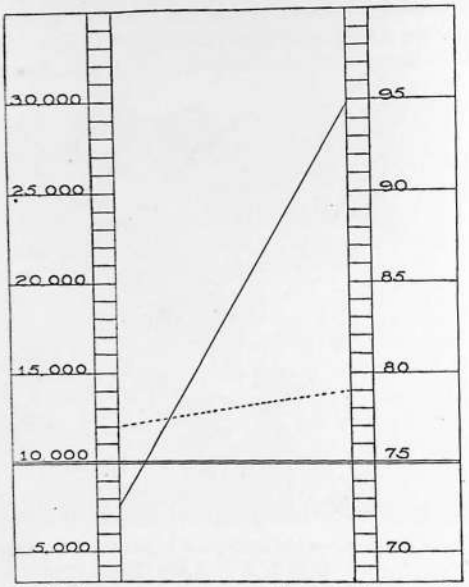


Fig. 143.—Gibson chart with blood count in 2 cases of appendicitis: Dotted line represents a mild case with prompt recovery; the continuous line, a very virulent streptococcic case with poor resistance, peritonitis, and early death.

− (90 − 70) = − 18. Under normal conditions the index will be zero or near it. With low resistance the index is a minus quantity; with disproportionately high resistance it is a plus quantity.

[1] Wilson, L. B.: Value of Sondern's Differential Leukocyte Resistance Line in the Diagnosis and Prognosis of Acute Appendicitis: Collected Papers of The Mayo Clinic, 1905–1909, p. 280. Also Walker, O. J.: Index of Body Resistance in Acute Inflammatory Processes as Indicated by Examination of the Blood, Jour. Am. Med. Assn., *72*:1453–1457 (May 17), 1919.

It is a matter of observation that in the absence of acute infectious disease or of inflammation directly in the blood stream (for example, phlebitis, sigmoid sinusitis, septic endocarditis), a neutrophilic percentage of 85 or more points very strongly to gangrene or pus formation somewhere in the body. On the other hand, excepting in children, where the percentage is normally low, pus is uncommon with less than 80 per cent of neutrophils.

Exceptions to these rules occur chiefly in moribund cases, in children, and in typhoid and tuberculous infections.

Normally, the cytoplasm of leukocytes stains pale yellow when films are subjected to fumes of iodine and mounted in syrup of levulose. In suppurative and septic conditions the cytoplasm of many of the neutrophils stains diffusely brown, or contains granules which stain reddish brown with iodine. This is called iodophilia. Extracellular iodine-staining granules, which are present normally, are more numerous in iodophilia. Iodophilia was at one time considered a useful sign of suppuration, but is now little valued.

Arneth's Classification of Neutrophils.—Arneth groups the neutrophilic leukocytes into five classes according to the number of lobes which their nuclei possess. The forms which fall into each class and the average normal percentages as given by Arneth are indicated in the following list:

Class 1. One round or indented nucleus; 5 per cent.
Class 2. Two nuclear divisions; 35 per cent.
Class 3. Three nuclear divisions; 41 per cent.
Class 4. Four nuclear divisions; 17 per cent.
Class 5. Five or more nuclear divisions; 2 per cent.

This is really a classification of neutrophils according to their age, the youngest cells being included in Class 1. Among these youngest cells are the myelocytes and metamyelocytes which do not appear in normal blood.

The percentages are fairly constant in the same individual in health, but may show considerable variations in disease, even when the leukocyte count remains unchanged. An increase of the lower classes at the expense of the higher is known as a "shift of the neutrophilic blood picture to the left." The opposite condition is a "shift to the right." In order to simplify comparison, many workers in this country use an index number obtained by adding the first, second, and one half of the third classes. The average normal "Arneth index" is, accordingly, about 60. Briggs found variations between 51 and 65 in normal individuals.

The clinical value of an Arneth count is not definitely determined. It appears to have greater usefulness in prognosis than in diagnosis. Most pathologic conditions which produce any change cause a shift to the left, that is, a high index. Among these are acute infectious diseases, pyogenic infections (appendicitis, and so forth), and tuberculosis. In tuberculosis

the Arneth count is regarded as having definite prognostic value, the higher the index, the more serious being the outlook.

A low index occurs in pernicious anemia. In a series of twenty-three examinations in 12 cases of pernicious anemia Briggs found an average index of 40.29; lowest, 16.5; highest, 51.25. Eight consecutive cases of severe secondary anemia (malignant disease, syphilis, nephritis, repeated hemorrhages, and so forth) gave an average index of 68.23, only 1 case (a case of syphilis with index of 39) falling below normal limits.

For the Arneth count thin well-stained blood films are essential. Wright's stain may be used, but hematoxylin-eosin is better since it brings out the nuclear structure more clearly. Nuclear parts which are joined by more than a thread should be counted as one.

Schilling Count.—The original Arneth count has been modified, and criticized by many workers. One of these, Schilling,[1] has developed a much simpler classification which has become popular in America during the last few years The technic described by this author can be applied in the course of an ordinary differential count by observation of the following forms of leukocytes: basophils, eosinophils, myelocytes, "juvenile" cells (metamyelocytes, having many of the characteristics of myelocytes, but with indented or bean-shaped nuclei), single-lobed neutrophils (called by the author "stab" cells), neutrophils with more than one lobe in the nucleus, lymphocytes, monocytes. A differential count with this classification constitutes a Schilling "hemogram."

HEMOGRAM (AFTER SCHILLING)

	Leukocyte count.	Basophils.[1]	Eosinophils.[1]	Myelocytes.[1]	Juveniles (metamyelocytes of Pappenheim).[1]	Stab nuclears (neutrophils with a single lobe).[1]	Segment nuclears (neutrophils with two or more lobed nuclei).[1]	Lymphocytes.[1]	Monocytes.[1]	Thick drop observations and remarks.
Normal limits	5000 to 8000	0 to 1	2 to 4	0	0 to 1	3 to 5	51 to 67	21 to 35	4 to 8	

[1] Per cent.

The significant dividing line in the Schilling count lies in the neutrophil group between the "stab" cells and the segmented nucleus cells. In the normal blood smear there are no myelocytes, nor "juvenile" forms, and only 3 to 5 per cent of neutrophils with single-lobed nuclei, and with 50

[1] Schilling, Viktor: Das Hämogramm in der Poliklinik. I. Biologische Kurven der Leukocytenbewegung als Grundlage der praktischen Bewertung einmaliger Blutuntersuchungen, Ztschr. f. klin. Med., *99*:232–247, 1924. Also Schilling, Victor (trans. by Gradwohl, R. B. H.): The Blood Picture and Its Clinical Significance, 7th and 8th revised edition, St. Louis, C. V. Mosby Co., 1929, 408 pp.

to 65 per cent of other neutrophils. A "shift to the left" is seen when the number increases in the percentage to the left of the dividing line. A **regenerative shift to the left** with a high total white count occurs in acute sepsis, appendicitis, and so forth. In this condition myelocytes and juvenile forms appear and there is an increase in stab cells. The prognosis in such conditions may be made by subsequent counts; a continued or increased shift to the left is unfavorable, while a shift toward the right is an encouraging sign. There is also a **degenerative shift to the left** with a diminished total white count. This condition may be found in tuberculosis, and especially in typhoid fever. There is a marked increase in the percentage of single-lobed nuclears, with no appearance of myelocytes, nor of juvenile forms.

Filament and Nonfilament Count.—A simple, practical method of studying the significance of the appearance of the nuclear structure of neutrophils has been proposed by Farley, St. Clair, and Reisinger.[1] They used the criterion of Cooke and Ponder in their classification of types of neutrophils (Fig. 144): "If there is any band of nuclear material except this chromatin filament connecting the different parts of a nucleus, that

Fig. 144.—Cooke and Ponder classification of neutrophils.

nucleus cannot, for the purposes of the count be said to be divided." The *method* consists of making smears on cover slips preferably, as they must be thin. Stain with Wright's stain. Count 100 leukocytes, including lymphocytes, monocytes, eosinophils, basophils, and neutrophils. In counting the neutrophils, simply distinguish between Class I of Cooke and Ponder, the *nonfilament* type, and classes II, III, IV, and V, the *filament* neutrophils. Distinct myelocytes, or myeloblasts, are counted as such. *The upper limit of normal for young forms (nonfilament) is 16 per cent.* The average for normal adults is 8 per cent. An increase in the percentage of nonfilament polymorphonuclear neutrophils of more than 16 per cent may indicate infection, even though the number of leukocytes is not increased. In fact, the filament and nonfilament count may be interpreted the same, as regards the "shift to the left," as the Schilling, or Arneth counts. It is true that the percentages given do not agree entirely with the corresponding percentages which were reported by Schilling. The method is much simpler than the method of Schilling and has grown in favor in the United States.

[1] Farley, D. L., St. Clair, H., and Reisinger, J. A.: The Normal Filament and Nonfilament Polymorphonuclear Neutrophil Count: Its Practical Value as a Diagnostic Aid, Am. Jour. Med. Sci., *180*:336-344 (Sept.), 1930.

Döhle's Inclusion Bodies.—In 1911 Döhle called attention to the occurrence of certain peculiar bodies within the cytoplasm of the neutrophils in cases of scarlet fever (Fig. 146). Their nature has not been definitely

Name of cell	Appearance of cell	Schilling	Arneth	Pons–Krumbhaar	Cooke–Ponder	Filament–Non-filament
Myeloblast		Myeloblast	Class 1	Myeloblast	One lobe	Non-filament
Premyelocyte		Premyelocyte	Class 1	Premyelocyte	One lobe	Non-filament
Myelocyte		Myelocyte	Class 1	Myelocyte	One lobe	Non-filament
Metamyelocyte		Juvenile	Class 1	Metamyelocyte	One lobe	Non-filament
Young neutrophil		Band	Class 1	Non-segmented	One lobe	Non-filament
Adult neutrophil (two lobes)		Segmenter	Class II	Segmented	Two lobes	Filament
Adult neutrophil (three lobes)		Segmenter	Class III	Segmented	Three lobes	Filament
Adult neutrophil (four lobes)		Segmenter	Class IV	Segmented	Four lobes	Filament
Adult neutrophil (five lobes)		Segmenter	Class V	Segmented	Five lobes	Filament

Fig. 145.—Schematic outline of all cells in the neutrophilic series showing their positions in the various classifications of neutrophilic immaturity. (Johnson in Kracke and Parker, A Textbook of Clinical Pathology, The Williams and Wilkins Co.)

determined. The typical "inclusion bodies" are about the size of micrococci or a little larger; some of them are pear shaped, others appear like short rods or like cocci lying in pairs. Smaller, discrete, punctiform granules are sometimes seen, but have not the same significance. It now seems

well established that typical inclusion bodies have considerable diagnostic value. They are apparently found in many or even the majority of the neutrophilic leukocytes in every case of scarlet fever early in the disease. Upon the other hand, a few may be found in many cases of diphtheria, pneumonia, and some other infectious diseases, but never in German measles and rarely in measles.

Fig. 146.—Döhle's inclusion bodies in leukocytes. From a case of scarlet fever. Pyronine-methyl-green stain (× 1500) (from a slide prepared by L. W. Hill).

The inclusion bodies can be seen in preparations stained with Wright's stain, but long staining with pyronine-methyl-green is preferable. With the latter stain nuclei are purplish and the bodies bright red.

Rosenthal,[1] using the technic which has been described on page 256, studied the pathologic changes that occur in polymorphonuclear leukocytes

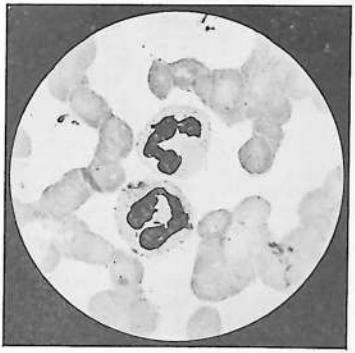


Fig. 147.—Appearance of polymorphonuclear neutrophils with Jenner-Giemsa stain in a normal control smear. Note the absence of "toxic" granules. (Kugel and Rosenthal, in Am. Jour. Med. Sci., May, 1932, Lea and Febiger, Publishers.)

during the progress of infections. In pneumonia the degenerative signs are irregular, "moth-eaten" cell outlines. The neutrophilic granules are replaced by large, dark, irregular, basophilic granules which may fill the entire cell.

[1] Kugel, M. A., and Rosenthal, Nathan: Pathologic Changes Occurring in Polymorphonuclear Leukocytes during the Progress of Infections, Am. Jour. Med. Sci., *183*:657–667 (May), 1932.

Irregular bluish clumps of Döhle bodies, or a diffuse bluish cytoplasm may be found in the myoblasts. Vacuoles may also be present (see Figs. 147 and 148).

(*d*) **Eosinophilic Leukocytes, or "Eosinophils"** (Frontispiece).—The structure of these cells is similar to that of the polymorphonuclear neutrophils, with the striking difference that, instead of fine neutrophilic granules, their cytoplasm contains coarse round or oval granules having a strong affinity for acid stains. They are easily recognized by the size and color of the granules which stain bright red with stains containing eosin (Fig. 150, B). Their cytoplasm has

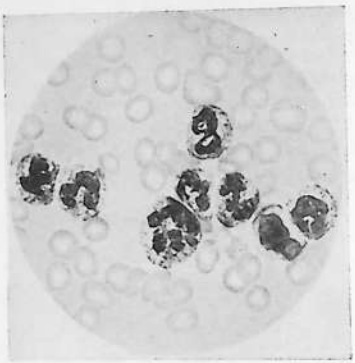


Fig. 148.—Acute lobar pneumonia (Jenner-Giemsa stain). "Toxic" granules in all segmented and nonsegmented polymorphonuclear neutrophils. Note anisocytosis of the leukocytes and also large polymorphonuclear neutrophils. (Kugel and Rosenthal, in Am. Jour. Med. Sci., May, 1932, Lea and Febiger, Publishers.)

generally a faint sky-blue tinge, and the nucleus stains somewhat less deeply than that of the polymorphonuclear neutrophil.

Eosinophils are formed in the bone marrow from eosinophilic myelocytes. Their normal number varies from 50 to 400 for each cubic millimeter of blood, or 1 to 4 per cent of the leukocytes. An increase is called *eosinophilia*, and is better determined by the actual number than by the percentage.

Marked eosinophilia—above 6 or 7 per cent—is probably always pathologic. It occurs in a variety of conditions, the most important of which are: Infection by animal parasites; bronchial asthma; myelogenous leukemia; scarlet fever; many skin diseases; anaphylactic conditions; and certain cases of chronic gonorrhea.

(*a*) Eosinophilia may be a symptom of *infection by any of the worms* and from a diagnostic viewpoint this is its most important indication. It is fairly constant in trichiniasis, uncinariasis, filariasis, and echinococcus disease, and reaches its highest figure in the first-named condition where the eosinophils usually range between 10 and 30 per cent of all the leukocytes, but may go much higher. An unexplained marked eosinophilia, especially if associated with muscular pains, warrants examination of a portion of muscle for *Trichinella spiralis* (p. 575).

(*b*) *True bronchial asthma* commonly gives a marked eosinophilia during and following the paroxysms. This is helpful in excluding asthma of other origin. Eosinophils also appear in the sputum in large numbers.

(*c*) In *myelogenous leukemia* there is almost invariably an absolute increase of eosinophils, although, owing to the great increase of other leukocytes, the percentage is usually diminished. Dwarf and giant forms are often numerous.

(*d*) *Scarlet fever* is frequently accompanied by eosinophilia, which may help to distinguish it from measles.

(*e*) Eosinophilia has been observed in a large number of *skin diseases*, notably pemphigus, prurigo, psoriasis, and urticaria. It probably depends less upon the variety of the disease than upon its extent.

(*f*) Eosinophilic cells are usually increased to a variable degree in tuberculin reactions and subacute and chronic anaphylactic conditions in general, notably in hay fever. Watkins and Berglund[1] have pointed out an increase in numbers of eosinophilic cells in pernicious anemia after a diet of liver.

(*e*) **Basophilic Leukocytes or "Mast Cells"** (Frontispiece).—In general, these resemble polymorphonuclear neutrophils, except that the nucleus is less irregular (usually merely indented or slightly lobulated) and that granules are larger and have a strong affinity for basic stains. They are easily recognized (Figs. 149 and 150, C). Sometimes one sees cells from which most of the granules have disappeared owing to their ready solubility in water, leaving clean-cut openings in the cytoplasm, which then takes a mauve color. With Wright's stain the granules are deep purple, while the nucleus is somewhat paler and is often nearly or quite hidden by the granules, so that its form is difficult to make out.

[1] Watkins, C. H., and Berglund, Hilding: Analysis of Morphological Blood Changes in Pernicious Anemia Following Administration of Liver, Proc. Soc. Exper. Biol. and Med., *25*:206–209 (Dec.), 1927.

There is some uncertainty as to the origin of the basophilic leukocytes. Most authorities believe that they originate in the bone marrow from basophilic myelocytes. They are least numerous of the leukocytes in normal blood, rarely exceeding 0.5 per cent, or 25 to 50 for each

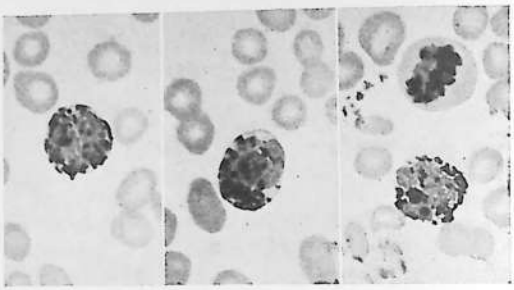
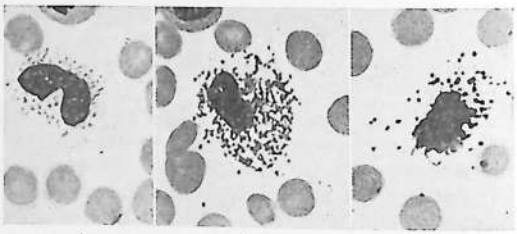


Fig. 149.—Basophilic leukocytes. At the right is also a normoblast undergoing mitosis (photographs, × 1000).

cubic millimeter. A notable increase is limited almost exclusively to myelogenous leukemia, where they are sometimes very numerous.

(2) **Abnormal Varieties.**—(*a*) **Myelocytes** (Frontispiece and Fig. 151) are large mononuclear cells whose cytoplasm is filled with gran-

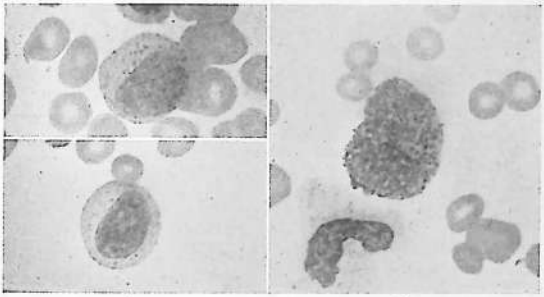


A B C

Fig. 150.—Ruptured leukocytes, showing relative size of granules: A, Neutrophilic; B, eosinophilic; C, basophilic (photographs, × 1000).

ules. Typically, the nucleus occupies about one half of the cell, and is round or oval, or is indented, with its convex side in contact with the periphery of the cell. It stains rather feebly and rarely contains nucleoli. The average diameter of this cell (about 15.75 μ) is greater than that of any other leukocyte, but there is much variation in size

among individual cells. Myelocytes are named according to the character of their granules—neutrophilic, eosinophilic, and basophilic myelocytes. These granules are identical with the corresponding granules in the leukocytes just described. They are, however, often less distinct and less sharply differentiated by the various stains than those of the corresponding polymorphonuclear cells. In some the granules are few in number, the cells departing but little from the structure of the parent myeloblast. Such cells may be called "promyelocytes." In young neutrophilic myelocytes there is a tendency to relatively large granules which take a purple color with Wright's stain. These finally give place to true neutrophilic granules. Sometimes only a portion of the cytoplasm near the nucleus is filled with granules, the periphery, or at least one edge, retaining the smooth



A B

Fig. 151.—Myelocytes from blood of myelogenous leukemia: A, Neutrophilic; B, eosinophilic (photographs, × 1000).

basophilic cytoplasm of the parent myeloblast (Plate I). Although the occurrence of two kinds of granules in the same cell is rare, a few basophilic granules are sometimes seen in young eosinophilic myelocytes. The basophilic myelocyte is usually small; and its nucleus is commonly so pale and so obscured by the granules that the cell is not easily distinguished from more mature forms.

The small neutrophilic cell with a single small, round, deeply-staining nucleus which is sometimes encountered, must not be confused with the myelocyte. Such atypic cells probably result from division of polymorphonuclear neutrophils.

Myelocytes are the bone marrow cells from which the corresponding granular leukocytes are developed. They, in turn, are derived from certain nongranular cells of the bone marrow, the

myeloblasts. Their presence in the blood in considerable numbers is diagnostic of myelogenous leukemia. The neutrophilic form is the least significant. A few of these may be present in very marked leukocytosis or any severe blood condition, as pernicious anemia. In the anemia of malignant disease they suggest bone marrow metastasis. Eosinophilic myelocytes are found practically only in myelogenous leukemia, where they are often very numerous. The basophilic variety is less common, and is confined to long standing, severe myelogenous leukemia.

(*b*) **Myeloblasts.**—These are the parent cells of the myelocytes, from which they differ chiefly in the absence of specific granules. They are about the size of myelocytes. Their round or oval nuclei are poor in chromatin, have a finely reticular or so-called "sievelike" structure, and contain several rather indistinct nucleoli which are generally pale blue with the usual stain and are outlined by a ring of denser chromatin (Plate I and Fig. 152). The cytoplasm, which is generally not abundant, is basophilic, staining pure blue with Wright's stain. In some preparations it is characteristically smooth in texture; in others it is finely reticular.

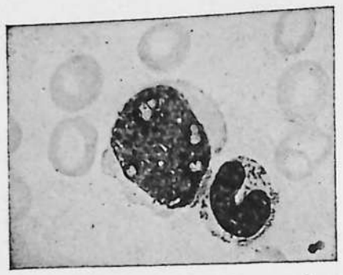


Fig. 152.—A myeloblast and a neutrophilic leukocyte. From a case of myelogenous leukemia. Wright's stain (photograph, × 1000).

Myeloblasts appear in the blood in large numbers in acute myelogenous leukemia and the terminal stages of chronic myelogenous leukemia, when the bone marrow reverts to the embryonic type. Their number is, therefore, important in prognosis. They may be indistinguishable morphologically from the lymphoblasts of acute lymphatic leukemia, but can usually be distinguished by the peroxidase reaction. In almost all advanced cases of myelogenous leukemia all stages of transition between the myeloblast and myelocyte may be found. For the modern opinion regarding the characteristics of the myeloblast the reader is referred to the excellent review and original study by Downey.[1]

Peroxidase Test.—The technic of Goodpasture, which we have found most satisfactory, is as follows:

1. Prepare dried films on slides or covers in the usual way.

[1] Downey, Hal: The Myeloblast—Its Occurrence Under Normal and Pathological Conditions, and Its Relations to Lymphocytes and Other Blood Cells, Folia hematol., *34*:65–102, 145–187 (June and Aug.), 1927.

2. Cover the film with a measured amount of Goodpasture's stain and let stand one minute.

3. Add an equal amount of water and let stand three or four minutes.

4. Rinse well in water, dry by blotting or by waving over a low flame, and examine with an oil-immersion objective. By this method nuclei are clear red, cytoplasm and platelets are pale pink, and red corpuscles a buff color. Cells which give the peroxidase reaction—polymorphonuclear neutrophils, eosinophils, myelocytes, myeloblasts, and occasionally monocytes and lymphoblasts—contain sharply defined deep blue granules. Such granules are lacking in lymphocytes.

Goodpasture's stain is as follows:

Alcohol	100.00 c.c.
Sodium nitroprusside	0.05 Gm.
Benzidine C. P. (Harmer)	0.05 "
Basic fuchsin	0.05 "
Hydrogen peroxide	0.5 c.c.

Dissolve the nitroprusside in 1 or 2 c.c. of water, mix with the alcohol, and then add the other ingredients. In order to secure satisfactory staining of the nuclei we have found it necessary to double the amount of fuchsin, owing probably to a difference in strength of dye.

Goodpasture's stain remains good only a few days. Beacom has found that it will give satisfactory results for eight months if made up without the peroxide. A freshly made 1 : 200 dilution of hydrogen peroxide is then used in place of water for diluting the stain on the slide. This solution is made with sufficient accuracy by adding 2 drops of hydrogen peroxide to 15 c.c. distilled water.

(*c*) **Lymphoblasts.**—In acute lymphatic leukemia there appears in the blood a high percentage of very young cells of the lymphocytic series. To these the name lymphoblasts is given. In many cases at least they are indistinguishable from the large or immature lymphocytes previously described as occasionally occurring in the blood of normal adults and frequently in that of children. Azure granules are sometimes seen in the cytoplasm, and the nucleus generally shows nucleoli (Plate I). At times the nucleus is curiously lobulated and the name "Rieder's cell" is then applied. Figure 1 of Plate IX shows several Rieder's cells.

Lymphoblasts are sometimes morphologically indistinguishable from myeloblasts, but do not give the peroxidase reaction.

(*d*) **Türk's Irritation Leukocytes.**—These are large, mononuclear, nongranular cells with dense, opaque, strongly basophilic cytoplasm which often contains vacuoles (Plate I). With Wright's and similar stains the cytoplasm stains almost as intensely as the nucleus, although of a different color, being deep blue, while the nucleus is deep purplish

red. The nuclear chromatin shows no tendency to radial arrangement. Nucleoli are usually present.

The nature of the irritation leukocytes is not definitely known and at present no diagnostic importance can be ascribed to them. Some believe them to be pathologic myeloblasts. Considerable numbers have been found in the blood in conditions associated with irritation of the bone marrow, notably primary and secondary anemia, leukemia, and malaria, and in the leukocytosis of pneumonia.

(*e*) **Plasma Cells.**—Morphologically very similar to Türk's irritation leukocyte, the nucleus of the plasma cell may show a tendency toward radial or "wheel-like" arrangement of its chromatin (Plate I).

Plasma cells are considered by some hematologists to be an altered form of the lymphocyte, the same cell which appears so frequently at

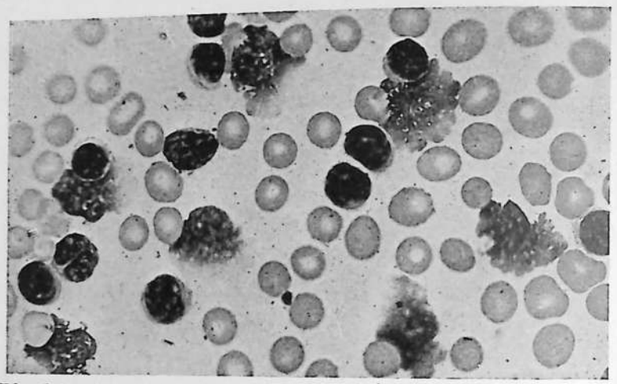


Fig. 153.—Blood in chronic lymphatic leukemia, showing many ruptured lymphocytes (photograph, × 750).

the site of chronic inflammation of certain types. They are extremely rare in the circulating blood and have no diagnostic significance.

(*f*) **Degenerated Forms.**—These are frequently met, but have no significance unless present in large numbers. They include (*a*) vacuolated leukocytes and (*b*) bare nuclei from ruptured cells. The former are found most frequently in toxemias and leukemia. A few of the latter are present in every blood smear, but are especially abundant in leukemia (Fig. 153). They vary from fairly well-preserved nuclei without cytoplasm to mere strands of palely stained nuclear substance arranged in a coarse network—the so-called "basket cells" (Fig. 154).

Occasionally in lymphatic leukemia frayed-out nuclei without cytoplasm exceed the usual lymphocytes in number. In such cases some writers infer involvement of the bone marrow, holding that the naked

nuclei represent very fragile bone marrow cells which have gone to pieces in the circulation. In many cases, at least, it seems more likely that such nuclei only represent fragile lymphocytes which have been broken in making the smear.

(*g*) **Atypic Forms.**—Leukocytes which do not fit in with the above classification are not infrequently met, especially in high-grade leukocytosis, pernicious anemia, and leukemia. They are always more abundant in childhood. The nature of many of them is not clear, and their number is usually so small that they may be classed as "undetermined" in making a differential count. Cooke[1] has described three types of large cells, which are found in pernicious anemia, and which he called "macropolycytes." They are about 50 per cent larger by

Fig. 154.—Two remnants of degenerated nuclei in a blood film. They are often called "basket cells" (photograph, × 1000).

measurement of their diameters than are normal cells. One type resembles a megakaryocyte, and has a gnarled nucleus and azurophilic granules. Another type has open meshwork in the nucleus, and oxyphilic granules. The third type, which Cooke called Type I, is a large neutrophil, and may be found occasionally in normal individuals, and in patients with acute infections, as well as in patients who have pernicious anemia.

3. Blood Platelets.—These are not colored by hematoxylin and eosin. With Wright's and similar stains they appear as spheric or ovoid, reddish to violet, granular bodies, 2 to 4 μ in diameter. Occasionally a platelet as large as a red corpuscle is seen. When well

[1] Cooke, W. E.: The Macropolycyte, Jour. Lab. and Clin. Med., *19*:453–462 (Feb.), 1934.

stained a delicate pale blue, hyaline, ground substance can be distinguished. In ordinary blood smears they are usually clumped in masses. A single platelet lying upon a red corpuscle may easily be mistaken for a malarial parasite, while unusually large and oval platelets are occasionally mistaken for estivo-autumnal crescents (Plate I and Fig. 155).

Blood platelets are being much studied at present, but, aside from the facts mentioned under their enumeration (p. 243), little of clinical value has been learned. They have been variously regarded as very young red corpuscles (the "hematoblasts" of Hayem), as disintegration products of leukocytes, as remnants of extruded nuclei of erythrocytes, and as independent nucleated bodies. At present the accepted explanation of their origin is that of J. H. Wright, who regards them as detached portions of the cytoplasm of certain giant cells (megakaryocytes) of the bone marrow. The megakaryocyte illustrated in Plate I, 38, is magnified about 500 diameters. If it were drawn to the scale of the other cells it would be four times as large. The giant cells push cytoplasmic processes into the capillaries, and these break off and appear in the circulating blood as platelets.

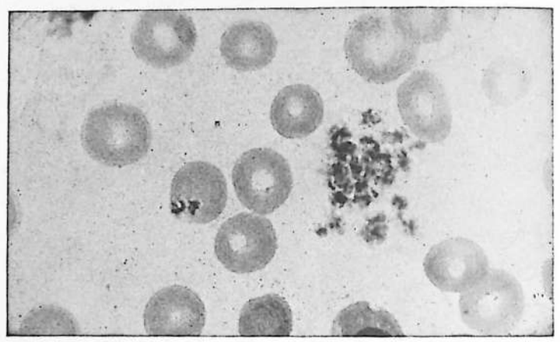


Fig. 155.—A cluster of blood platelets and two platelets lying upon a red cell and simulating malarial parasites (photograph, × 1000).

The megakaryocytes themselves occasionally get into the blood stream, particularly in conditions in which the bone marrow is under intense strain as in myelogenous leukemia. Most of them are too large to pass the capillaries and are arrested in the lungs, but the smaller cells may pass the lungs and appear in the peripheral circulation. They are rarely numerous.[1]

[1] For a description of these cells as they appear in blood films and an excellent colored plate see Minot, G. R.: Megakaryocytes in the Peripheral Circulation, Jour. Exper. Med., *36*:1–7 (July), 1922.

C. Hematopoiesis

A complete discussion of how blood cells are formed in embryonic and adult life belongs in the province of histology and is of little practical importance in clinical laboratory diagnosis. However, there has been an increasing interest in hematology, as evidenced by the recent appearance of several excellent monographs on the subject. In adult life the bone marrow, lymphoid structures and connective tissues contain the precursors of the mature cells found in the circulating blood in normal individuals, and of the immature cells found in the blood in pathologic conditions. Hematologists are not agreed as to whether the several cells of the circulating blood have a common origin from one type of cell—the unitarian theory of Downey, Ferrata, Pappenheim and many others—or from two or more primitive blood cells, as advocated by the polyphyletists, represented by Piney, Naegeli, Schilling, Rosenthal, Osgood, Sabin, Cunningham and Doan, to mention only a few of the well-known hematologists supporting the latter view.

Plate I has been carefully arranged as an adaptation of a chart prepared originally by Downey and his associates to illustrate development of cells from the parent or "stem" cells. It may be used for teaching any theory of formation of blood cells, if used in conjunction with the charts in Figs. 156 and 157. The cells depicted in Plate I were painted by Dr. Elaine Limbert from preparations stained with Wright's stain. The light source was a Zeiss microscope lamp, with an ammonia-cupric oxide filter. This blue-white light simulates daylight, and eosin stained cells appear to be pink rather than bright red, as they do when yellow, unfiltered light is used in a microscope lamp.

Figure 156 shows the cells in outline form, and with numbers referring to those on Plate I, but with no names given to these cells. By filling in the chart the reader can adapt Plate I to any of the polyphyletic theories of the formation of blood cells. Many polyphyletists claim that an erythrocyte arises from an endothelial cell (1A), probably only a different shape of a cell of the reticulum, or from the undifferentiated mesenchyme (Naegeli). Some hematologists name the stem cells "megaloblasts" (2), "lymphoblasts" (3), "monoblasts" (4), and "myeloblasts" (5). The dualists claim that there are two stem cells, "lymphoblasts" (3), precursors of lymphocytes and "myeloblasts" (2, 4, 5), from which all other cells are derived. The trialists differ from the dualists chiefly in deriving the monocytes directly from the reticulo-endothelial cells (1).

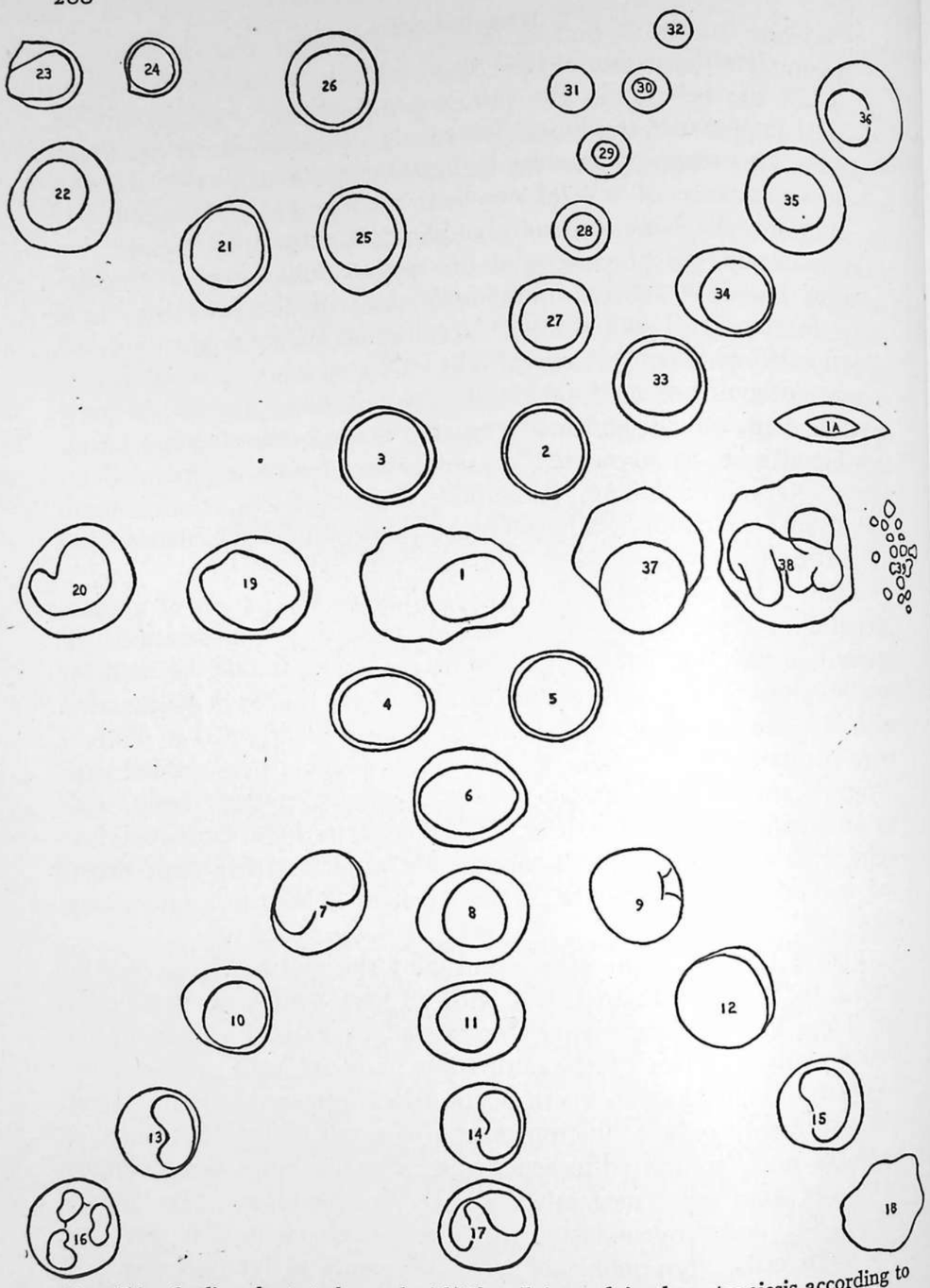


Fig. 156.—Outline chart, to be used with Plate I, in studying hematopoiesis according to polyphyletic theories.

Neo-unitarian Theory.—Figure 157 contains the names of the blood cells according to the neo-unitarian theory, as taught by

Downey. The monophyletic school claims that the stem cells (2, 3, 4, 5) are morphologically identical and that all blood cells are derived

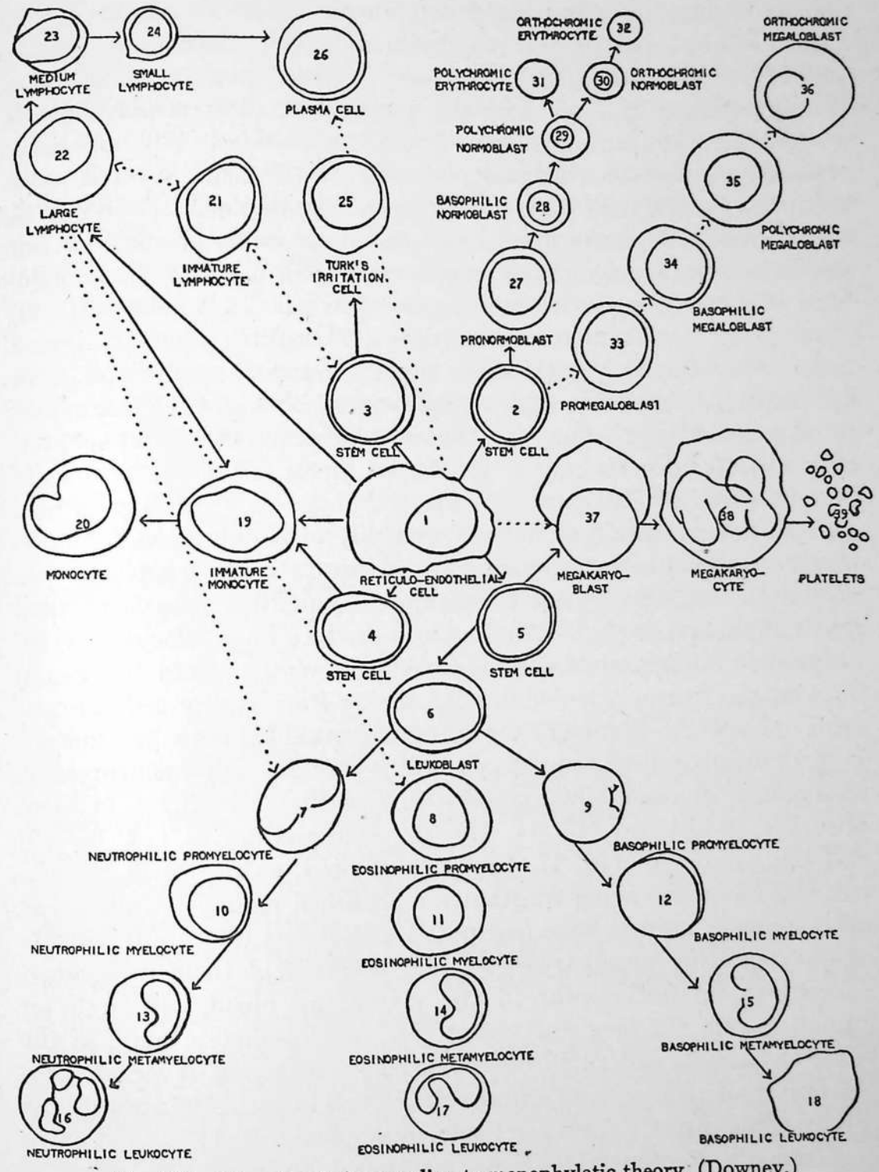


Fig. 157.—Hematopoiesis according to monophyletic theory. (Downey.)

from these stem cells. The arrows indicate the development of cells from the precursors, all cells originating in the reticulo-endothelial cells (1). The mature cells found in normal blood are represented on

the outer edge of the figure (16, 17, 18, 20, 22, 23, 24, 32, 39 and occasionally 26). All of the other cells are immature forms that are found in the circulating blood only in pathologic conditions, or in reticulo-endothelial tissues. An erythrocyte (32), developing from a parent cell (1), through a stem cell (2), may have as precursors a pronormoblast (27), a basophilic normoblast (28), a polychromic normoblast (29) and an orthochromic normoblast (30). A polychromic normoblast (29) may, by losing its nucleus, form a polychromic erythrocyte (31) in the presence of pathologic conditions. A megaloblast (36), according to this theory, is not a normal cell but develops from a polychromic megaloblast (35), a basophilic megaloblast (34), a promegaloblast (33) and a stem cell (2). A monocyte (20) develops from an immature monocyte (19) which is derived from a reticulo-endothelial cell (1) directly, or through a stem cell (4), or even from a lymphocyte (22). Lymphocytes (22, 23, 24) come directly from reticulo-endothelium (1). There is evidence that lymphocytes, under stimulation in cases of disease, may produce "immature lymphocytes" (21) or stem cells (3), and that the latter may in turn develop into mature lymphocytes. A study of lymph nodes obtained from patients with myelogenous leukemia, and a study of experimental metaplasia and tissue cultures (Maximow, Lang, Bloom) have revealed that lymphocytes (22, 23, 24) may also produce promyelocytes (7, 8). Stages of dedifferentiation of lymphocytes are indicated by dotted lines on the figure. A leukoblast (6) arising from a stem cell (5) contains no specific granules, though azure granules may be present. It is a precursor of a promyelocyte which contains either neutrophilic, eosinophilic or basophilic granules (7, 8 or 9). From these, in turn, arise the myelocytes (10, 11, 12), the metamyelocytes (13, 14, 15) and the leukocytes (16, 17, 18). The myeloid leukocytes in the circulating blood are being constantly replenished mainly by mitosis of myelocytes in normal bone marrow. The platelets (39) are fragments of disintegrating megakaryocytes (38). Because of their size, megakaryocytes do not appear in the circulating blood, although an immature cell, the megakaryoblast (37), is occasionally found in the blood of patients suffering from myelogenous leukemia. A megakaryoblast normally comes from a stem cell (5), and in pathologic conditions it may come directly from a reticulo-endothelial cell (1).

Osgood's Nomenclature.—As mentioned on page 286, there is much confusion concerning nomenclature and classification of cells of the blood and bone marrow. Osgood[1] has proposed a new nomenclature

[1] Osgood, E. E.: A Textbook of Laboratory Diagnosis. Ed. 3, Philadelphia, The Blakiston Company, 1940, pp. 153–162.

and has attempted to use one word to describe a cell to which a number of names have been applied by different authors. He realized that usage will decide which term is finally used for these cells. Osgood's classification is as follows:

In the lymphocyte series, he proposed that the term, *lymphoblast* be used for the stem cell, *prolymphocyte* for the large lymphocyte, and *lymphocyte* for the small lymphocyte. In the monocyte series, he used *monoblast* for the stem cell, *promonocyte* for the immature monocyte, and *monocyte* for the large mononuclear, endothelial leukocyte. The greatest change in nomenclature is in the granulocyte series. Osgood proposed *granuloblast* for the stem cell, *progranulocyte S* and *progranulocyte A* for promyelocytes, *granulocyte* for myelocytes, *metagranulocyte* for metamyelocytes, *rhabdocyte* for Schilling's stab cell, and *lobocyte* for filamented polymorphonuclear leukocytes. He also classified cells in the plasmacyte series as *plasmablast* for the stem cell, *proplasmacyte* for lymphoblastic or myeloblastic plasma cells or Türk's irritation form, and *plasmacyte* for plasmacytoid lymphocytes. In the erythrocyte series he proposed the terms *karyoblast* for the stem cell, *prokaryocyte* for orthochromatic, basophilic or polychromatic normoblasts, *karyocyte* for macronormoblasts, *metakaryocyte* for normoblasts, *reticulocyte* for reticulated erythrocytes and *akaryocyte* for the normal erythrocyte. The platelets or thrombocytes he divided into *megalokaryoblast*, *promegalokaryocyte*, *megalokaryocyte* and *platelets*.

D. Examination of Bone Marrow

Histologic studies of bone marrow have been the basis for much knowledge regarding hematopoiesis. The histologic technic for such studies does not properly belong among clinical laboratory methods; however, aspiration of bone marrow may yield interesting material for clinical laboratory study.

Adopting the technic of Schleicher and Sharp[1] for preparing and staining bone marrow, Beizer[2] suggested a simple method and has obtained some interesting results.

Method.—Place in a small test tube the amount of heparin that may be carried on the point of a 20-gauge hypodermic needle. Dissolve this in 0.1 c.c. of distilled water. Remove the water by evaporation in a drying oven, thus leaving a fine coating of heparin on the bottom of the tube. The Klima and Rosegger needle (Fig. 158) is

[1] Schleicher, E. M., and Sharp, E. A.: Rapid Methods for Preparing and Staining Bone Marrow. Jour. Lab. and Clin. Med. *22*:949–951 (June) 1937.

[2] Beizer, Lawrence: The Antemortem Examination of Bone Marrow. Thesis, University of Minnesota Graduate School, December, 1941.

very satisfactory, although similar needles with a guard of some sort may also be used. Set the guard so that the needle may be inserted the proper depth and sterilize the needle by dry heat. Also sterilize a 5 c.c. syringe with dry heat. Cleanse the skin over the upper two-thirds of the sternum and prepare the skin as for an operation, with some antiseptic. Anesthetize the skin with a drop or two of a solution of procaine hydrochloride. Make a small nick through the cuticle. Introduce the needle with a screwdriver-like rotary motion through the bone. There will be a characteristic "give" to the point of the needle when entering the bone marrow. Remove the stylet from the needle and attach the dry, sterile syringe. Aspirate 1 to 1.5 c.c. of material. Place the aspirated material in the tube containing the small amount of heparin. Stopper the tube and agitate by tipping end

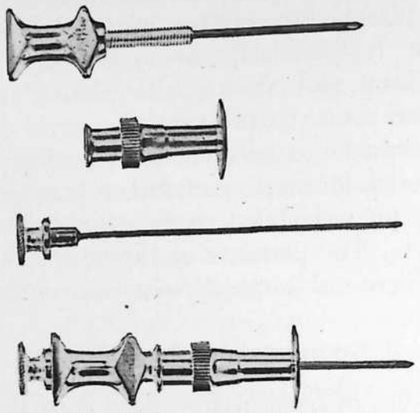
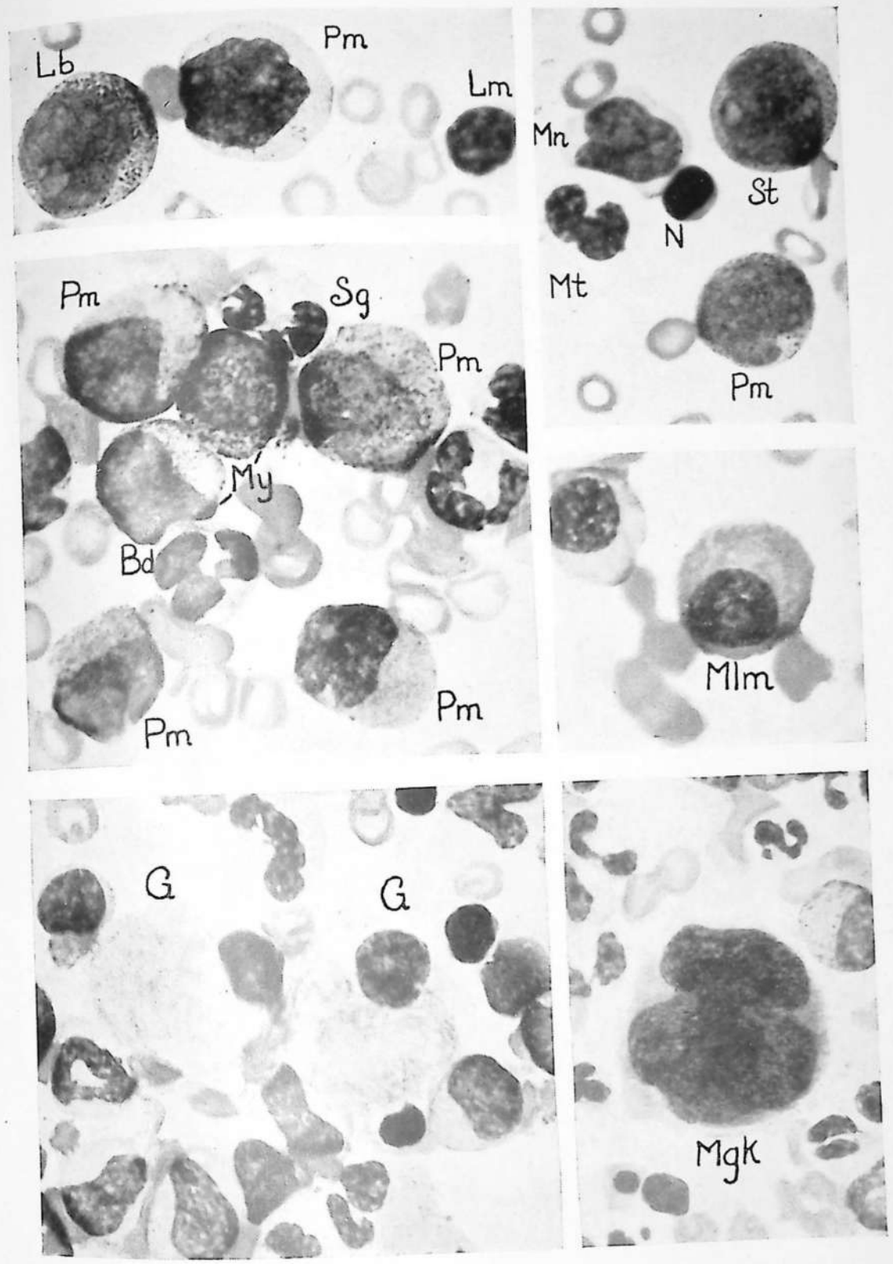


Fig. 158.—Klima-Rosegger needle for sternal puncture.

to end several times. Follow the usual technic for making leukocyte counts. Make an estimation of the total number of nucleated cells in the heparinized bone marrow. Make smears in the usual manner and stain them with Wright's stain, followed by Giemsa stain. Plate VIII shows cells from normal and pathologic bone marrow prepared in this manner.

Sternal aspiration is diagnostic in cases of untreated pernicious anemia, in acute myelogenous leukemia and in many instances of chronic myelogenous leukemia. When positive evidence is found, it is of use in the diagnosis of lymphatic leukemia, monocytic leukemia, reticulo-endotheliosis, multiple myeloma, Gaucher's disease and malignant metastasis. Sternal aspiration may serve in the diagnosis of aplastic anemia, infectious mononucleosis, agranulocytosis, congenital hemolytic icterus and hemorrhagic purpura by excluding positive



Bone marrow corpuscles (× 950). Pm, Promyelocyte; Lb, leukoblast with azurophilic granules; Lm, lymphocyte; Mn, monocyte; Mt, metamyelocyte; St, stem cell; N, normoblast; Sg, segmented neutrophil; Bd, band form; My, myelocyte; Mlm, myeloma cell; G, Gaucher cell; Mgk, megakaryocyte.

evidence of other blood dyscrasias. However, the combination of symptoms which most frequently suggests bone marrow studies is anemia, leukopenia and thrombocytopenia. A sternal aspiration is an important aid in the differential diagnosis of leukopenic leukemia, aplastic anemia and idiopathic thrombocytopenic purpura.

E. Vital Staining

(1) **Erythrocytes.**—On the assumption that ordinary staining of dried and fixed blood film gives the reactions of dead cells and does not necessarily indicate the condition of the living blood, attempts have been made to stain blood cells in the living state. The information yielded by this so-called vital or "supravital" staining has to do chiefly with certain "reticulated" red corpuscles, which contain a coarse skein or network of granular filaments. Sometimes, apparently depending upon variations in technic of staining, there are no definite filaments, but only a number of discrete coarse granules scattered irregularly through the cell. The granulofilamentous substance stains sharply with many of the basic dyes when these are applied to the living cells, but completely fails to stain in the usual dried films.

As to the nature of the reticulum little is known definitely. Some workers connect it with polychromatophilia, others with mitochondria. In any case reticulation is apparently a characteristic of very young red corpuscles, and the number of these in the circulating blood is probably the best available index of the activity of blood regeneration. An increase may be interpreted as indicating both an excessive demand for new cells and a competent bone marrow; when the demand lessens or the bone marrow fails, the number of reticulated red cells falls.

In healthy medical students we have found the reticulated cells to range from 0.3 to 1 per cent of all the red corpuscles, usually 0.6 to 0.8 per cent. In young infants the percentage is two to four times as high. Some writers give higher figures: 0.5 to 2 per cent for adults, 5 to 10 per cent for infants. In the various anemias associated with increased activity of the bone marrow they range from 2 to 20 per cent or even higher, the percentage running parallel with the varying activity of blood regeneration and bearing no necessary relation to the red cell count. The percentage is notably high in hemolytic jaundice.

Of the various dyes which have been used for vital staining, brilliant cresyl blue appears to be by far the most satisfactory. The most satisfactory results are obtained with Grübler's dye, but at least one dye of American make has proved equally good.

The following method is very satisfactory for staining reticulocytes. Make a saturated solution of brilliant cresyl blue, by placing a very small amount of dye in the bottom of a small tube (Wassermann tube) and adding distilled water to dissolve as much of the dye as possible. If the American-made dye is used, better results may be obtained if the dye is dissolved in 0.9 per cent saline solution. Place a drop of the patient's blood on one end of a clean slide as in making an ordinary blood film. Place beside it a drop of the cresyl blue solution, using a glass rod. Wipe off the excess stain from the glass rod, and mix the blood and the stain thoroughly. The mixture will be a light green color. Then spread the blood in the usual manner, as illustrated on page 250 (Fig. 127), and stain with Wright's stain. The reticulocytes will have the appearance shown in Fig. 159. A percentage differential count may be made.

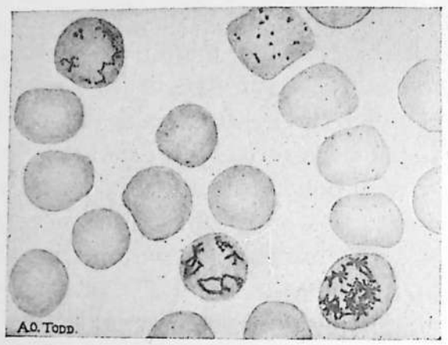


Fig. 159.—Reticulated red corpuscles, drawn from two slides stained as described in the text. The red corpuscles were yellowish green, the reticulum and granules, blue (× 1000).

Another method which gives excellent results, but which takes considerably longer time to carry out, is also given:

1. In a centrifuge tube with conical tip place the following solutions, and mix:

1 per cent brilliant cresyl blue in 0.85 per cent sodium chloride solution, freshly filtered	5 drops
1 per cent neutral potassium oxalate in 0.85 per cent sodium chloride solution	25 "

2. Prick the finger or ear deeply and allow 2 or 3 drops of blood to fall into the stain. Mix gently and let stand ten to twenty minutes.

3. Centrifugalize for a few moments and by means of a capillary pipet remove most of the supernatant fluid, leaving a volume equal to the volume of corpuscles. Mix the sediment with the remaining fluid, transfer a small drop to a clean slide spread in the usual way, and dry in the air.

The sediment must be sufficiently concentrated to allow of quick drying in order to avoid distortion of the corpuscles and to give films with the red cells lying close together but not overlapping. Also the sediment must be well mixed to prevent unequal distribution of the reticulated cells owing to a possible difference in their specific gravity.

4. Examine with an oil-immersion lens and calculate what proportion of the red corpuscles show reticulum or granules. As a basis for calculation at least 1000 cells (better 3000) should be examined for reticulation, and these on several different portions of the slide. To obviate the difficulty of counting large fields with a confusing number of cells, the field of view may be divided by cementing four short pieces of a hair across the diaphragm of the ocular in such a way as to form a small rectangle in the center.

In preparations made as above described the leukocytes and platelets are colored shades of blue and the red corpuscles yellowish green. The reticulated corpuscles are often decidedly larger than their fellows. The network and granules are deep blue and stand out distinctly (Fig. 159). The only serious source of error will be particles of stain adhering to the surface of the corpuscles. The preparations retain their color well if kept from the light.

When desired the films may also be stained with Wright's stain which, combined with the brilliant cresyl blue, gives beautiful preparations which are quite satisfactory.

(2) **Leukocytes.**—Florence Sabin has introduced a simple method of studying the leukocytes in the living condition which opens a new and fertile field for research, and also promises to become a useful clinical procedure, although sufficient facts have not yet been accumulated to give it clinical value at present. For details the reader is referred to the papers of Sabin and her coworkers.[1]

A similar method has long been in use for vital staining of red corpuscles, but the concentration of dye which stained the red cells satisfactorily injured the white. Moreover, in the case of the red cells it was not necessary to keep the cells alive during the examination.

Technic.—1. Carefully clean slides and covers as follows: Soak in bichromate cleaning fluid three or four days; place in running water two or three hours, separating slides occasionally; rinse in three or four changes of

[1] Sabin, Florence R.: Studies of Living Human Blood-Cells, Bull. Johns Hopkins Hosp., *34*:277–288 (Sept.), 1923. Sabin, F. R., Austrian, C. R., Cunningham, R. S., and Doan, C. A.: Studies on the Maturation of Myeloblasts into Myelocytes and on Amitotic Cell Division in the Peripheral Blood in Subacute Myeloblastic Leucemia, Jour. Exper. Med., *40*:845–871 (Dec.), 1924. Sabin, F. R., Cunningham, R. S., Doan, C. A., and Kindwall, J. A.: The Normal Rhythm of the White Blood Cells, Bull. Johns Hopkins Hosp., *37*:14–67 (July), 1925. Cunningham, R. S., Sabin, F. R., Sagiyama, S., and Kindwall, J. A.: The Rôle of the Monocyte in Tuberculosis, Bull. Johns Hopkins Hosp., *37*:231–280 (Oct.), 1925.

distilled water, and soak overnight; store in 80 per cent alcohol. Before use wipe with a clean towel and polish slides with jeweler's rouge applied on a piece of silk.

2. Flame the slides and flood with a very dilute solution of the selected dye in absolute alcohol, drain off excess, and place the slides upright until dry. The film must be very thin and evenly spread. Prepared slides may be stored until needed, but must be kept from dust. Many stains will serve. Sabin prefers neutral red (specify for vital staining) as it is relatively nontoxic and is also an indicator, showing the chemical reaction of the parts of the cell which take the stain. The very dilute solution with which the slides are spread is prepared by mixing 0.4 c.c. of a 1 per cent solution of neutral red in absolute alcohol with 10 c.c. absolute alcohol. When it is desired also to bring out the mitochondria of the cells, 3 drops of saturated solution of Janus-green in absolute alcohol may be added to each 2 c.c. of the diluted solution of neutral red.

3. Receive a very small drop of blood from a puncture on a perfectly clean cover, invert upon the prepared slide, and immediately seal with vaselin of high melting point or with a mixture of vaselin and paraffin. The slide must not be cold but need not be warmed.

4. Within ten minutes place the slide on a warm stage or better, in a micro-slide incubator. Examine at once with the oil-immersion objective. Normally the cells remain alive for at least an hour, sometimes three or four hours.

Each lot of cleaned slides and each new bottle of diluted stain should be tried out with normal blood to insure uniform conditions. Leukocytes are very sensitive to the least trace of acid that may be left on the slides and to an excess of the dye. Any coloring of the nucleus indicates injury to the cell.

Appearance of Leukocytes.—*Polymorphonuclear neutrophilic leukocytes* are constantly moving about with their characteristic ameboid motion and are readily seen. Their cytoplasmic granules, which are numerous, of small size and pale red, are constantly streaming through the cytoplasm. As the cell moves about, the nucleus with its several lobes, is usually in the rear part. In addition to the granules, the cytoplasm of most of these cells contains one or more rounded bodies which take the stain slowly. These are larger than the granules and are presumably digestive vacuoles, indicating phagocytic activity. They vary in color, depending on the chemical reaction of the contents. Certain leukocytes are rounded and motionless, the nucleus is structureless and nearly fills the cell, and the granules, although distinctly visible, do not take the stain. These are interpreted as dead or dying neutrophils. Their number varies at different hours, being usually greatest near the middle of the day. The number may be greatly increased by faulty technic (excessive heat, pressure of the cover, and so forth).

The cytoplasmic granules of *eosinophils* and *basophils* stain with the neutral red. The basophilic granules are somewhat smaller than the eosinophilic, and differ among themselves in size and depth of staining. Digestive vacuoles are apparently absent from both cells. Eosinophils are actively motile, basophils slightly so.

The younger *monocytes*, with oval nuclei, are rounded and practically nonmotile. Their cytoplasm contains very fine salmon-colored granules which are grouped around a clear spot, the centrosphere. With these fine granules are a variable number of larger red bodies, apparently digestive vacuoles. The older cells, with lobulated or saddle-shaped nuclei, are more irregular in shape, usually elongated, and are sluggishly motile. The red vacuoles are usually numerous and may displace or at least obscure the fine granules.

The cytoplasm of *lymphocytes* is clear excepting for a few small vacuoles which take the red stain, and, when Janus-green is added, a clump of blue mitochondria opposite the nucleus. The nucleus is oval or indented, seldom round. The large and small lymphocytes show no locomotion. Those of intermediate size move very slowly, and the nucleus is then at the front end and its shape changes.

XII. SPECIAL BLOOD PATHOLOGY

Because of the special functions of the blood and because of its circulation in all parts of the body, every local or generalized disease will produce some change in it, even though this may be inappreciable by our present methods. The great majority of blood changes are, therefore, purely secondary and have no claim to be classed as blood diseases. There are a few blood conditions (for example, malaria) which may be regarded as true blood diseases, and still others of which the etiology is so obscure and in which the blood changes are so conspicuous and so dominate the clinical picture that they are commonly spoken of as blood diseases. In a general way, they fall into four groups. In the first group, the characteristic feature is a change in the amount of hemoglobin and number of erythrocytes, either diminution (anemia) or increase (erythremia); in the second group, the leukocytes are chiefly affected (leukemia and leukopenia); in a third group, the clotting mechanism is distributed (hemorrhagic diseases); and in a fourth group, there are animal parasites living in the blood (malaria and others).

A. Anemia

For the sake of simplicity anemia may be characterized as a deficiency of hemoglobin. The number of red corpuscles is diminished also, and decrease of blood volume and changes in the composition of the plasma are usual, but the hemoglobin loss is the most conspicuous

change. *An estimation of hemoglobin is the most sensitive index of the existence and grade of anemia, and the most practicable;* but the normal variations dependent upon age and sex must be considered, as well as the inaccuracies of the instruments in common use. The appearance of the patient is often deceptive, since the color of the skin depends as much upon the capillary circulation as upon the color of the blood.

Factors which Determine the Blood Picture in Anemia.—Red corpuscle formation and red corpuscle destruction go on continuously within the body. In health there is such a balance between the two processes that the red corpuscles and the hemoglobin are maintained at a remarkably constant level. Any disturbance of this "hemogenic-hemolytic balance" results in an increase or a decrease of the hemoglobin and the red cell count. When the balance is upset by deficient blood formation, on the one hand, or by excessive destruction or actual loss of blood upon the other, the result is anemia; and, in any given case, the red cells and hemoglobin will rise or fall as the hemogenic-hemolytic ratio fluctuates. The red corpuscle count and the hemoglobin estimation therefore tell us no more than the grade of the anemia, that is, the resultant of the two opposing processes of hemogenesis and hemolysis. We can, in a certain crude way, get at the real conditions by a study of urobilin excretion, particularly in the feces or duodenal contents (p. 476), which serves as an index of blood destruction, and by a count of the percentage of reticulated red corpuscles (Vital Staining, p. 293), which furnish a guide to the activity of blood regeneration. *The most information regarding types of anemia is to be obtained from a careful microscopic study of a well-stained blood film.*

In addition to simple diminution of hemoglobin and red corpuscles in anemia certain qualitative changes appear and may be very marked in the severer cases. In general these are an expression of the strain put upon the erythroblastic bone marrow, which, when hard pushed, tends to react excessively and sometimes in an abnormal manner, and therefore puts out variable numbers of immature and imperfect red corpuscles (poikilocytes, polychromatophilic cells, nucleated red cells, and so forth). The presence of immature forms may be thought of partly as representing a lowering of the threshold whereby they overflow more easily into the circulating blood. In some anemias, owing probably to a toxic injury to the erythroblastic tissue, the type of blood regeneration takes on some of the characters of that seen in fetal life, and megaloblasts appear in great numbers in the bone marrow and even pass over into the circulating blood.

Many classifications of diseases characterized by anemia have been proposed but none of them are entirely satisfactory. The following

classification of Ottenberg[1] lists most of the conditions in which anemia occurs:

I. **Deficiencies**

(*A*) Iron Deficiencies
1. Loss of blood
   (*a*) Acute
   (*b*) Chronic
   (*c*) Hookworm anemia
2. Hypochromic anemia
   (*a*) Chlorosis
   (*b*) Simple hypochromic anemia
   (*c*) Achlorhydric anemia
   (*d*) Hypochromic anemia of pregnancy
3. Simple nutritional anemia of infants (on exclusive milk diet)
   (*a*) Anemia of premature infants

(*B*) Deficiency of "Antianemic Principle"
1. Pernicious anemia
2. Sprue
3. "Pregnancy pernicious anemia"
4. Diphyllobothrium anemia (certain cases)

(*C*) Nutritional Deficiencies
1. Avitaminoses: beriberi, pellagra, scurvy, rickets
2. Loss of bile, or of pancreatic secretion
   (*a*) Biliary fistula
   (*b*) Pancreatic or duodenal fistula
3. Failure of intestinal absorption
   (*a*) Chronic diarrhea, sprue (some cases), celiac disease
   (*b*) Stenosis of small intestine
4. Nutritional anemia of adults
5. Certain infantile anemias (von Jaksch, Cooley)

II. **Injury to the blood making organs** (Interference with regeneration of blood)

(*A*) Toxic Destruction of Marrow
1. Aplastic anemia secondary to:
   (*a*) Roentgen rays, radium, thorium
   (*b*) Benzene, arsphenamine, nitrobenzene, trinitrotoluene
   (*c*) Lead, mercury and so forth
2. "Primary" aplastic anemia (toxic agent not yet known)

[1] Ottenberg, Reuben: Reclassification of the Anemias, Jour. Am. Med. Assn., *100*: 1303–1311 (Apr. 29), 1933.

(*B*) Mechanical Replacement of Bone Marrow

1. Osteosclerosis
   (*a*) Osteosclerotic anemia
   (*b*) Marble bone disease (Albers-Schönberg)
2. Gaucher's disease and other lipoid deposits in bone marrow (Niemann-Pick disease, Schüller-Christian syndrome)
3. Leukemia and Hodgkin's disease
4. Metastatic new growths in bone marrow

(*C*) Interference with Regeneration of Blood at Some Intermediate Stage

1. Diseases of the spleen
   (*a*) Banti's disease ("splenic anemia")
   (*b*) Sclerosis or thrombosis of the splenic vein
2. Diseases of the liver
   (*a*) Cirrhosis
   (*b*) Prolonged obstructive jaundice

III. **Disintegration of blood** (Hemolysis)

(*A*) Caused by Hereditary Defects of Red Blood Cells Themselves

1. Hemolytic icterus
2. Sickle cell anemia

(*B*) Toxic Destruction of Blood

1. Infections
   (*a*) Bacteria of all varieties, especially those invading the blood: hemolytic streptococcus, Staphylococcus aureus, Streptococcus viridans (bacterial endocarditis)
   (*b*) Protozoa: malaria, kala-azar, syphilis
   (*c*) Acute febrile hemolytic anemia (cause unknown)
2. Intestinal worms (Diphyllobothrium)
3. Cancer (including leukemia and allied diseases)
4. Nephritis—azotemia
5. Extensive burns
6. Hemolytic poisons
   (*a*) Serum hemolysins (paroxysmal hemoglobinuria, incompatible transfusion)
   (*b*) Chemicals: saponin, pyridine, tolylendiamine, pyrogallol, snake venom, phenylhydrazine and potassium chlorate

In the diagram (Fig. 160) there is an attempt to present graphically a simple classification of anemia according to hemoglobin content

and the size of the erythrocyte. Thus, anemia may be *hypochromic*, the cell having less than the normal amount of hemoglobin; anemia, in which the individual cell has a normal amount of hemoglobin, is indicated as *normochromic* on the diagram. (Some authors have called this type "hyperchromic" anemia, but there is some question whether a cell ever contains more than a normal amount of hemoglobin for the size of the cell.[1]) The erythrocytes may be small, or *microcytic* normal, called *normocytic* on the diagram; or they may be larger than normal, *macrocytic*. The lines connecting words describing the hemoglobin content of the erythrocyte, and those describing the size of the cells represent the combinations of characteristics that occur in various types of anemia. The names placed on these lines are those of definite

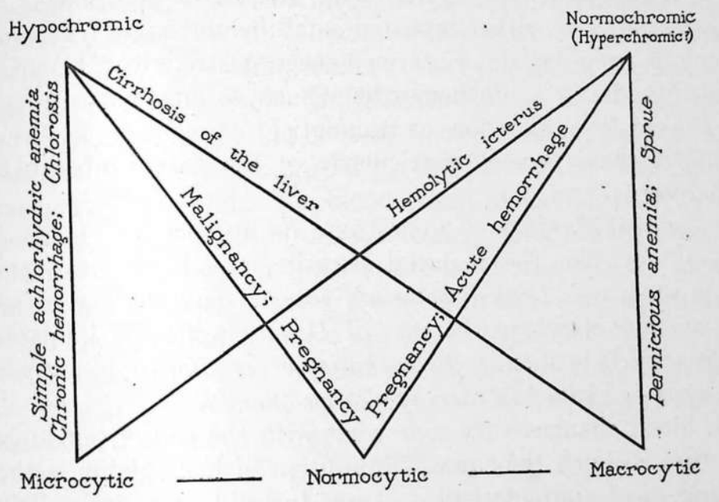


Fig. 160.—Diagram illustrating the classification of the anemias.

conditions or diseases that produce anemia of the type described by combining the two characteristics connected by the lines; thus acute hemorrhage causes a normochromic normocytic anemia—a loss of hemoglobin resulting from a loss of normal erythrocytes, while chronic hemorrhage tends to produce a hypochromic microcytic anemia. The conditions named on the diagram will be considered in detail later, though there are other conditions which produce anemia which can be classified according to this scheme. Toxic types of anemia may occur in any of the groups, and in many conditions there are combinations that cannot be shown on a simple diagram.

[1] Wintrobe, M. W.: Volume and Hemoglobin Content of Red Blood Corpuscle; Simple Method of Calculation, Normal Findings, and Value of Such Calculations in Anemias, Am. Jour. Med. Sci., *177*:513–523 (Apr.), 1929.

The more important conditions which produce "secondary" or symptomatic anemia are listed below. In many clinical cases two or more of these operate together:

(*a*) *Poor nutrition*, which usually accompanies unsanitary conditions, such as poor and insufficient food.

(*b*) *Acute infectious diseases*, especially rheumatism and typhoid fever. The anemia is often more conspicuous during convalescence.

(*c*) *Chronic infectious disease;* tuberculosis, syphilis, leprosy, chronic suppuration.

(*d*) *Chronic exhausting diseases*, as chronic nephritis, cirrhosis of the liver, and gastro-intestinal disease, especially when associated with atrophy of gastric and duodenal glands.

(*e*) *Chronic poisoning*, as from lead, arsenic, or phosphorus.

(*f*) *Hemorrhage*, either repeated small hemorrhages (chronic hemorrhage), as from gastric or uterine cancer, gastric ulcer, hemorrhoids, uterine fibroids; or acute hemorrhage such as may occur in typhoid fever, tuberculosis, abortion, or traumatism.

(*g*) *Malignant tumors*, particularly in the gastro-intestinal tract and the uterus.

(*h*) *Animal Parasites.*—Some cause no appreciable change in the blood; others, like the malarial parasite, the hookworm, and the "fish tapeworm," *Diphyllobothrium latum*, may give rise to very severe anemia, which in the case of *D. latum* closely simulates progressive pernicious anemia. *Examination of feces for the ova of parasites should never be omitted in cases of obscure anemia.*

The blood changes vary somewhat with the cause, but in general the picture is much the same. Diminution of hemoglobin is the constant and most characteristic feature. In mild cases it is slight and is the only blood change to be noted; in these cases the normal hemoglobin variations dependent upon age and sex must be taken into account as well as the inaccuracies of the usual clinical hemoglobinometers. A reading which is normal for a girl of seven years would mean anemia for a man. In moderate cases of anemia, the value for the hemoglobin ranges from 10 to 12 Gm. per 100 c.c. of whole blood; in severe cases, it is from 6 to 8 Gm. per 100 c.c. of blood; while in extreme cases, it may be as low as 2.5 Gm. per 100 c.c. of whole blood, or even lower. Red corpuscles are diminished in all but very mild cases, while in the severest cases the count is sometimes as low as 1,000,000 for each cubic millimeter. The color index is generally reduced to about 0.7 or 0.8, but may rarely fall to 0.5.

Although the number of leukocytes bears no direct relation to the anemia, polymorphonuclear leukocytosis is common, being due to the same cause as is the anemia.

Stained films show no appreciable changes in very mild cases. In moderate to severe cases variations in size and shape of the red cells occur; while scattered polychromatophilic cells are found, and even occasional normoblasts. Very severe cases show the same changes to greater extent, with addition of basophilic granular degeneration and the presence of normoblasts in small or considerable numbers. However, the student must be cautioned that, even when "very numerous," these abnormal cells can usually be found only after a search; they are not found in every field, as many seem to expect. The number of normoblasts bears no direct relation to the severity of the anemia, but rather serves as a rough index of the regenerative activity of the bone marrow. At times, particularly in acute posthemorrhagic anemia, great numbers of normoblasts may appear rather suddenly—a so-called *blood crisis*—and this is often followed by rapid increase in the red count. The finding of more than five nucleated red cells for each hundred leukocytes seen during the differential count is rather arbitrarily taken to indicate a blood crisis. Megaloblasts may be encountered in severe cases of anemia, but in interpreting reports one should know the criteria adopted in the particular case for identification of the megaloblast. They are especially numerous and may even exceed the normoblasts in severe anemia of Diphyllobothrium infestation, which gives a blood picture identical with that of pernicious anemia, and in the anemia of malignant disease, presumably with metastases in the bone marrow.

In the anemia of lead poisoning a striking feature and one of much diagnostic significance is the exceptionally large number of red cells which show blue granulation with the polychrome methylene-blue-eosin stains (Fig. 161). These granulated cells may be found in chronic and acute lead poisoning even when little or no anemia is apparent, while in cases with marked anemia they may be so numerous that several appear in every microscopic field. Polychromatophilic cells are generally more numerous than in the usual anemias.

1. Acute posthemorrhagic anemia, or *normochromic normocytic* anemia, deserves more extended mention. The greatest amount of blood which can be lost at one time with recovery depends upon the age and health of the individual, therapeutic measures, and other factors, but is generally taken to be about one half the total blood volume. Immediately after a large hemorrhage the red cell count will be the same as before. Within a few hours the volume of blood is, to

a great measure, restored by means of fluids from the tissues, and the process of dilution continues more slowly for a day or two. At the same time the red cell count and hemoglobin percentage fall to a figure roughly corresponding to the amount of blood lost. Active regeneration of corpuscles begins within a short time, probably within twenty-four hours. The new corpuscles tend to be deficient in hemoglobin, hence the color index is low, sometimes strikingly so. Some of the newly-formed cells show polychromatophilia, and a few normoblasts commonly reach the circulation. Occasionally blood crises occur, with subsequent improvement in the blood picture. There is moderate neutrophilic leukocytosis. Blood platelets are strikingly increased, even up to 1,000,000 for each cubic millimeter.

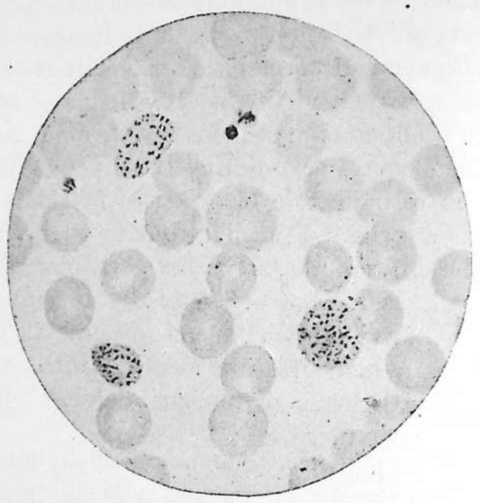


Fig. 161.—Blood from a case of secondary anemia due to lead poisoning. In this small area note three red corpuscles showing basophilic granular degeneration (photograph, × 1000).

The time required for restoration of the number of corpuscles is about a month in the case of a large hemorrhage in a previously healthy person. After a loss of 500 to 600 c.c.—an amount frequently given by donors in transfusion cases—the number of corpuscles is usually made up in one or two weeks, although the hemoglobin percentage lags behind. Giffin and Haines[1] have shown that professional donors, who are usually between the ages of twenty and forty, may give blood every four to six weeks for two years or more with no apparent ill effects, and even with improvement in general health and

[1] Giffin, H. Z., and Haines, S. F.: A Review of a Group of Professional Donors, Jour. Am. Med. Assn., *81*:532–534 (Aug. 18), 1923.

increase in weight. In some, however, repeated bleedings are followed by mild anemia to which women show a greater tendency than do men; and in a very few, probably because of preexisting weakness of the blood-forming tissues, marked chronic anemia may develop.

2. Progressive Pernicious Anemia (Addison's Anemia).—This is a *normochromic macrocytic* anemia. The essential feature is excessively active destruction of red corpuscles by an unknown poison, together with active, although abnormal, regeneration of red corpuscles by the bone marrow. The course of the disease and the blood picture vary with the varying rate of blood destruction, with the functional sufficiency of the hemogenic bone marrow, and with the bone marrow threshold for immature cells. As a terminal event the bone marrow may become exhausted, giving a blood picture similar to that of aplastic anemia. Characteristic of the clinical course is a striking tendency to remissions, which may occur spontaneously or may be induced by one or another therapeutic measure. At such times hemolysis is greatly reduced, and the blood picture approaches the normal. There may be one or several of these remissions, lasting from a few weeks to many months or even years. Stockton has reported a case with remission of twelve years. While in some cases diagnosis can be made with considerable certainty from a study of repeated blood examinations, yet, as a rule, a careful analysis of all clinical and laboratory data is required, and even this may sometimes be indecisive. Of the laboratory findings the most significant for diagnosis are the blood picture, the great increase of urobilin in urine and feces, and the absence of free hydrochloric acid from the gastric juice.

Castle and Strauss[1] and their colleagues have investigated the relation of diet and gastric disorders to anemia. The evidence is that their hypothesis that pernicious anemia is a peculiar type of deficiency disease is correct. This condition results not from an inadequate diet, but from a failure of a normal function of the stomach to manipulate proteins in the production of a factor necessary for the activity of bone marrow. The epoch making experimental work of Whipple and his associates, in the administration of liver, and various other substances to dogs, which were made anemic by repeated bleedings, led Minot and Murphy to use liver in various forms in the treatment of pernicious anemia. The results have been so remarkable that the entire picture of the course of the disease, in cases in which

[1] Castle, W. B., Clark, C. W., Strauss, M. B., and Townsend, W. C.: The Relationship of Disorders of the Digestive Tract to Anemia, Jour. Am. Med. Assn., *97*:904–907 (Sept. 26), 1931.

patients have been treated in this manner, has been changed. While the classic description must still be presented, the student at the present time probably will not often see the terminal stages of this so-called "pernicious" disease.[1]

Pernicious anemia is practically never seen at the onset of the disease, so that red cells are generally very low when the first examination is made—usually 1,500,000, or less, for each cubic millimeter in dispensary work, more frequently about 2,500,000 in private practice. There seems to be a tendency for the red cells to fall rapidly to a certain level, which varies with the case, usually between 1,000,000 and 1,500,000 for each cubic millimeter, and to remain fairly stationary near this level. However, a few cases have been reported which terminated fatally with the red corpuscles above 2,500,000 for each cubic millimeter, while, upon the other hand, counts as low as 150,000 have been recorded. In remissions, the red count often rises with surprising rapidity, for example, from 1,000,000 to 3,000,000 within a few days.

The average hemoglobin value in active cases is about 3 to 4.5 Gm. per 100 c.c. In more than two thirds of the cases the hemoglobin loss is apparently less than the loss of red cells on account of the macrocytosis: The color index is therefore high, usually 1.1 to 1.5, sometimes as high as 2. A color index of 0.8 or below speaks against a diagnosis of progressive pernicious anemia, although it may occur in mild cases, or in the early stage of a remission when the blood is rapidly regenerating, as following transfusion. To be of any value, however, color-index determinations must be based upon an accurately standardized hemoglobinometer (p. 229). The volume index is high, and is even more significant than is the color index.

In stained films red corpuscles show marked variations in size and shape. There is decided tendency to large oval forms, and despite the presence of microcytes the average size of the corpuscles is generally strikingly increased. In some cases even the majority exceed 11 μ in diameter, a circumstance of great diagnostic significance. A few of these large cells show endoglobular degeneration. Another striking feature of the red cells is that while an occasional corpuscle may show marked pallor, the great majority evidently contain their full amount of hemoglobin. Polychromatophilic red cells and cells showing basophilic granular degeneration, especially the former, are

[1] The student should familiarize himself with the literature of Dr. Whipple and his associates, and with the clinical reports of Dr. Minot and Dr. Murphy. The Nobel prize in Medicine for 1934 was awarded to these Americans jointly for their very valuable work in this field.

numerous in some cases, scarce in others. Nucleated red corpuscles are probably present in every case, though not always continuously. Contrary to the impression usually gained by students who in their class work have studied only slides from selected cases, nucleated red corpuscles are not often numerous, a search of fifteen minutes to an hour or more being generally required to find them. As a rule, the presence of a large number is a point against the diagnosis of pernicious anemia, except during a blood crisis, which is an infrequent incident. The diagnostic weight given megaloblasts by different workers varies, but in general less importance is ascribed to them than formerly. It depends in a large measure upon the criteria adopted for identifying these cells (p. 261). The young megaloblasts, larger than polynuclear leukocytes, nearly always polychromatophilic, and with large delicately reticular nuclei, are indeed very rarely seen in other conditions; but in probably half of the cases of pernicious anemia they are found only after tedious search or not at all. Sometimes they are unevenly distributed, few or none being found on one slide, while on another, made at the same time, they may be readily found. In many cases of pernicious anemia only the older megaloblasts, with small, condensed nuclei, can be found in any length of time which is practicable in clinical work; and these have less significance, although still very suggestive (Fig. 162).

The leukocyte count may be normal or moderately high, but is commonly diminished to about 3000 or 4000 for each cubic millimeter, and is sometimes much lower. The decrease affects chiefly the neutrophils so that lymphocytes are relatively increased. In some cases a decided absolute increase of lymphocytes occurs. Neutrophilic leukocytosis, when present, is due to some complication. Occasional neutrophilic myelocytes can usually be found. Heck and Watkins[1] in a study of 50 well-proved cases demonstrated that increased segmentation of the nucleus of the polymorphonuclears is the rule (Fig. 163).

Vital staining (p. 293) will generally demonstrate a marked increase in the number of reticulated red corpuscles which usually range between 1 and 5 per cent in this disease, but may at times reach 20 per cent of all the red corpuscles. This percentage is a useful index of the activity of blood regeneration, and it should be considered in connection with the urobilin excretion, the index of hemolysis, since it is upon the balance between hemogenesis and hemolysis that the welfare of the patient depends. Resistance of the red cor-

[1] Heck, F. J., and Watkins, C. H.: The Neutrophil in Pernicious Anemia, Am. Jour. Clin. Path., *3*:263–269 (July), 1933.

puscles to hypotonic salt solutions (p. 327) is not much changed, but is usually slightly increased, thus showing that the excessive hemolysis is not due to increased fragility of the red cells as it is in the case of hemolytic jaundice.

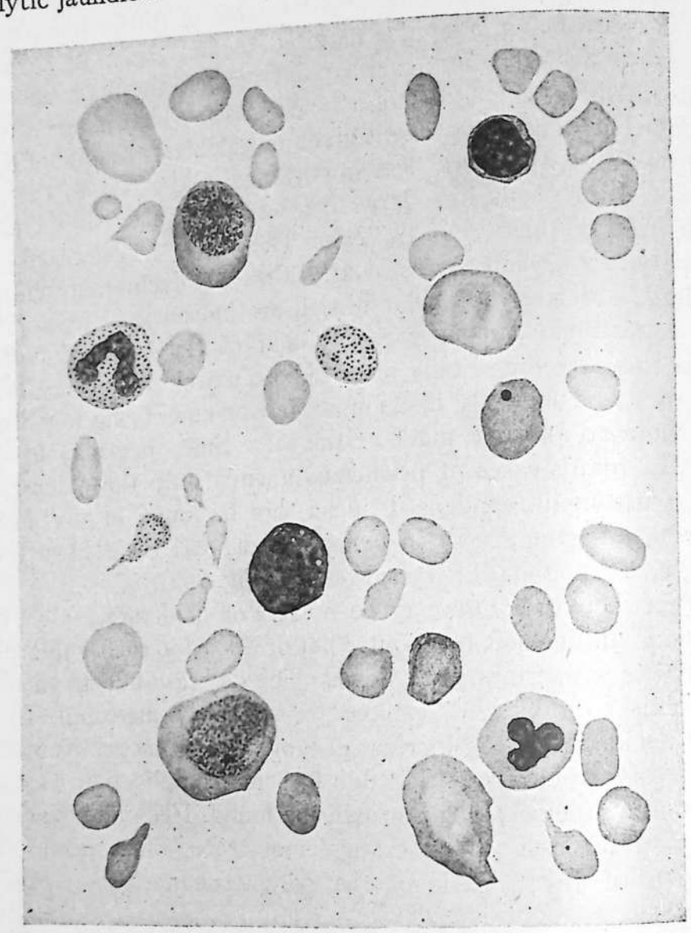


Fig. 162.—Blood cells in pernicious anemia. Note variations in size and shape of the red corpuscles; three megaloblasts, one with irregular, deeply stained nucleus; red corpuscles showing grade, of polychromatophilia, basophilic granular degeneration, and one nuclear particle; one lymphocyte and one polymorphonuclear neutrophil. All drawn from actual cells on two slides. Wright's stain; × 800 (0.8 mm. = 1 micron).

As a rule blood platelets are diminished, even below 50,000 in some cases. The decrease is apparent in the stained film, where it may be difficult to find any. Coagulation time is delayed.

To sum up the blood findings in typical cases of pernicious anemia: The red cells are greatly diminished, the color index and volume index

high, leukocytes normal or slightly diminished, platelets reduced. Many macrocytes, and a few megaloblasts are present in stained films.

The tropical disease **sprue** should be mentioned in connection with anemia of this type. It is a disease of unknown etiology, which is characterized by sore mouth, and tongue, and by gastro-intestinal symptoms. There is usually, but not always, a copious diarrhea, with frothy, bulky stools. Subsequently, a *normochromic macrocytic* anemia may develop.

3. Anemia During Pregnancy.—A toxic factor, the result of pregnancy, may account for a *normochromic normocytic* anemia. The bone marrow function is impaired and there is a lack of production

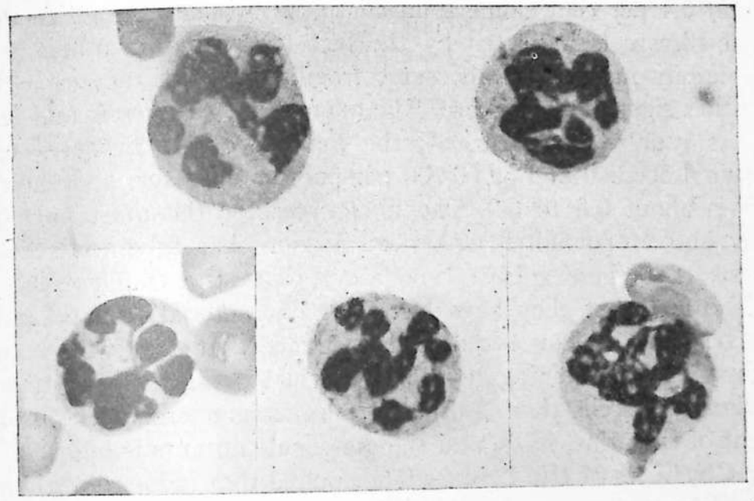


Fig. 163.—Increased nuclear segmentation of neutrophils in pernicious anemia (photographs, × 1450).

of normal cells containing the normal amount of hemoglobin. There is usually some anisocytosis and polychromatophilia, and an increase in reticulocytes. Evidence of toxicity is shown in a moderate "left shift" of the neutrophils, with swelling of the nuclei, cloudiness of the chromatin, and irregularity in distribution of the specific granulation, and intensification of the staining reaction of the individual granules. After parturition, the blood usually regenerates promptly and returns to normal.

A second type of anemia in pregnancy is characterized chiefly by marked hypochromasia—a *hypochromic normocytic* anemia. All other features are practically the same as in type I. It is likely that this type of anemia represents an aggravation of a previous tendency to

anemia. It is corrected rather promptly with the administration of ferric citrate, or ferric ammonium citrate in large doses.

In malignancy the anemia is usually of the *hypochromic normocytic* type.

4. Hemolytic Jaundice (Congenital Familial Icterus).—This is a rare, chronic, generally hereditary, disease, which is characterized by periods of excessive hemolysis, and which results, in part at least, from abnormal fragility of the erythrocytes. There is marked splenomegaly, and usually rather definite icterus.

A striking feature, from the laboratory point of view, is the lowered resistance of the erythrocytes to hypotonic saline solution; in a typical, well-marked case, initial and complete hemolysis occurs in 0.5 and 0.4 per cent saline solution (p. 327). The familial tendency of this disease is indicated by the fact that other members of the same family may reveal the same fragility of erythrocytes without any other signs of the disease. The anemia, *normochromic* and *microcytic*, is usually very marked; the number of erythrocytes varies between 1,500,000 and 3,000,000 per cubic millimeter, and the color index is about 0.8 to 0.9. The microcytes for the most part stain solidly and are definitely spherical. Haden[1] has advanced the hypothesis that these spherical microcytes are the fragile cells, and that the fact that they have lost their characteristic shape and no longer appear as biconcave disks is evidence that they will be easily destroyed. Vital staining reveals a distinctly higher percentage of reticulated red cells than is usual in pernicious anemia. The urobilin content of the urine, feces, and the duodenal contents is high.

5. Cirrhosis of the Liver.—The anemia that is found in cirrhosis of the liver is usually *hypochromic macrocytic*, with a color index which is less than 1, although Cheney[2] reported a few cases of the *normochromic macrocytic* type in which the color index was greater than 1.

6. Simple Achlorhydric Anemia.[3]—This *hypochromic microcytic* anemia usually affects middle-aged women. It is characterized by pallor, weakness, atrophied tongue, brittle fingernails, achlorhydria, and a color index which is less than 1. The hemoglobin content is low, 4 to 10 Gm. per 100 c.c. of blood, while the erythrocyte count

[1] Haden, R. L.: The Mechanism of the Increased Fragility of the Erythrocytes in Congenital Hemolytic Jaundice, Am. Jour. Med. Sci., *188*:441–449 (Oct.), 1934.

[2] Cheney, Garnett: Morphology of Erythrocytes in Cirrhosis and Other Disorders of the Liver, Calif. and West. Med., *39*:90–97 (Aug.), 1933.

[3] Meulengracht, E.: Simple Achylic Anemia, Acta. Med. Scand., *78*:387–426, 1932. Damashek, William: Primary Hypochromic Anemia; Clinical Features, Jour. Am. Med. Assn., *100*:540–548 (Feb. 25), 1933.

ranges from 1,500,000 to 4,700,000 per cubic millimeter. The cells are chiefly microcytes. The leukocyte count is usually about normal, though leukopenia, and low platelet counts have been reported. The first description of the syndrome is credited to Faber in 1909. It was named "simple achylic anemia" by him in 1924. There have been numerous names proposed for the disease; among these are the following: Chronic microcytic anemia, idiopathic hypochromic anemia, chronic chlorosis, achylia chloranemia, essential hypochromic anemia, simple achylic anemia, primary hypochromic anemia, and simple achlorhydric anemia. This last name is preferred by several authors.

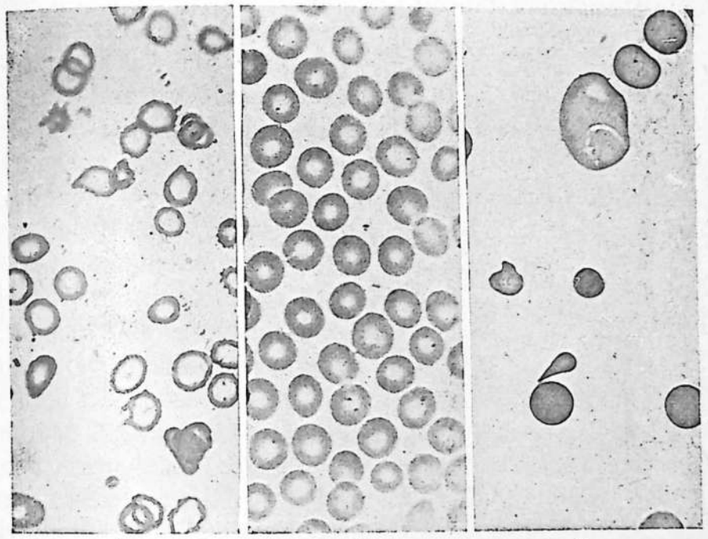


Fig. 164.—Red blood corpuscles in chlorosis (A) and pernicious anemia (C) contrasted with those of normal blood (B). In a well-marked case of chlorosis the red corpuscles are pale and ringlike; in pernicious anemia they are rich in hemoglobin, and show marked variations in size and shape. The megalocyte in the upper part of the figure is especially characteristic of pernicious anemia. Wright's stain; photographs (× 750).

All investigators of this condition report a remarkable response to the administration of iron salts in large doses.

Chronic hemorrhage is also characterized by *hypochromic microcytic* anemia as is the rare disease, **chlorosis.** The latter disease has been described in all of the textbooks for many years as a profound anemia which affects adolescent girls, and which produces a peculiar greenish pallor. The color index may be only 0.4 or 0.5, as a result of the marked hypochromia (Fig. 164). The cell count is usually rather high, and nearly all of the cells are of the microcytic type. This disease is not often seen in the United States at present.

7. Sickle Cell Anemia.—In 1910 J. B. Herrick[1] described a peculiar form of anemia in which a large proportion of the red corpuscles were crescentic or fusiform in shape. This anemia is now recognized as a clinical entity, and many cases are on record, usually in Negroes. Instances of this type of anemia have been reported among Greeks, Italians and Sicilians; in one case that was reported the patient was a boy of Scotch-Irish parentage. It is a hereditary and familial disease, present and recognizable very early in infancy. There is probably a congenital defect of the red corpuscles which renders them susceptible to certain hemolytic poisons, and to phagocytosis, although resistance to hypotonic salt solutions appears to be normal or in some cases slightly increased. The disease appears in two forms, latent and active. While transitions from one to the other may occur, these are not merely stages in the development of the disease.

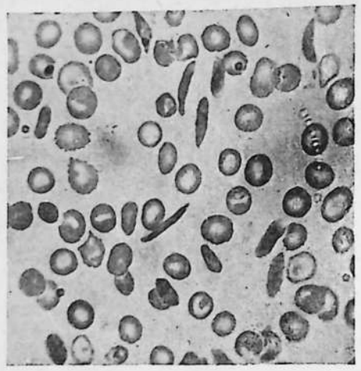


Fig. 165.—Blood in sickle-cell anemia, active form, stained film. The diagnosis is best made from unstained wet preparations, in which, after a few hours, the number of crescentic and stellate forms is greatly increased. (Photograph about × 500.) (Courtesy of V. P. Sydenstricker.)

In the latent form, which is much the more common, there is no definite anemia. The usual routine blood examination shows no more than anisocytosis with many microcytes, and, on careful search, a few endothelial leukocytes containing phagocytized red cells. The diagnosis is established only by the study of wet preparations sealed with vaselin, and kept in a warm place. After eighteen or twenty-four hours these show great numbers of the remarkable crescentic fusiform and stellate red corpuscles which are characteristic of the disease, and also active phagocytosis of red cells by monocytes.

In the active form, which is most active in children, anemia is marked. Red corpuscles usually number 1,500,000 to 3,000,000 in each cubic millimeter. Many sickle-shaped and elongated corpuscles are present in stained films (Fig. 165), and phagocytosis of red cells by monocytes, and even neutrophils, is always present. These characteristics become much more marked in sealed wet preparations which stand for some hours. There are many microcytes and numerous normoblasts. The hemoglobin content is proportionately high, so that this disease may be classed as a *normochromic microcytic* anemia. Vital staining shows a great increase of reticulated red corpuscles, which reach 15 to 40 per cent of the total number. Moderate to marked leukocytosis is the rule with nearly normal differential count, and ordinarily a shift of Arneth's formula to the right; a few macrocytes may be present. There is much urobilin in urine and feces, showing excessive blood destruction. The acidity of gastric contents is low, but hydrochloric acid is not completely absent. For a complete bibliography of this interesting disease the student is referred to the report of Diggs.[1] Of interest are the observations on this disease by Sharp and Schleicher.[2] The method of study described by Beck and Hertz[3] is briefly as follows:

Let a drop of blood fall in a small tube containing 0.2 to 0.5 c.c. of a mixture of equal parts of physiologic salt solution and a 3 per cent solution of sodium citrate. Mix the blood thoroughly through the solution and add a sufficient quantity of paraffin oil to make a layer 1 cm. thick. Make sure no bubbles of air are under the oil. Let the preparation stand for twenty-four hours, then introduce 0.2 to 0.5 c.c. of 10 per cent solution of neutral formalin in physiologic salt solution. This is most easily prepared by adding 0.85 Gm. of sodium chloride to 100 c.c. of 10 per cent neutral formalin. Thoroughly mix by forcing the liquids in and out of the pipet several times. Place a few drops on a slide and cover with a cover glass. Examine the moist preparation for sickle cells.

Diggs and Pettit,[4] in comparing various methods, advocated as the most convenient the stasis method of Scriver and Waugh.[5] The steps of this method are illustrated in Fig. 166 (p. 314). They have found that when the preparations are made by this method the results may be read immediately; consequently, this is the method of choice for routine work.

8. Acute Aplastic Anemia.—There is a rare and rapidly fatal anemia of obscure etiology which is apparently the result of more or less complete failure of blood formation, the red bone marrow being

[1] Herrick, J. B.: Peculiar Elongated and Sickle-shaped Red Blood Corpuscles in a Case of Severe Anemia, Arch. Int. Med., *6*:517–521 (Nov.), 1910.

[1] Diggs, L. W.: The Blood Picture in Sickle Cell Anemia, South. Med. Jour., *25*:615–620 (June), 1932.

[2] Sharp, E. A., and Schleicher, E. M.: Hematologic Observations on Sickle Cell Anemia, Am. Jour. Clin. Path., *6*:580–590 (Nov.), 1936.

[3] Beck, J. S. P., and Hertz, C. S.: Standardizing Sickle Cell Method and Evidence of Sickle Cell Trait, Am. Jour. Clin. Path., *5*:325–332 (July), 1935.

[4] Diggs, L. W., and Pettit, V. D.: A Comparison of Methods Used in the Detection of the Sickle-Cell Trait. Jour. Lab. and Clin. Med. *25*:1106–1111 (July), 1940.

[5] Scriver, J. B., and Waugh, T. R.: Studies on a Case of Sickle-Cell Anaemia. Canad. M. A. Jour. *23*:375–380 (Sept.), 1930.

found at autopsy to have almost wholly disappeared, even from the flat bones and the bodies of the vertebrae. To this the name "aplastic anemia" is applicable. The failure appears to involve red blood corpuscles first, then leukocytes, and finally platelets.

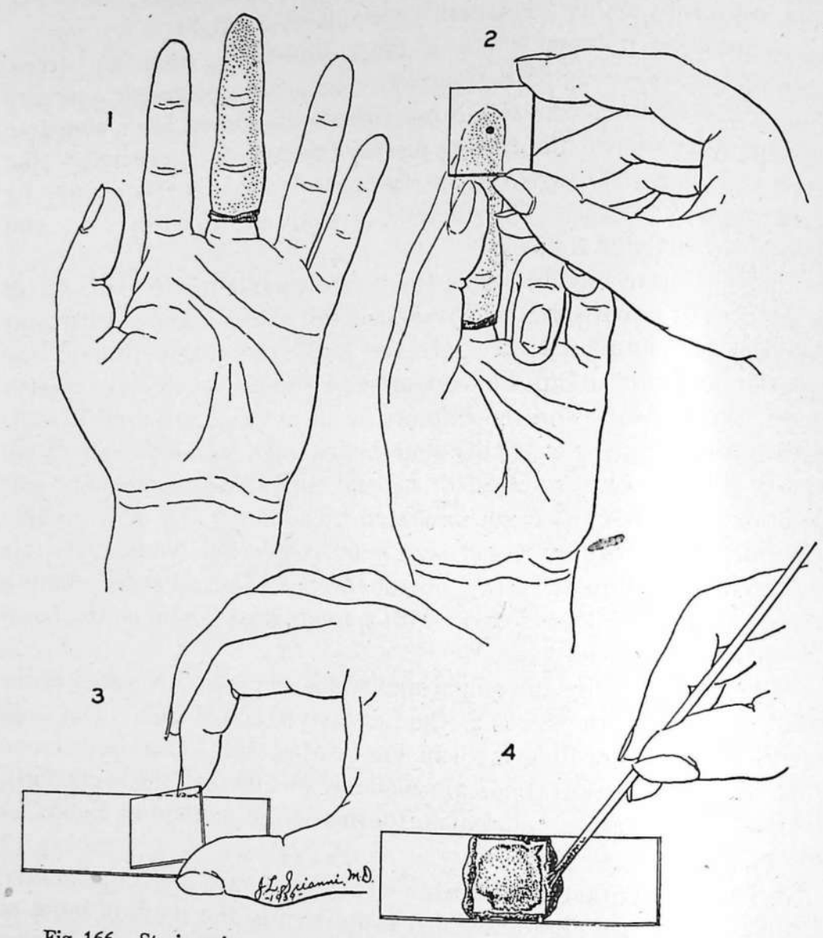


Fig. 166.—Stasis moist preparation for the detection of the sickle-cell trait. 1. Rubber band constriction of finger for five minutes. 2. Collection of drop of blood from finger. 3. Cover slip preparation. 4. Sealing preparation with petrolatum (Diggs and Pettit, J. Lab. and Clin. Med. Vol. 25, C. V. Mosby Co.).

The disease is most frequent between the ages of sixteen and thirty; its duration is only about two or three weeks. The red corpuscles sink to 1,000,000 for each cubic millimeter or less, and hemoglobin likewise falls rapidly. The leukocytes generally are low. Platelets are diminished, and when they are very low symptoms like those of purpura haemorrhagica appear.

In contrast to other conditions which give so low a red cell count, stained films show little that is abnormal unless some foci of active bone marrow remain, in which case a few normoblasts and other cells indicating attempted blood regeneration may be found.

Reticulated red cells are rare; resistance of the red cells to salt solutions of various strengths is about normal. Bleeding time and coagulation time are somewhat prolonged.

Aplasia of the bone marrow, with a similar blood picture, may occur secondarily in leukemia and other conditions.

9. Acute Hemolytic Anemia.—Lederer,[1] in 1925, described an acute anemia which is rare, but which is now recognized as a clinical entity. Giordano and Blum[2] presented an excellent review of the literature up to the time of reporting 3 cases of this disease. It usually affects children and young adults, and is characterized by an acute onset, severe *normochromic macrocytic* anemia with marked erythrocytic destruction, marked leukocytosis, or, at times, a leukemoid reaction, hyperbilirubinemia, often hemoglobinemia and marked regeneration of blood with reticulocytosis. The prognosis is grave, although there is a tendency to spontaneous recovery. Blood transfusion often brings about a spectacular and nearly immediate cure. This form of therapy may be the chief aid at times in the differential diagnosis of acute hemolytic anemia, pernicious anemia, and acute leukemia.

10. Paroxysmal Nocturnal Hemoglobinuria with Hemolytic Anemia (Marchiafava-Micheli Syndrome).—Another form of anemia is accompanied by paroxysmal nocturnal hemoglobinuria. This disease is characterized by macrocytic anemia, hypochromia, with corpuscles of normal shape. The fragility test is normal. There is a persistent reticulocytosis and a leukopenia with relative lymphocytosis and a moderate degree of thrombocytopenia. The sternal marrow shows hyperplastic erythropoietic elements. There is a recurring fever and a predisposition to thrombosis and a slight icterus. One of the most striking signs is nocturnal hemoglobinuria which is increased by an accumulation of carbon dioxide in the circulating blood. The disease terminates fatally. For a recent discussion of this syndrome and

[1] Lederer, Max: A Form of Acute Hemolytic Anemia probably of Infectious Origin, Am. Jour. Med. Sci., *170*:500–510 (Oct.), 1925. Three Additional Cases of Acute Hemolytic (Infectious) Anemia, Am. Jour. Med. Sci., *179*:228–236 (Feb.), 1930.

[2] Giordano, A. S., and Blum, L. L.: Acute Hemolytic Anemia (Lederer Type) Am. Jour. Med. Sci., *194*:311–326 (Sept.), 1937.

bibliography, see the article by Buell and Mettier.[1] See page 754 for a test for the demonstration of the hemolysis that occurs in this syndrome.

B. Polycythemia

Secondary increase of red corpuscles and hemoglobin due to chronic heart disease, concentration of blood in severe diarrheas, and other causes, is known as erythrocytosis, and has been discussed (p. 217). In addition, a rare "idiopathic polycythemia," possibly somewhat analogous to leukemia, is recognized, and is known as erythremia or polycythemia vera. There is marked hyperplasia of the red bone marrow, thought by some to be of the nature of a malignant tumor arising from the erythroblast, and the spleen is generally much enlarged. Patients exhibit a peculiar and striking cyanotic cast of countenance. Minot and Buckman have observed an apparent transition from erythremia to myelogenous leukemia in 3 cases, and believe that the two diseases are intimately related.

The red corpuscles of the circulating blood number 7,000,000 to 12,000,000. The highest counts recorded are 15,500,000 and 15,900,000. Hemoglobin ranges from 110 to 150 per cent; and the color index is moderately low. Macrocytes, microcytes, polychromatophilic red cells, and normoblasts may be met, but are not prominent. Moderate polymorphonuclear leukocytosis is the rule. The blood coagulates rapidly. As would be expected its viscosity is very high. The total volume of the blood is two to three times the normal. Resistance of red corpuscles to hypertonic salt solutions has been found increased when tested. R. J. Pickard has noted an increased resistance of the red cells to antihuman hemolytic amboceptor.

C. Hemoconcentration

In contradistinction to polycythemia and to a persistent erythrocytosis which is found in persons living in high altitudes, there occurs under certain conditions a rapid increase in the relative erythrocytic content of the blood. This is called hemoconcentration. Moon[2] has given a comprehensive review of the literature on the occurrence and clinical significance of this phenomenon. The simplest method of detecting hemoconcentration is the demonstration of a sudden increase in hemoglobin content. This may be confirmed by an increased

[1] Buell, Arthur, and Mettier, S. R.: Paroxysmal Nocturnal Hemoglobinuria with Hemolytic Anemia (Marchiafava-Micheli Syndrome). Jour. Lab. & Clin. Med. *26*:1434–1439 (June), 1941.

[2] Moon, V. H.: The Occurrence and Clinical Significance of Hemoconcentration. Ann. Int. Med. *13*:451–475 (Sept.), 1939.

erythrocyte count and by a comparative increase in the cellular volume as demonstrated by the hematocrit method. In cases of burns there is marked hemoconcentration as the fluid portion of the blood leaks into the tissues. Hemoglobin content has been found to be reduced after hemorrhage and increased during shock. In shock there is reduction in the plasma volume resulting in hemoconcentration. In anaphylactic shock the same hemoconcentration as is found in surgical shock has been noted by some investigators. Hemoconcentration occurs several hours before blood pressure sinks to critical levels. Studies of blood concentration made early may show a rising curve that will act as a warning signal of the more serious circulatory failure that will follow unless active supportive treatment is immediately carried out.

D. Leukemia

Two types of the disease are commonly distinguished. Atypical cases are not rare, especially in children. The disease is characterized by hyperplasia and overactivity of the leukoblastic bone marrow (myelogenous leukemia) or of the lymphoid tissue (lymphatic leukemia), together with overflow of many immature leukocytes, and excessive numbers of the adult normal types into the circulating blood. By some it is regarded as a neoplasm with metastases in the blood stream. An acute and a chronic form of each of the two types occur, although chronic cases are by far the more common. In general, the more acute the process the larger the proportion of very immature cells which reach the circulation.

1. Chronic Myelogenous Leukemia.—Owing to its insidious onset, the disease is rarely recognized until well established. By this time, except in rare instances, the diagnosis is easily made from the blood alone, usually at the first glance at the stained film. Not infrequently the existence of the disease is first revealed by a blood examination made with some other diagnosis in view. A significant clinical feature, which may be the first sign to arouse suspicion, is great enlargement of the spleen; because of this the name splenomyelogenous leukemia is sometimes used. The duration of the disease is variable; it is usually fatal within two to five years, but cases which were under observation for a much longer period are on record.

The most striking feature of the blood picture is an enormous increase in number of leukocytes, which is usually evident at the first examination. The leukocyte count in ordinary cases lies between 100,000 and 400,000 in each cubic millimeter, while counts over 1,000,000 have been met. In exceptional cases, upon the other hand, the counts may not rise above 50,000. The height of the count is not

necessarily an index of the severity of the disease. There is little tendency to progressive increase as the disease advances, and there may even be a decided fall in the count in the terminal stages. During spontaneous remissions, during intercurrent infections, and during treatment with *x*-ray or benzol, it may fall to normal.

While these enormous leukocyte counts are approached in no other disease except lymphatic leukemia, and a rare case of extremely high-grade neutrophilic leukocytosis, the diagnosis, particularly during remissions, depends more upon qualitative than quantitative leukocytic changes (Plate IX, Fig. 2). Although all varieties are increased, the characteristic and conspicuous cell is the myelocyte, and diagnosis cannot be made in its absence. This cell never appears in normal blood, rarely in leukocytosis or lymphatic leukemia, and only occasionally in pernicious anemia. In chronic myelogenous leukemia it usually constitutes more than 20 per cent of all the leukocytes. DaCosta's lowest case had 7 per cent. The neutrophilic form is generally much more abundant than the eosinophilic, which, however, is the more characteristic, as it occurs practically nowhere else. Both show considerable variation in size. Myeloblasts may be present in small or moderate numbers at any time, and in the terminal stage may be abundant. A marked increase in their number is of grave significance, indicating a tendency to a more acute course. Very constant, though not of much diagnostic significance, is a marked absolute, and often a relative increase of eosinophilic and basophilic leukocytes. Polymorphonuclear neutrophils are absolutely increased, although relatively decreased. Many atypical and degenerated leukocytes, which may be listed as "unidentified," may be found.

When the patient is first seen the red cell count may be normal with only slight deficiency of hemoglobin. Sooner or later, however, a definite anemia develops, the red cells generally falling below 3,500,000. Accurate estimation of hemoglobin is difficult in some cases, owing to the cloudiness produced by the great number of leukocytes. The color index is moderately low. In stained films the red cells show the usual changes seen in severe secondary anemia, excepting that nucleated red corpuscles are commonly very numerous. In fact, no other disease gives so many or offers so good an opportunity for their study. They are chiefly of the normoblastic type, and often all stages in their aging can be followed, from large polychromatophilic, immature forms which resemble megaloblasts to small orthochromatic forms with pyknotic nuclei. Mitotic figures are common. True megaloblasts are present in some cases, but it is often difficult to decide whether a given cell is a megaloblast or an exceptionally young

PLATE IX

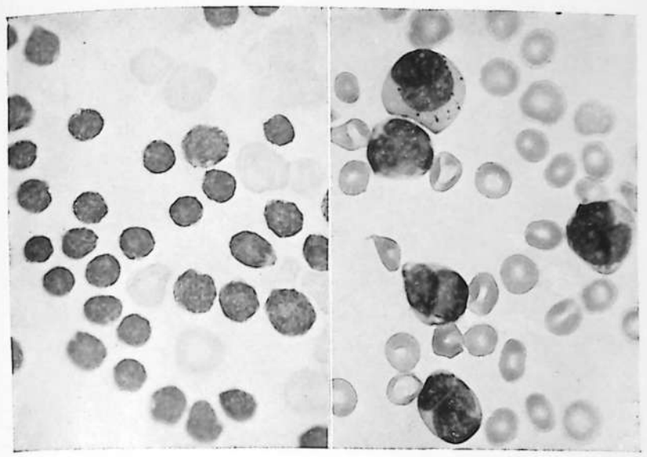


Fig. 1.—Blood in lymphatic leukemia; × 700. On the left, chronic form of the disease; on the right, acute form (courtesy of Dr. W. P. Harlow).

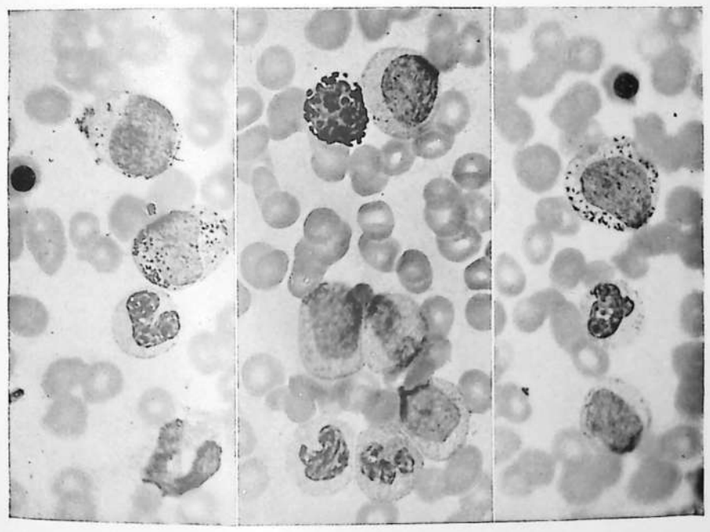


Fig. 2.—Blood in chronic myelogenous leukemia. Wright's stain; × 700.

normoblast. Blood platelets are variable, but generally greatly increased.

2. Acute Myelogenous Leukemia.—This is a rare form of leukemia which rapidly progresses to a fatal termination, usually within three to eight weeks. Prominent clinical symptoms are irregular fever, and a marked tendency to hemorrhages from gums and mucous membranes and into the skin. In some cases there is a throat condition closely resembling Vincent's angina, and containing the typical spirochete.

The leukocyte count is not so high as in chronic myelogenous leukemia, seldom exceeding 100,000 in each cubic millimeter; and it may be below 10,000. The predominating leukocyte is the most immature member of the bone marrow series, the myeloblast. Since, morphologically, this cell closely resembles the large lymphocyte of acute lymphatic leukemia, it may be impossible to distinguish between the two types of leukemia by the blood examination. In general, the peroxydase reaction (p. 282) will serve to identify the myeloblast, but in some cases the cells are so very immature that the test fails. In most cases it is best to be content with a diagnosis of "acute leukemia," leaving the question of the precise nature open until cells of a more mature type are found. When the case is of myelogenous origin, a sufficient number of myelocytes can usually be found to put one upon the right track. Eosinophilic myelocytes would, of course, be especially significant.

The accompanying anemia, which develops rapidly, is generally severe, the red cells sometimes falling to 1,000,000; and in some cases, when the leukocyte count is low, there may be difficulty in excluding pernicious anemia.

3. Chronic Lymphatic Leukemia.—The course of the disease extends over years, sometimes as many as ten. An outstanding feature in most cases is a generalized enlargement of the lymph nodes.

The leukocyte count is high, but less so than in chronic myelogenous leukemia. Counts of 100,000 are about the average, but in exceptional cases may go as high as 1,000,000. Upon the other hand, the count may be much lower, even as low as 15,000. Very rarely the leukocytes do not exceed the normal, and the name "aleukemic leukemia" or "leukopenic leukemia" may be applied. In such cases the diagnosis must be based upon the clinical findings, and the differential leukocyte count. Lymphocytes constitute 90 to 98 per cent of the total number (Plate IX, Fig. 1). As a rule, most of them resemble the small mature lymphocyte seen in normal blood, but large immature forms, like those in the blood of children, may be numerous in

some cases, while atypical forms with deeply indented nuclei are common. Very frequently the lymphocytes appear to be abnormally fragile, since many, even the majority, may be ruptured in thin films (Fig. 153). During remissions, and sometimes near the end, the leukocyte count may fall below normal, but the percentage of lymphocytes remains high. Occasionally a stray myelocyte is encountered.

The red cells and hemoglobin may stand at a high normal level for a long time, but sooner or later anemia develops, and, as a rule, is very marked. The color index is moderately low. The red cells show the usual changes of a severe secondary anemia. Erythroblasts are seldom abundant. Platelets are decreased in most cases.

4. Acute Lymphatic Leukemia.—This is rare, but is more frequently seen than is acute myelogenous leukemia, which it closely resembles in its symptomatology and clinical course. Even a study of the blood will not serve to distinguish the two in all cases, owing, as has been previously explained, to the close morphologic similarity between the lymphoblast and the myeloblast. In contrast to the chronic leukemias, which are diseases of adult life, acute leukemia is most frequent in childhood and youth.

As in all forms of leukemia the number of leukocytes varies in different cases, and fluctuates in the same case. The count may exceed 100,000 in each cubic millimeter in some cases; while in others it never goes above 15,000. At times it may fall below 5000 (leukopenic leukemia). Large, immature lymphocytes (lymphoblasts) predominate in the great majority of cases (Plate IX, Fig. 1), although there is usually a sufficient percentage of ordinary lymphocytes to prevent confusion with acute myelogenous leukemia. Many of the lymphocytes, especially the large ones, are atypical, the form with lobulated nucleus (Rieder's form) being especially frequent.

Red cells fall rapidly, even to 1,000,000 in each cubic millimeter; and there is great loss of hemoglobin with usually a low color index. Normoblasts may be found.

In addition to the forms of leukemia that have been described there are also some rare conditions, which are characterized by leukocytosis, and which should be mentioned.

Hay and Evans,[1] and others have described an **eosinophilic leukemia.** In these cases there is evidence of stimulation of bone marrow as revealed by the marked leukocytosis, and the high percentage of eosinophils. Intestinal parasites have never been found in these

[1] Hay, John, and Evans, W. H.: Acute Eosinophilic Leukemia and Eosinophilic Erythroleukemia, Quart. Jour. Med., *22*:167–189, 1929.

cases, and this cause for the eosinophilia is thus excluded. The disease usually progresses with an increase in the number of eosinophils, an increase in the splenomegaly, and glandular enlargement. This condition may be considered as a myelogenous leukemia, with the predominance of a single, mature type of cell. Eosinophilic leukemia is usually chronic, though an acute type of the disease has been reported.

Another rare disease, which is characterized by an abnormal increase in the number of leukocytes, is **monocytic leukemia.** Both acute and chronic forms have been reported. There is a marked increase in the percentage of monocytes. The names of Doan and Wisemann[1] are associated with the studies on the monocytes, and especially with the study of this condition. They expressed the opinion that the monocyte represents an independent entity, with a life cycle and function which are distinct from those of other leukocytes. Watkins and Hall[2] summarized the current opinions concerning two types of monocytic leukemia. The Schilling type is a variant of leukemic reticulo-endotheliosis in which there is a transformation of reticulo-endothelial cells into monocytes. On the other hand, it is probable that the so-called Naegeli type of monocytic leukemia is a variant of myelogenous leukemia. These same authors[3] also have reported an interesting case in which the blood picture changed from that of a typical myelogenous leukemia to one of monocytic leukemia of the Naegeli type.

A condition to be differentiated from acute leukemia is **infectious mononucleosis.** This disease is undoubtedly the "glandular fever" which was described by Pfeiffer[4] in 1889. It appears in epidemics, often among school children or older students, and frequently affects persons who live in the same house. It usually appears in the fall, winter, or spring months. The clinical characteristics are fever, adenopathy, abdominal pain, splenomegaly, and leukocytosis in which there is a preponderance of mononuclear cells. Longcope,[5] following the work of Cabot, Sprunt, and Evans, and others, studied 10 cases, and in his report in 1922 he used the term "infectious mononucleosis." Since then, this syndrome has become more generally recognized as a

[1] Doan, C. A., and Wisemann, B. K.: The Monocyte, Monocytosis, and Monocytic Leukosis: A Clinical and Pathological Study, Ann. Int. Med., *8*:383–416 (Oct.), 1934.

[2] Watkins, C. H., and Hall, B. E.: Monocytic Leukemia of the Naegeli and Schilling Types. Amer. Jour. Clin. Path. *10*:387–396 (June), 1940.

[3] Hall, B. E., and Watkins, C. H.: Myelogenous Leukemia Changing to Monocytic Leukemia: (report of a case). Amer. Jour. Clin. Path. *11*:443–459 (May), 1941.

[4] Pfeiffer, Emil: Drüsenfieber, Jahrb. f. Kinderh., *29*:257–267, 1889.

[5] Longcope, W. T.: Infectious Mononucleosis, Am. Jour. Med. Sci., *164*:781–808 (Dec.), 1922.

distinct entity. Downey and McKinlay[1] pointed out the characteristics which distinguish acute lymphadenosis, as they called it, from acute lymphatic leukemia. The leukocytosis is not so pronounced as in leukemia, but there may be from 20,000 to 40,000 leukocytes per cubic millimeter. The value for the mononuclear cells may be 60 per cent or more, and may be as high as 99 per cent. These may be ordinary lymphocytes, or there may be many larger cells, which have deeply staining nuclei and a nongranular cytoplasm, which are more or less filled with vacuoles, and which present a foamy appearance. The leukocytosis persists only a few days, and the mononuclear cells gradually disappear. Complications may arise, as in other acute infections, but the disease is self limited, and is very seldom fatal. An interesting confirmatory laboratory diagnostic procedure is the demonstration of a heterophile antibody in the blood of a patient at the height of an attack of infectious mononucleosis (see p. 755). The cause of this disease, which is apparently infectious, is unknown.

E. Leukopenia

Leukopenia as a finding in various diseases is discussed on page 238. There are somewhat rare conditions in which marked leukopenia is considered a most grave prognostic sign.

1. Agranulocytosis.—Schultz[2] described this condition, and stressed the septic sore throat which usually accompanies this disease. The first cases were called "agranulocytic angina." There is fever, severe prostration, and a marked reduction in the number of neutrophils. The relative lymphocyte count is high, but there is absolute leukopenia. In severe cases, the number of leukocytes may be less than 1000 per cubic millimeter, and the granulocytes may almost entirely disappear. The disease in this severe form is often fatal, though many cases of recovery have been reported. The condition has been classified as granulocytopenia, or agranulocytosis. Kastlin[3] presented a very good review of the literature up to 1927, and it was

[1] Downey, Hal, and McKinlay, C. A.: Acute Lymphadenosis Compared with Acute Lymphatic Leukemia, Arch. Int. Med., *32*:82–112 (July), 1923. These authors also thought that it should be differentiated from a highly infectious type of glandular fever occurring in children.

McKinlay, C. A.: Infectious Mononucleosis. Part I. Clinical Aspects, Jour. Am. Med. Assn., *105*:761–764 (Sept. 7), 1935.

Downey, Hal, and Stasney, Joseph: Infectious Mononucleosis. Part II. Hematologic Studies, Jour. Am. Med. Assn., *105*:764–768 (Sept. 7), 1935.

[2] Schultz, Werner: Ueber eigenartige Halserkrankungen, Deutsch. med. Wchnschr., *2*:1495–1496 (Nov.), 1922.

[3] Kastlin, G. J.: Agranulocytic Angina, Am. Jour. Med. Sci., *173*:799–813 (June), 1927.

felt at that time that the disease probably was not a distinct clinical entity, but rather the sign of a very severe infection which also affected the bone marrow. Since then, there have been many reports in the literature which indicate that the use of certain drugs is a very probable etiologic factor in producing this effect in the bone marrow. While the barbiturates have been mentioned, Kracke and Parker[1] in a review of the literature, and also by means of experimental work on rabbits, have presented circumstantial evidence that in susceptible individuals the disease may be produced by the use of certain benzene ring drugs. Amidopyrine, occasionally acetphenetidin, and dinitrophenol have been thus incriminated. These investigators further point out the large number of cases which have occurred among people of the medical group, nurses, doctors, and their families, who are prone to self-medication with these drugs. The agent which is directly responsible, according to their experimental work, would appear to be quinone, or perhaps pyrocatechin.

Bone marrow studies made by several hematologists indicated that in some cases of "malignant neutropenia" there was a proliferation of myeloblasts, a paucity of myelocytes, and nearly a complete absence of segmented forms. While hyperplasia was present there was apparently a deficiency of some specific factor necessary for maturation. This type may be a distinct entity which should be distinguished from agranulocytosis with aplasia and from leukocytic degeneration.

2. Hypoleukocytic Angina.—Rosenthal and Kugel[2] have described a new form of severe leukopenia. In this condition, which they called hypoleukocytic angina, the clinical syndrome is similar to that of agranulocytic angina. There is a decrease in the number of leukocytes, but there is a high percentage of nonsegmented neutrophils which reveal toxic granules.

F. Hemorrhagic Diseases

A marked tendency to hemorrhage from the mucous membranes and into the tissues and skin, with the formation of ecchymoses, is a prominent feature of a number of conditions. In some the hemorrhagic tendency is manifestly a symptom of a recognized disease. In another group, including purpura haemorrhagica, hemophilia, and melena neonatorum, the bleeding tendency appears to be the primary feature of the disease. The immediate cause in both groups appears

[1] Kracke, R. K., and Parker, F. P.: The Etiology of Granulopenia with Particular Reference to Drugs Containing the Benzene Ring, Am. Jour. Clin. Path., *4*:353–469 (Nov.), 1934.

[2] Rosenthal, Nathan, and Kugel, M. A.: Hypoleukocytic Angina, an Unusual Form of Infectious Leukopenia, Jour. Lab. and Clin. Med., *19*:344–349 (Jan.), 1934.

to be a disturbance of the clotting mechanism, whereby the coagulation time is prolonged or the clot is imperfectly formed. The causes underlying the defective clotting are not fully understood, but differ in the different diseases. There may be: (*a*) Deficiency in number of platelets, leading to the formation of a nonretractile clot, as in purpura haemorrhagica, aplastic anemia, and lymphatic leukemia; (*b*) alteration of their function, leading to deficiency of available prothrombin, as in hemophilia, melena neonatorum, and aplastic anemia; (*c*) deficiency in amount of fibrinogen, as in cirrhosis, and some acute destructive diseases of the liver with hemorrhagic symptoms; (*d*) lack of available calcium, as in obstructive jaundice, in which the blood calcium appears to be bound by the bile pigment, or (*e*) excess of antithrombin, which may be a factor in hemorrhagic septicemia and other conditions.

A hemorrhagic tendency may also result from weakness of the small vessels and from emboli. The *Rumpel-Leede phenomenon* may indicate weakness of small vessel walls. Apply a tourniquet not too tightly about the upper arm. In ten minutes time multiple petechiae may appear below the constriction in certain hemorrhagic diatheses. If the small vessels are normal, there will be few or no minute subcutaneous hemorrhages.

Only the primary hemorrhagic diseases will be taken up in detail.

1. Purpura Haemorrhagica.—The most striking and significant feature is an enormous decrease in number of blood platelets. They range between 40,000 and 75,000 in each cubic millimeter in mild cases, and are reduced to 15,000 or even 10,000 in severe cases. The cause of the platelet loss is obscure and probably varies in different cases. Coagulation time is usually normal or nearly so, but the clot is soft and lacks retractility (p. 199). The bleeding time is very much prolonged, generally an hour, sometimes longer, and furnishes a direct index of the tendency to bleed. In some cases at least there may be abnormalities in other coagulation factors, and there is practically always toxic injury of the capillary endothelium which makes these vessels very fragile.

The severe anemia which develops is due to the loss of blood and differs from ordinary posthemorrhagic anemia chiefly in the number of platelets, which is increased in the latter condition. In general there is slight or moderate neutrophilic leukocytosis.

2. Hemophilia (*Bleeder's Disease*).—This is an interesting sex-linked hereditary tendency to hemorrhage confined to males, but transmitted by females who do not themselves show any tendency to abnormal bleeding. There are, however, many cases in which hereditary transmission cannot be established. The cause of the disease is

apparently a congenital abnormality of the blood platelets which are normal in number, but physiologically defective.[1]

Howell and Cekada[2] showed that the platelets are more resistant than normal, and, contrary to the opinion formerly held, there is no change in the quantity or quality of the prothrombin. Nevertheless the so-called "prothrombin time" is five to twenty-five times the normal. This may be due to the slowness with which thromboplastin is liberated from the platelets. The coagulation time, when blood is taken from a vein, is about five to fifteen times the normal, that is, from one to five hours or longer. When blood is secured from a skin puncture, the coagulation-accelerating substances of the tissue juice may reduce the coagulation time nearly or quite to normal. When once formed, the clot is firm and has normal retractile power. The bleeding time by Duke's method (p. 204) is usually normal, which at first sight seems inconsistent with the well-known tendency of hemophiliacs to bleed from wounds. The difference apparently lies in the opportunity for admixture with tissue juice, which is relatively less in the case of large wounds or wounds of the mucous membranes.

Quick[3] stated that in a true case of hemophilia the prothrombin time by his method (p. 201) should be practically normal. Centrifugation of samples of oxalated blood at both high and low speeds yields plasma which differs in coagulation time on recalcification. The Rumpel-Leede phenomenon (p. 324) should not be pronounced.

3. Melena Neonatorum.—The condition is not well understood. In some cases, at least, the platelets have not been reduced and the bleeding time has been only slightly prolonged, but prothrombin time and coagulation time have been delayed.

Quick's[4] *classification of hemorrhagic diseases due to defects in the coagulation mechanism of the blood.*

I. **Diminished prothrombin** (prothrombinopenia)

(*A*) Lack of Vitamin K

(*a*) Dietary origin—absence of bacteria in intestines: (1) hemorrhagic disease of the newborn.

(*b*) Faulty absorption—lack of bile salts. (1) obstructive jaundice; (2) biliary fistula; (3) sprue.

[1] Minot, G. R., and Lee, R. I.: The Blood Platelets in Hemophilia, Arch. Int. Med., *18*:474–495 (Oct.), 1916.

[2] Howell, W. H., and Cekada, E. B.: The Cause of the Delayed Clotting of Hemophilic Blood, Am. Jour. Physiol., *78*:500–511 (Nov.), 1926.

[3] Quick, A. J.: The Diagnosis of Hemophilia. Amer. Jour. Med. Sci. *201*:469–474 (Apr.), 1941.

[4] Quick, A. J.: A Classification of Hemorrhagic Diseases Due to Defects in the Coagulation Mechanism of the Blood. Amer. Jour. Med. Sci. *199*:118–132 (Jan.), 1940.

(*B*) Liver Damage—Faulty Utilization of Vitamin K
- (*a*) Post-anesthesia liver damage
- (*b*) Chloroform poisoning
- (*c*) Acute yellow atrophy

(*C*) Toxins
- (*a*) Toxic sweet clover disease of cattle

II. **Changes in concentration of calcium in blood** (No clinical or experimental hemorrhagic conditions known which are attributable to changes of the calcium concentration.)

III. **Deficiency of thromboplastin** (thromboplastinopenia)

(*A*) Diminished Number of Platelets
- (*a*) Thrombocytopenia (this disease is best classified under "defective vascular response" since the vascular dysfunction predominates)

(*B*) Increased Resistance of Platelets
- (*a*) Hemophilia

IV. **Decreased fibrinogen** (fibrinogenopenia)

(*A*) Acquired Fibrinogenopenia
- (*a*) Nutritional deficiencies
- (*b*) Diseases of blood forming organs
- (*c*) Severe liver damage
- (*d*) Snake bites (Black snake of Australia)

(*B*) Congenital

V. **Anticoagulants in the blood**

(*A*) Liberation of Heparin into the Blood
- (*a*) Peptone shock
- (*b*) Anaphylactic shock

Quick, using this classification and the Morawitz theory for coagulation, stated that a prolonged coagulation time by the Lee and White method may be due to diminished prothrombin, thromboplastin or the presence in the blood of an anticoagulant such as heparin. Fibrinogen is present if the blood ultimately clots.

G. Diseases Due to Blood Parasites

The discussion of bacteria that may be found in the blood is included in the chapter on Bacteriologic Methods. Five of the animal parasites, which have been found in the blood, are of clinical interest. These are: (1) *Borrelia recurrentis*, the spirochete of relapsing fever; (2) trypanosomes; (3) the parasites of malaria; (4) filarial larvae; and (5) larvae of *Trichinella spiralis*. These parasites are described in the chapter on Animal Parasites.

XIII. MISCELLANEOUS METHODS

1. Fragility of Red Corpuscles (Resistance to Hypotonic Salt Solution).—Destruction of red corpuscles goes on continually within the body. In certain pathologic conditions this destruction is greatly accelerated, leading usually to anemia; and it is then of great practical interest to ascertain whether the excessive hemolysis is referable chiefly to increased fragility of the red cells, as is typically the case in hemolytic jaundice, or to an excessively toxic hemolytic agent acting upon red cells of comparatively normal resisting power, as is usual in pernicious anemia. The resistance of the red cells can be measured by subjecting them to the action of various harmful agents. In clinical work hypotonic salt solution is generally used.

Method of Sanford.—1. Prepare a stock solution containing 0.5 Gm. chemically pure and freshly dried sodium chloride in 100 c.c. of distilled water. Weigh on a delicate balance and measure in a volumetric flask.

2. Arrange a series of 12 small test tubes in a rack and number them 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, and 14.

3. With a capillary pipet place in each tube the number of drops of the stock 0.5 per cent salt solution indicated by the number on the tube. To insure equality in size of drops the pipet must always be held at the same angle.

4. With the same pipet add to each tube the number of drops of distilled water required to bring the volume in each tube to 25 drops. Mix well. The percentage strength of the salt in any tube may then be found by multiplying its number by 0.02.

5. Obtain 1 or 1.5 c.c. of the patient's blood from a vein with a small dry sterile syringe and No. 21 needle, and immediately expel 1 drop into each of the tubes. Mix by inverting (Fig. 167).

If some time must elapse before the blood can be added, it may be mixed with citrated salt solution and the corpuscles washed with 0.7 per cent salt solution before use. In this case make a 50 per cent suspension of the cells and add 1 drop to each of the tubes in the rack.

6. Prepare a similar set of tubes, using the blood of a normal person as a control. *This should not be omitted.* The normal control serves as a test of the accuracy of the salt solution, and at the same time gives a definite standard for the interpretation of slight changes in fragility.

7. Let the tubes stand two hours at room temperature. At the end of

that time the corpuscles will have settled to the bottom and hemolysis may be recognized by the color of the supernatant fluid: Faintly pink if hemolysis is partial ("initial hemolysis"); red, with little or no sediment, if it is complete.

With normal blood, hemolysis usually begins in the tube containing 0.44 or 0.42 per cent salt solution and is complete in that containing 0.34 per cent. When a control is used a variation of 0.02 or 0.04 may be considered quite definite. Sanford found the average figures for initial and complete hemolysis in 23 cases of hemolytic jaundice to be 0.478 and 0.413 respectively; in chronic obstructive jaundice, 0.396 and 0.31. In secondary and pernicious anemia the figures vary only slightly from the normal, with a tendency to slight increase of resistance. In purpura resistance is normal.

2. Viscosity.—It is evident that variations in the viscosity of the blood must markedly influence the load carried by the heart, but

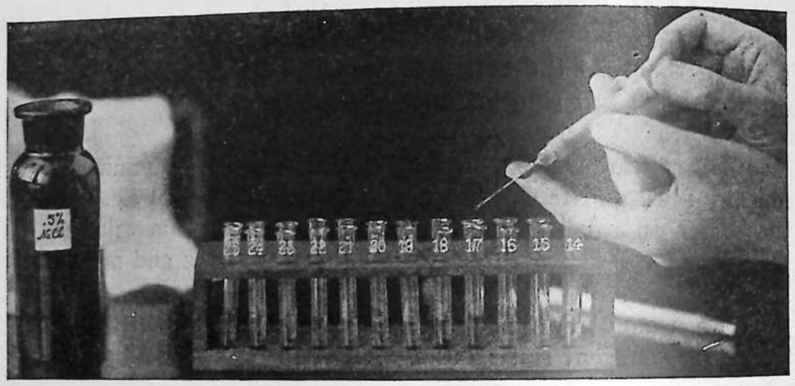


Fig. 167.—Set-up for fragility test; adding the blood.

viscosity estimations have proved of comparatively little value. The greatest field would seem to be in suggesting need for treatment when high viscosity is throwing an excessive burden upon an already weakened heart.

Compared with distilled water, the normal viscosity is about 4.5. It is reduced in primary and secondary anemia (roughly proportional to the grade of anemia), nephritis, cardiac lesions with edema, and usually in leukemia and malaria. It is increased in polycythemia, diabetes mellitus, icterus, and usually in pneumonia. Profuse sweating without an opportunity to replace the water lost appears to raise viscosity by about 25 to 30 per cent. Measurement of viscosity is comparatively simple if one has a suitable instrument. The Hess instrument is one of the best and is accompanied by directions for use (Fig. 168).

3. Sedimentation Speed of Red Corpuscles.—An increased tendency to sedimentation of red corpuscles in shed blood in certain pathologic conditions, particularly inflammation, has long been recognized as an interesting phenomenon explaining the well-known "buffy-coat" of coagulated blood. Within the last few years, following the work of Fahraeus, and others, with blood which has been rendered noncoagulable by heparin, citrate, or oxalate, the rate of sedimentation has been actively studied and applied clinically.

It has been found that the corpuscles settle more rapidly in the blood of women than of men, and very much more rapidly in pregnancy after the third or fourth month. Increased speed of sedimentation is also seen in tuberculosis, where it increases with the activity of the disease; in cancer, where it more or less closely parallels the degree of malignancy; and in localized acute inflammations, where the rate appears to increase with the leukocyte count.

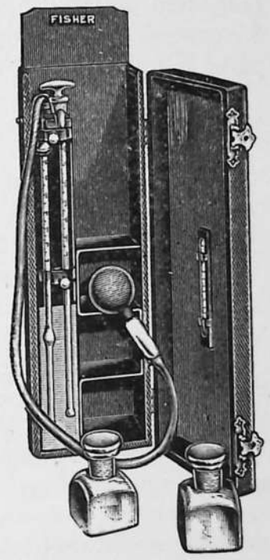


Fig. 168.—New model Hess viscosimeter.

The cause of the phenomenon is not clear. It is apparently connected with the ratio of albumin, globulin, and fibrinogen in the plasma, or with the concentration of cholesterol. Yardumian[1] reported on the physicochemical factors influencing the sedimentation rate, and concluded that it is inconceivable that a phenomenon influenced by many variable factors should be of appreciable diagnostic and prognostic value.

Since a variety of methods have been used the figures of different workers are not comparable. Some record the time required for the corpuscles to settle to an arbitrarily fixed point. Others note the height of the corpuscular layer after the blood has stood for a definite time, or make several readings at stated intervals and plot a curve. The height of the column of blood is an important factor as is also the size of the bore of the sedimentation tube. It is very important that the tube be kept as vertical as possible, as a slight inclination hastens sedimentation. Temperature also affects the rate, the optimum being between 22° and 27° C. It has been demonstrated repeatedly that the concentration of the erythrocytes in the blood is a very important

[1] Yardumian, K.: Physicochemical Factors Influencing the Red Cell Sedimentation Rate, Am. Jour. Clin. Path., 7:105–119 (Mar.), 1937.

factor in the sedimentation rate, and a corrected estimation should be made in anemia. Wintrobe and Landsberg[1] have made a critical review of sedimentation methods, have investigated the factors affecting the rate, and have developed a simple, satisfactory method.

Determination of Sedimentation Rate (Wintrobe and Landsberg Method).—This method requires a special hematocrit tube, which is illustrated in Fig. 169. This tube is 110 mm. long, has a uniform bore of 3 mm., and has a flat bottom. It is graduated on one side from 0 to 10 cm. in 1 mm. divisions with white numerals, and on the other side it is graduated from 10 cm. to 0 in 1 mm. divisions with red numerals. The only *reagent* is the anticoagulant, which is a mixture of dry ammonium oxalate and potassium oxalate (6 mg. of the former and 4 mg. of the latter for each 5 c.c. of blood). The tubes in which the blood is to be placed may be prepared in advance by placing in them the proper amount of the oxalates in solution, and then evaporating the water by placing the tubes in a hot-air oven.

Fig. 169.—Wintrobe hematocrit tube.

Method.—1. Withdraw 5 c.c. of venous blood in a dry syringe and place in a tube or bottle containing the anticoagulant described above.

2. With a capillary pipet, fill a Wintrobe hematocrit tube to the 10 cm. mark.

3. Place the filled tube in an exactly vertical position, at room temperature, and observe the point on the mm. scale to which the corpuscles fall during exactly one hour.

4. Centrifugalize the tube until packing of erythrocytes is complete; then read the volume of packed erythrocytes.

5. Correct the rate according to the volume of cells by referring to the chart for correction (Fig. 170).

Find the horizontal line which represents the sedimentation in millimeters for one hour. Follow this across the chart until it intersects the vertical line which represents the blood cells volume per cent. Follow the nearest curved line until it intersects the heavy line at 42 c.c. per 100 c.c., if the patient is a woman, or the line at 47 per cent if the patient is a man. Then, at the point of intersection, read the value on the horizontal line for the corrected sedimentation rate. The normal average sedimentation in one hour by this method is 3.7 mm. for healthy men, and 9.6 mm. for women, with a maximal range from 0 to 9 mm. for men, and 0 to 20 mm. for women.

The normal figures given here are not to be correlated with those which have been obtained with other methods.

[1] Wintrobe, M. M., and Landsberg, J. W.: A Standardized Technique for the Blood Sedimentation Test Am. Jour. Med. Sci., *189:*102–115 (Jan.), 1935.

Westergren Method.—Because of its simplicity the Westergren[1] method is widely used, and the following technic has been found to be satisfactory. The *apparatus* required is a Westergren tube, and rack (Fig. 171). It is also convenient to use a tube with a mark indicating 5 c.c. This may be a graduated centrifuge tube, or a plain test tube with a mark etched on it at the 5 c.c. level. The only *reagent* is a 3.8 per cent solution of sodium citrate.

Method.—1. Transfer with a graduated pipet exactly 0.5 c.c. of the 3.8 per cent solution of sodium citrate to a tube with the mark at the 5 c.c. level.

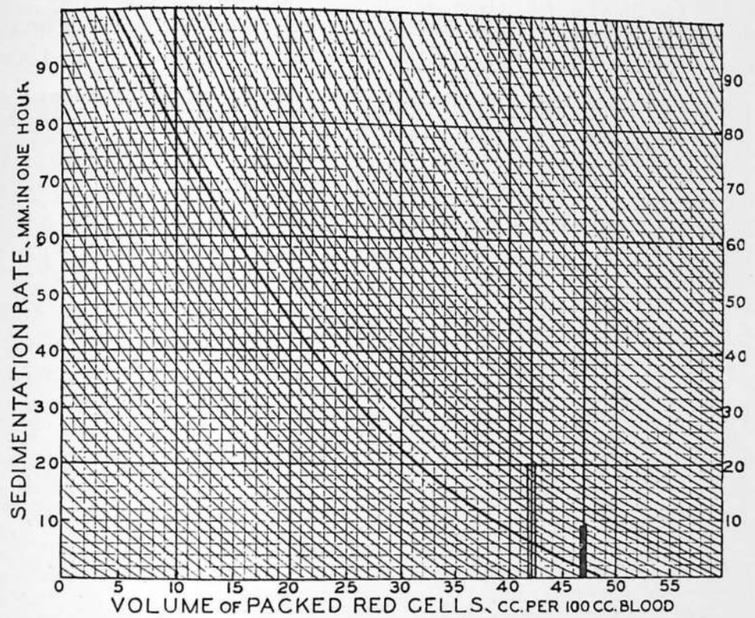


Fig. 170.—Chart for correcting sedimentation rate for variations resulting from differences in the concentration of red corpuscles as measured by volume of packed red cells. The logarithmic curve on which the chart is based is heavily outlined. The mean normal volume of packed red cells for men (47 c.c.) and for women (42 c.c.) is also heavily outlined and the range of normal sedimentation is represented by solid and open columns for each sex, respectively. (Wintrobe and Landsberg, in Am. Jour. Med. Sci., January, 1935, Lea and Febiger, Publishers.)

2. Withdraw 5 c.c. of venous blood in a dry syringe, and place exactly 4.5 c.c. of it in the tube containing the anticoagulant. The tube, now filled to the 5 c.c. mark, is inverted two or three times to mix thoroughly the anticoagulant with the blood.

3. Fill a Westergren tube exactly to the 0 mark and place it in the rack. The bottom of the tube must be pressed firmly against the rubber stopper in the base of the rack before removing the finger from the top of the tube.

[1] Westergren, Alf: Studies of the Suspension Stability of the Blood in Pulmonary Tuberculosis, Acta med. Scand., *54:*247–282, 1921.

The tube must be held firmly by the clip at the top of the rack in an *exactly vertical* position. The rack is constructed to hold twelve or more tubes.

4. Read the fall of the corpuscles in millimeters in exactly sixty minutes. It may be of interest also to note the fall in thirty minutes.

With this method the normal sedimentation rate of men's erythrocytes is 0 to 15 mm. in one hour. The rate for healthy women is 0 to 20 mm. in one hour.

It would seem best to report the results by any method merely as "Normal," "Fast," or "Very Fast."

4. Blood and Plasma Volume.—There have been many methods devised for the estimation of the total blood volume. The classical

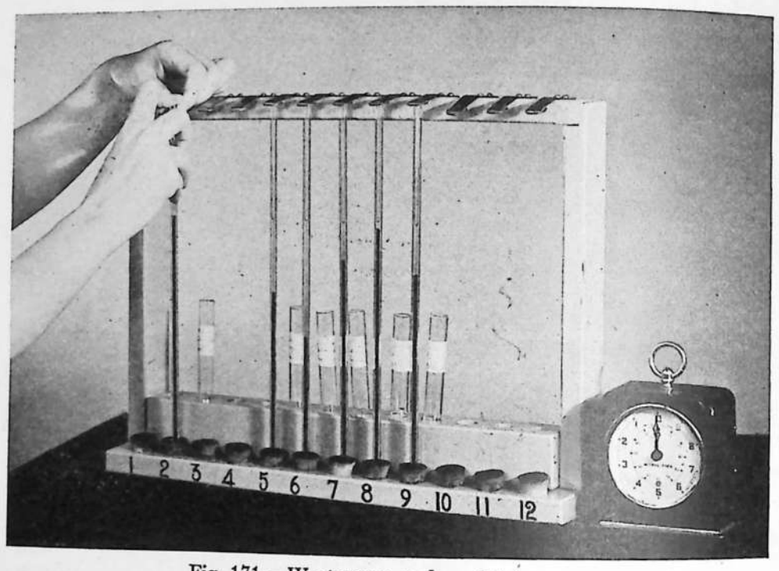


Fig. 171.—Westergren rack and tubes.

experiments of Welcker giving one thirteenth of the body weight as the traditional figure for blood volume cannot, of course, be applied clinically. It also seems that this figure is too low, and that the newer dye methods would indicate that the value is more nearly one eleventh of the body weight. The range in normal subjects is from 72 to 100 c.c. of blood and from 43 to 59 c.c. of plasma for each kilogram of body weight.

Method for the Determination of Plasma and Blood Volume.[1]—A known amount of slowly excretable dye is injected into an unknown amount

[1] Keith, N. M., Rowntree, L. G., and Geraghty, J. T.: A Method for the Determination of Plasma and Blood Volume, Arch. Int. Med., *16*:547–576 (Oct.), 1915. Rowntree, L. G., and Brown, G. E.: The Volume of the Blood and Plasma in Health and Disease, Philadelphia, W. B. Saunders Co., 1929, 219 pp.

of fluid and its dilution determined. It is estimated that there is approximately 50 c.c. of plasma for each kilogram of body weight. Therefore by multiplying 50 c.c. by the number of kilograms of the patient's body weight the theoretic plasma volume is first determined. By dividing this theoretic volume by 200 (a factor determined by experiment) the number of cubic centimeters of the solution of the dye to be injected is determined.

Materials and Stock Solutions Used.—1. Dissolve 375 mg. of biologically tested Congo red in 25 c.c. of fresh, triple distilled water. Heat to boiling to sterilize and cool rapidly before injecting. This makes a 1.5 per cent solution. Although Congo red is usually nontoxic, every fresh bottle should be tested by injecting a solution of the dye intravenously into a dog. If the solution is toxic it should, of course, be discarded.

2. Use a calibrated Record syringe. Sterilize by boiling for ten minutes in distilled water, wrap the barrel and plunger in a sterile towel and dry in a hot-air oven which has been heated to 110° C., but in which the heat has been turned off. Such syringes may be prepared in advance and kept wrapped in sterile towels. Sterilize needles by placing them in small stoppered test tubes and baking in a hot-air oven at 180° C.

3. Prepare a solution of 1.1 per cent sodium oxalate as an anticoagulant.[1]

4. For each test use four 15-c.c. calibrated centrifuge tubes or, preferably, the volume index tubes described on page 231.

Procedure.—1. In each of the four centrifuge tubes place exactly 1 c.c. of 1.1 per cent sodium oxalate solution, measured with a 1-c.c. pipet.

2. Weigh the patient, and calculate the number of cubic centimeters of dye to be injected by using the formula:

$$\frac{50 \times \text{wt. in kg.}}{200} = \text{number of cubic centimeters to be injected.}$$

3. Insert a needle in the vein of one arm and take at least 10 c.c. of blood in the usual manner for venipuncture. Place exactly 5 c.c. of blood in each of two centrifuge tubes containing 1 c.c. of oxalate solution, for standard plasma color. Without removing the needle from the vein, change syringes and inject the proper amount of dye with a sterile, calibrated Record syringe.

4. In three minutes withdraw 10 c.c. of blood from the opposite arm, using a clean, dry syringe, and place exactly 5 c.c. in each of two remaining centrifuge tubes.

5. Centrifugalize all four tubes for thirty minutes in a high-speed centrifuge (2500–3000 revolutions per minute); take the hematocrit reading and calculate the ratio of red blood cell volume to total volume. For example, if the red blood cell volume equals 2 c.c., the ratio equals two fifths or 40 per cent.

[1] The strength of the sodium oxalate solution was originally 1.6 per cent. It has been found, however, that 1.1 per cent oxalate solutions are more nearly isotonic. See Graff, Samuel, and Clarke, H. T.: Determination of Plasma Volume, Arch. Int. Med., *48*:808–820 (Nov.), 1931.

6. Prepare the standard and unknown for colorimetric comparison.

(*a*) To prepare the standard mix 2 c.c. of plasma without dye, 2 c.c. of 1/200 dilution of the same Congo red that has been used for injection, and 4 c.c. of physiologic sodium chloride solution (0.85 per cent).

(*b*) To prepare the unknown plasma, mix 2 c.c. of plasma containing the injected dye, and 6 c.c. of physiologic sodium chloride solution. Set the colorimeter cup containing standard at 10 and compare (*b*) with (*a*).

Calculation.—1. Calculate the total amount of oxalated plasma by subtracting the number of cubic centimeters of erythrocytes from 6, or from the exact reading of the meniscus level. Determine a factor for the correction of the dilution by the oxalate solution by subtracting 1 (the quantity of the oxalate solution) from the total quantity of oxalated plasma and then dividing the resulting number by the total number of cubic centimeters of oxalated plasma. For example, if the erythrocyte volume equals 2 c.c., and the meniscus level equals 6 c.c., then 6 minus 2 equals 4 c.c., the total amount of oxalated plasma, and 4 c.c. minus 1 c.c. equals 3 c.c. Thus the dilution factor is three fourths or 0.75.

2. Determine the percentage of the unknown expressed in per cent of the standard by dividing the reading of the standard, 10, by the reading of the unknown.

3. Determine the plasma percentage by subtracting the erythrocyte per cent from 100.

4. *Formula:*

$$\frac{\text{Number of cubic centimeters of dye injected} \times 200 \times 100 \times \text{dilution factor}}{\text{Per cent of unknown expressed in per cent of standard}} = \text{total plasma volume in cubic centimeters.}$$

$$\frac{\text{Total plasma volume} \times 100}{\text{Plasma per cent}} = \text{whole blood volume in cubic centimeters.}$$

Divide the total plasma volume, and also the whole blood volume by the patient's weight in kilograms to determine the plasma volume for each kilogram and the whole blood volume for each kilogram.

It should be stated that in **amyloidosis** Congo red rapidly disappears from the blood. In fact, the rapid disappearance from the blood makes the above technic for the injection of this dye also usable for a valuable test for the diagnosis of amyloid disease. In this condition 90 per cent of the dye injected will be gone from the blood in one hour in comparison to the 15 per cent that is ordinarily removed.

Paunz Test for Amyloidosis.—Paunz[1] has so modified the Congo red test that it becomes a simple qualitative test for the diagnosis of amyloidosis. Make a 0.6 per cent solution of Congo red (Grübler) and sterilize by boiling. Inject intravenously 10 c.c. for each 50 Kg. of body weight,

[1] Paunz, L.: Diagnosis of Amyloidosis by Means of Congo Red. Magyar Orvosi Archivum, *25*:448–456, 1924. Chem. Abs., *19*:1009 (Mar.), 1925. Németh, L.: Ueber den klinischen Wert des Nachweises der Amyloidose durch die Kongorotprobe, Klin. Wchnschr., *5*:1040–1041 (June 4), 1926.

or use the proportionate dose of the 1.5 per cent solution which is used in the blood volume test. At the end of one hour withdraw the blood, centrifugalize, and remove the serum. Add a few drops of hydrochloric acid and note the color. The presence of Congo red in the serum is shown by a bluish coloring of the coagulated proteins. This is the normal negative reaction. *If there is no bluish color after the addition of hydrochloric acid the test is positive for amyloidosis.*

XIV. THE BLOOD GROUPS

Untoward results which sometimes follow transfusion of blood are now known to be due in most instances to hemolysis or agglutination of either the donor's or recipient's blood corpuscles, or both. By a simple test, it is possible to ascertain whether the blood of any individual is suitable in this respect for transfusion into the veins of a given patient. This is known as "matching bloods," and it should always be done when transfusion is contemplated. The two factors to be considered are hemolysis and agglutination; but since hemolysis does not occur without agglutination, it is sufficient in practice to test for agglutination only. However, it should be kept in mind that hemolysis may mask agglutination and lead to error. Blood matching is also important in selecting donors for skin grafts.

There are two factors involved in isohemagglutination, an agglutinin in the plasma and an agglutinogen in the corpuscles which renders them agglutinable. It is an interesting fact that in respect to the presence or absence of these, every adult falls into one of several definite groups. For many years four groups, designated for convenience, I, II, III, and IV, have been recognized. To explain this grouping it is necessary to assume the existence of two separate agglutinins *a* and *b*, and the corresponding agglutinogens, *A* and *B*. A blood with a given agglutinin will agglutinate corpuscles which contain the corresponding agglutinogen, and will not affect other corpuscles. The four groups and their interrelationships are well shown by the simple chart devised by Sanford (Fig. 172). The figures there set down for the percentage of individuals in the different groups are those of Moss and are only approximately correct. There are probably more group B individuals than group AB. They vary somewhat with different races.[1]

While all individuals can be placed in one of the four groups, it has been shown by a number of observers that there are subgroups

[1] Hirschfeld, L., and Hirschfeld, H.: Serological Differences Between the Blood of Different Races, Lancet, *2*:675–679 (Oct.), 1919. Ottenberg, Reuben: A Classification of Human Races Based on Geographic Distribution of the Blood Groups, Jour. Am. Med. Assn., *84*:1393–1395 (May), 1925.

also, evidenced by the fact that occasionally blood is found with an agglutinin in its serum that acts on the corpuscles of the blood of certain individuals that are really in the same group. It has now been definitely established that group A can be subdivided into group A_1 or group A_2, and also group AB may be subdivided into A_1B and A_2B. While subgroups are interesting and may be used very rarely in medicolegal cases, there is probably not as much importance to be attached to the determining of subgroups as formerly was thought. Wiener, Oremland, Hyman and Samwick[1] stated that probably no case has yet been reported in which the transfusion of blood from a group A_1 individual into an A_2 patient, or vice versa, has proved to be responsible for a severe or fatal hemolytic reaction. For the methods of determining subgroups, the reader is referred to an article by Davidsohn.[2]

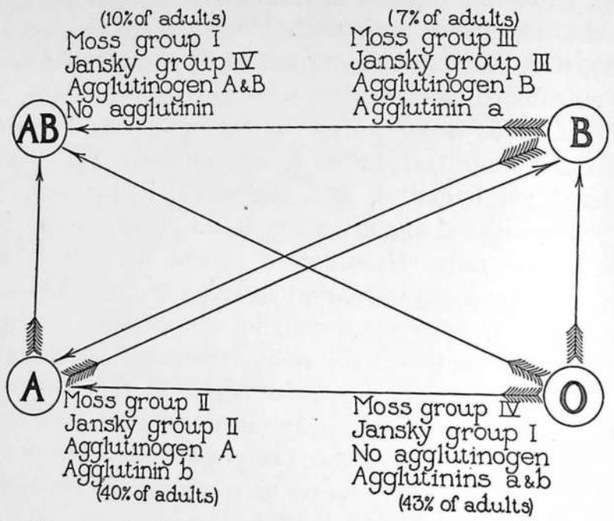


Fig. 172.—Diagram showing the interrelation of the four iso-agglutination groups. The serum of any group will agglutinate the corpuscles of those groups toward which its arrows point. Thus, serum of an individual belonging to Group O will agglutinate red corpuscles belonging to any other group, while the serum of Group AB lacks agglutinating power. Several subgroups have been discovered. The percentages are only approximately correct. Some studies give more B than AB individuals.

[1] Wiener, A. S., Oremland, B. H., Hyman, M. A., and Samwick, A. A.: Transfusion Reactions; Experiences with More Than Three Thousand Blood Transfusions. Amer. Jour. Clin. Path. *11*:102–121 (Feb.), 1941.

[2] Davidsohn, Israel: A Method for Recognition of Blood Subgroups A_1 and A_2 as a Means of Avoiding Transfusion Reactions. Jour. Am. Med. Assn. *112*:713–718 (Feb. 25), 1939.

The nomenclature of the four standard groups which is followed in this book is that of Landsteiner. Jansky's system, which *interchanges Moss Groups I and IV*, was at one time approved on grounds of priority by a joint committee of the American Association of Immunologists, the Society of American Bacteriologists, and the Association of Pathologists and Bacteriologists. The Moss system was, however, adopted by the American Society of Clinical Pathologists. The use of two systems side by side would be deplorable. It now seems best to name the groups by their agglutinogens: AB, A, B, and O for the Moss groups I, II, III, and IV, respectively, using the terms proposed by Landsteiner, the discoverer of blood groups.

The group to which an individual belongs is an inherited characteristic which appears to follow Mendel's law. The corpuscles show the group characteristic as a rule well developed at birth, except for subgroups A_2 and A_2B in which the agglutinogens A_2 may be weak at birth and may be difficult to detect.

Grouping may in some cases be utilized medicolegally. The theory, advanced by Bernstein is that blood groups are inherited as a series of three allelomorphs. Two dominants A and B, and a recessive O, are considered as the basis of the four groups. According to this theory paternity cannot be proved, but the impossibility of being the parent of a child in question might be established in some instances.[1] This point is discussed more fully on page 342.

It is generally held that an individual's grouping never changes. A few apparent exceptions to the rule have been reported, mostly among persons who have successfully withstood acute infections or among anemic patients who have recently been transfused. In most such cases errors in technic have been responsible, usually owing to weak typing serums. In some instances an agglutinin which had formerly been too weak for detection by the usual test became much stronger, thus causing an apparent change of group if only the agglutinating power of the serum was studied.

An individual's serum may be capable of agglutinating his own corpuscles when cooled in the ice-box or occasionally even at room temperature. This power is absent at body temperature. The "auto-agglutinin" is quite distinct from the iso-agglutinins. They belong to a group of so-called cold agglutinins which clump erythrocytes regardless of their blood group at low temperatures.

The discovery that 85 per cent of individuals had in their corpuscles agglutinogens for serum of laboratory animals immunized against the corpuscles of the rhesus monkey and that 15 per cent of individuals did not have this factor in their corpuscles has brought about very interesting speculations regarding the cause of some of the unexplained reactions that have occurred in transfusions with donors of apparently the proper group. This subject will be discussed further on pages 343–346.

[1] Current Comment; Blood Tests for Paternity, Jour. Am. Med. Assn., *87*:1834 (Nov. 27), 1926. Bernstein, F.: Ztschr. f. Indukt. Abstammungs- und Vererbungsl., *37*:237, 1925.

When transfusion is undertaken, the blood should be secured from an individual belonging to the same group as the patient. If such a donor cannot be found, as may easily happen if the patient belongs to either of the small groups, AB or B, blood of Group O may in emergencies be used for patients of any of the four groups, *provided that serum of the patient does not agglutinate the corpuscles of the donor, and that the blood be introduced very slowly*. The reason for this is found in the fact that although the donor's blood has the power of agglutinating the recipient's corpuscles in a test tube or on a slide, yet in actual transfusion, slowly carried out, the blood which is introduced mixes at once with the recipient's blood and is so greatly diluted that its power to injure the recipient's corpuscles is greatly reduced, if not completely lost. If, upon the other hand, blood of Groups AB, A, or B be used for a Group O patient, the introduced corpuscles are subjected to practically the full strength of the patient's blood, and all are agglutinated or hemolyzed with disastrous results for the patient. However, a patient of group AB may receive blood of any group provided that the same precautions are observed. When transfusion of patients with abnormally fragile red corpuscles—as in hemolytic jaundice—is undertaken, use of a donor of the same group is obligatory; and, moreover, the donor's blood should be matched directly with the patient's before each transfusion.

Technic of Blood Matching.—1. Obtain the following from each of the two persons whose blood is to be matched:

(*a*) *Red-cell Suspension.*—Puncture finger or ear, and let a large drop of blood fall directly into a small test tube containing 1 c.c. of a 1 per cent solution of sodium citrate in 0.85 per cent salt solution. Mix gently by inverting a few times.

(*b*) *Serum.*—Obtain a few drops of blood in a small tube or Lyon capsule (Fig. 353, p. 658). As soon as coagulation has taken place, gently loosen the clot from the wall of the tube. Let stand until serum has separated well. Separation of serum can be hastened by centrifugation.

2. Make vaselin rings on two slides, or use a cover slip on a hanging drop slide. In one mix 1 large drop each of the patient's serum and the suspension of the donor's corpuscles; in the other mix 1 large drop each of the patient's corpuscles and the donor's serum. Label the slides with a wax pencil.

3. Keep the slides at room temperature and every few minutes remix corpuscles and serum by tilting the slide.

4. At intervals examine for agglutination of red corpuscles with a low-power objective. When agglutination takes place the corpuscles gather into dense irregular clumps (Fig. 173). These are nearly always so large as to be seen with the unaided eye as brick-red granules, best viewed over a sheet of white paper; when clumping is not seen with the unaided eye, the result should be checked with the low-power objective of the microscope. Clumping is usually well marked within a few minutes, but it is safe to allow twenty minutes. If it does not occur within this time, it will not occur at all.

Sources of Error.—An important source of error is rouleau formation, which may or may not occur. Although the clumps are usually very small, this is not always easy to differentiate without close observation with the 4 mm. objective. In the case of rouleau formation the corpuscles can be seen to lie in rows within the groups (Fig. 89). Frequent remixing of the corpuscles and serum, as above directed, tends to break up rouleau and to

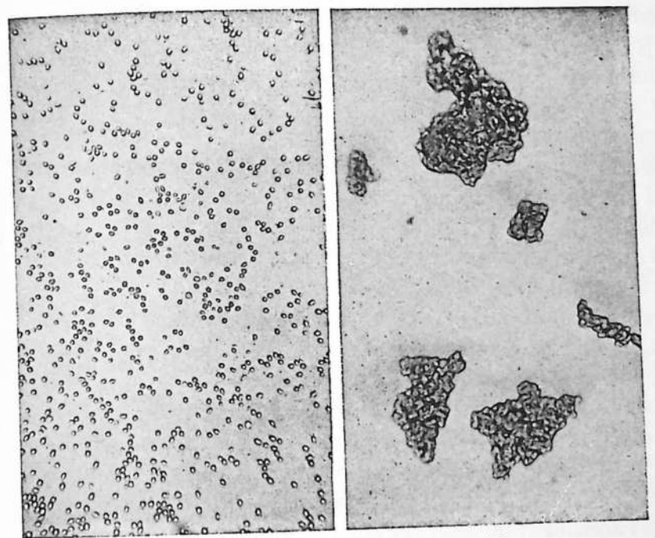


Fig. 173.—Matching bloods for transfusion: A, Corpuscles of a patient with serum of a prospective donor; no agglutination. B, Serum of patient with corpuscles of prospective donor; strong agglutination. The blood of the donor is therefore unsuited for use in this case (photographs, × 100).

favor agglutination. When in doubt, diluting serum with an equal amount of physiologic salt solution eliminates rouleaux formation, whereas clumping will be unaffected.

A still more important source of error lies in the fact that sera differ in their agglutinating titer and the same individual's serum may vary from time to time. Occasionally the agglutinating power may be so low as to result in a false negative reading. When results are in doubt the test should be repeated with two or three times the usual amount of serum. Especial care must be exercised in matching the blood of children because the group characteristics may not yet be fully developed. Occasionally because of the weak A_2 agglutinogens found in group A_2B, if additional care is not taken

by testing the patient's serum with the corpuscles of groups A and B, the individual may be wrongly placed in group B.

To Determine the Group to Which an Individual Belongs.—This is called **blood typing.** As has been indicated above, it is sufficient in a given case to test the blood of a series of prospective donors until one is found which matches the patient's blood, and such direct matching is obligatory in certain cases. In general it is preferable and it will be found much more convenient to determine in advance the grouping of a number of individuals who may be willing to serve as donors upon occasion. When an emergency arises, it is then only necessary to find the group to which the patient belongs in order to know at once the appropriate donor, a procedure which does not require more than fifteen minutes; but even here there should be a direct test of the donor's corpuscles against the patient's serum before the transfusion is done. Employment of the grouping and of the direct matching will help to eliminate errors.

The group to which an individual belongs is easily ascertained by testing his serum and corpuscles against the corpuscles and serum of an individual known to belong to Group A or B, using the simple method described above. Interpretation of results is made clear by Fig. 172. If, for example, the unknown blood agglutinates Group A blood and is not agglutinated by it, then the unknown must belong to Group O.

The same end may be accomplished by testing the corpuscles of the unknown against sera of both Groups A and B. As practiced by Vincent, a drop of each of the sera is placed on a slide, one at each end, and a drop of the suspension of unknown corpuscles or a loopful of the whole blood obtained directly from a skin puncture is mixed with each. Here also reference to Fig. 172 will make the interpretation clear. Agglutinating sera, if kept sterile, will remain active for months and may be kept on hand in small glass capsules (Fig. 352) the ends of which are to be sealed in the flame. The serums used for typing must have a sufficiently high titer of agglutinins to detect the weakly reacting corpuscles of subgroups A_2 and A_2B. It is advisable to titrate them in increasing dilutions with corresponding red blood corpuscles and not to use typing serums with a titer less than 1 : 20 for anti-B serum and 1 : 40 for anti-A serum. When serum is to be used for typing, the blood should be kept in the icebox over night before the serum is separated in order to remove cold agglutinins. The serum should be inactivated at 55° C. for thirty seconds to prevent hemolysis.

The surest way to avoid errors in blood grouping is to type the unknown erythrocytes with typing serums of high titers and to type unknown serums with 1 to 2 per cent fresh suspensions of erythrocytes of known groups. The use of serum of group O for typing in addition to the commonly employed serums of Group A and B will be a further check, because serum O should clump corpuscles of all groups except of group O.

A method which is both fast and reliable is to place a drop each of typing serum, of physiologic salt solution and of the 1 to 2 per cent suspension of erythrocytes in a narrow test tube (7 mm. inside diameter), shake the mixture, let it stand at room temperature for five minutes, then centrifuge it for one minute at about 600 revolutions per minute. A clearcut clumping is evidence of agglutination. If the result is doubtful or negative, a drop of the suspension should be viewed with the microscope. With this method, testing the corpuscles as well as the serum also will help to eliminate errors.

To demonstrate the agglutinogens A_1 and A_2 it is necessary to make use of group B serum in which the agglutinin for A_2 has been removed. To prepare this serum, wash known A_2 corpuscles and place approximately three times as much group B serum with these cells. After thoroughly mixing, allow them to stand at room temperature for thirty minutes and then centrifuge to remove the corpuscles. The serum should then be tested with known group A_2 corpuscles to be sure that all of the agglutinins for A_2 corpuscles have been removed. This serum will then demonstrate only agglutination with group A_1 corpuscles.

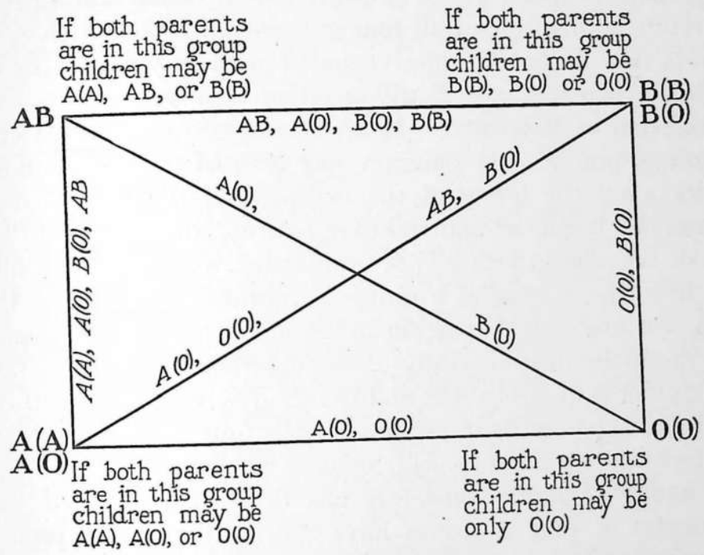


Fig. 174.—Diagram illustrating blood group inheritance according to Bernstein's theory.

Blood Groups and Heredity.—The diagram in Fig. 174 should be studied in connection with Fig. 172. An attempt has been made to show graphically Bernstein's theory regarding blood groups as inherited characteristics. Agglutinogens A and B are dominants, and O is recessive. There are six possible genotypes in the four groups indicated in the diagram: AB is made up entirely of dominant characteristics, and O(O) is made up entirely of recessive characteristics. Group A may have as its characteristics either all dominants A(A), or it may be grouped as A, but with both dominant and recessive inherited

characteristics A(O). Likewise group B may be considered either B(B) or B(O). The results of mating are indicated on the diagram. If both parents are in group AB, the offspring may inherit combinations of genes that will result in individuals of either group AB, A, or B, but not O. On the other hand, children of group O parents can be only in group O. It is also shown that group A parents may have either A or O children, and group B parents may have either B or O children. When the parents are in different groups, very interesting combinations may appear in the groups of the offspring, as indicated on the diagram. These combinations are given on the lines connecting the groups. Offspring of AB mated with O result in either A or B groups, but not in the group of either parent, while mating A with B may result in offspring of all four groups. AB mated with A or with B yields AB, A, or B, while O mated with A yields A or O. The offspring of O mated with B will be either B or O.

Exclusion of Paternity.[1]—In an attempt to exclude paternity in medicolegal practice, the diagram may be used to determine the possibilities when the group of the mother and the child are known. For example, if mother and child are both in group A, then the blood group of the alleged father is of no interest, as a group A child may result from the mating of a group A mother with any of the four groups. An analogous situation arises when both mother and child are in group B. Further study of the diagram will demonstrate that if the mother is in group AB, and the child is in either group A, or B, that the father may be in any one of the four groups. For exclusion tests the following table (p. 343) may be used with Figure 174.

M and N Agglutinogens.—It has been demonstrated that the erythrocytes of human beings have specific antigenic properties in addition to the factors A and B. They were discovered by Landsteiner and Levine[2] by means of immune serums produced in rabbits. They are the factors M and N. There are no normal isoagglutinins for them in human blood. They can be detected by means of immune serums of rabbits injected with corpuscles M or N respectively and then purified by adsorption to remove all antibodies except those which react with either factor M or N. The preparation of anti-M and anti-N testing fluids was well described by Davidsohn and Rosenfeld.[3]

[1] Landsteiner, Karl: Forensic Application of Serologic Individuality Tests, Jour. Am. Med. Assn., **103**: 1041–1044 (Oct. 6), 1934. Heise, H. A.: Some Medicolegal Aspects of Isoagglutinins, Am. Jour. Clin. Path., **4**: 400–409 (Sept.), 1934.

[2] Landsteiner, Karl, and Levine, Philip: A New Agglutinable Factor Differentiating Individual Human Bloods. Proc. Soc. Exper. Biol. & Med. *24*:600–602 (Mar.), 1927.

[3] Davidsohn, I., and Rosenfeld, I.: The Preparation of Anti-M and Anti-N Testing Fluids. Amer. Jour. Clin. Path. *9*:397–413 (July), 1939.

Group of child.	Group of mother.	Exclude as father, group.
O	O A B	AB
AB	AB	O
	A	A, O
	B	B, O
A	B	O
A	O	B O
B	A	O
B	O	A O

Based on these properties there are three types of cells, M, N, and MN, depending on which agglutinogen is present. Approximately 50 per cent of the white population are of type MN, 30 per cent of type M, and 20 per cent of type N. These characteristics are inherited as two allelomorphic genes, yielding genotypes M(M), N(N), and MN. The use of tests for M and N agglutinogens, in addition to blood grouping, increases considerably the chance of excluding paternity in the case of a wrongly accused man. It is emphasized by Landsteiner and others, however, that great caution is necessary in the performance and the interpretation of the test, particularly for the property, N, and that there are questions concerning technic that still require thorough study in order to exclude possible mistakes.

Rh Factor.—Landsteiner and Wiener[1] demonstrated that agglutinins developed in laboratory animals, either rabbits or preferably guinea pigs, against the red blood corpuscles of *Macacus rhesus* monkeys would also agglutinate the corpuscles of 85 per cent of human individuals regardless of iso-agglutinin groups. The agglutinogen in human corpuscles responsible for the reaction has been termed the *Rh factor*. The remaining 15 per cent of individuals lack this

[1] Landsteiner, Karl, and Wiener, A. S.: An Agglutinable Factor in Human Blood Recognized by Immune Sera for Rhesus Blood. Proc. Soc. Exper. Biol. & Med. *43*:223 (Jan.), 1940.

Landsteiner, Karl, and Wiener, A. S.: Studies on an Agglutinogen (Rh) in Human Blood Reacting with Anti-rhesus Sera and with Human Isoantibodies. Jour. Exper. Med. *74*:309–320 (Oct.), 1941.

factor. It is probable that some atypical reactions that have occurred in transfusions may be explained on the basis of the development in an Rh-negative individual of agglutinins for corpuscles containing the Rh factor. This may occur in patients who have had several transfusions with donors of the same group or with corpuscles from the "universal donor" group, or it may occur on the first transfusion in a pregnant woman, or a recently postpartum mother, in which it has been demonstrated that she is an Rh-negative individual, but the fetus has corpuscles that are Rh positive. As this is an inherited characteristic, in instances of this sort the father would also be Rh positive. In a large number of cases of erythroblastosis the condition has been shown to be due apparently to the fact that the mother's corpuscles did not contain Rh agglutinogen, while those of the fetus were Rh positive. The theory is that there is a leakage of agglutinogens through the fetal circulation into the mother's circulation which produces agglutinins in the mother's serum against corpuscles containing the Rh factor. These agglutinins then act upon the corpuscles of the fetus. Although this cannot account for all of the cases of erythroblastosis fetalis, Levine, Vogel, Katzin and Burnham[1] found that 91 per cent of 111 mothers of such infants lacked Rh agglutinogen in the corpuscles.

Preparation of Anti-Rh Serum.—1. Anesthetize a *Macacus rhesus* monkey and withdraw 10 c.c. of blood by cardiac puncture and place in a tube containing 2 c.c. of a sterile solution of 3.8 per cent sodium citrate.

2. Centrifuge and wash the corpuscles three times with physiologic salt solution and make a 50 per cent suspension of the corpuscles.

3. Inject intraperitoneally 2 c.c. of the suspension of corpuscles into each of eight to twelve guinea pigs. Repeat these injections five days later. Seven days after the last injection, test the serums of the guinea pigs to determine whether or not a sufficiently high titer of anti-Rh agglutinin has been developed when used with human corpuscles. Use group O human corpuscles from Rh-positive individuals, and also group O corpuscles that are Rh negative. If no anti-Rh agglutinin is present, a further series of injections is necessary.

4. Before determining the titer of the guinea pig serum, it will be necessary also to test the serum against known group A and B corpuscles. If agglutinins are present, they must be absorbed from the guinea pig serum with known A or B corpuscles which are also known to be Rh negative.

5. The titer of the serum must be determined. Make a 2 per cent suspension of the washed corpuscles of known Rh-positive and Rh-negative individuals. Separate suspensions should be made from at least two individuals of each type. Make dilutions of 1 : 4, 1 : 8, and 1 : 16 of the serum. Place twelve small test tubes in a rack, making four rows of three tubes each. Place 2 drops of each dilution of the serum in each of the four rows of tubes. Add 1 drop of the suspension of the known Rh-positive corpuscles to each of the tubes in two of the rows. Also add 1 drop of the suspension of the Rh-negative corpuscles to each of the tubes in the other two rows. Mix gently and allow to stand thirty minutes at room temperature. Observe signs of agglutination in the bottom of the test tube. Magnify the image of the bottom of the tube in a concave mirror (see Fig. 175). Shake gently and let stand for two hours at room temperature and observe again for agglutination. Rh-negative corpuscles precipitate in a sediment with a smooth edge. Positive Rh-factor corpuscles precipitate and are agglutinated in a wrinkled sediment with a serrated border. High titer serums may require further dilutions to demonstrate clear-cut Rh-negative factors. A satisfactory serum should give clear-cut positive and negative reactions in at least three successive serial dilutions.

[1] Levine, Philip, Vogel, P., Katzin, E. M., and Burnham, L.: Pathogenesis of Erythroblastosis Fetalis; Statistical Evidence. Science. *94*:371–372 (Oct. 17), 1941.

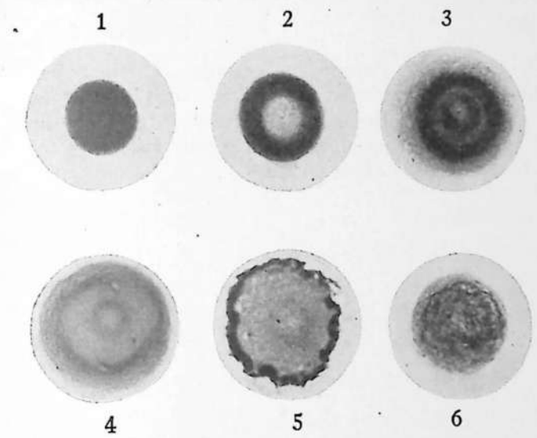


Fig. 175.—Negative reactions 1, 2 (15 per cent). Faintly positive reaction, 3, weakly positive reaction, 4, typical positive reaction, 5, 6 (85 per cent) (Landsteiner and Wiener, J. of Exper. Med., Vol. 74.)

Determination of the Rh Factor.—To determine the presence or absence of the Rh factor in the blood of an individual, it is necessary to have agglutinating serum of known type as described previously. Make a dilution of the serum of the proper titer as previously determined. Use 2 drops of diluted serum and one drop of the suspension of corpuscles of unknown type. Mix gently, let stand for thirty minutes, make a preliminary reading, shake gently and let stand again at room temperature for two hours. Make a final reading for Rh-positive or Rh-negative reactions.

A very satisfactory serum for testing for the Rh factor is often obtainable from women who have given birth recently to babies with erythroblastosis. It will, of course, be necessary to take into account the blood group of such serum. Iso-agglutinins must be removed. Wiener[1] suggested for this purpose the pooling of two parts of saliva from an A individual with one part of saliva from a B individual. This is placed in a boiling water bath for ten minutes. Centrifuge and pipet off the supernatant, opalescent fluid. This pooled saliva when added to human serum in the proportion of two parts of serum to one part of saliva, neutralizes the iso-agglutinins present in the serum. The iso-agglutinins may be removed also with group A and group B corpuscles. In using human serum for testing for the Rh factor, incubate in the water bath at 37° C. for thirty minutes, instead of at room temperature.

[1] Wiener, A. S.: Hemolytic Transfusion Reactions. I. Diagnosis, with Special Reference to the Method of Differential Agglutination. Amer. Jour. Clin. Path. *12*:189–199 (Apr.), 1942. II. Prevention with Special Reference to a New Biological Test. Ibid. *12*:241–252 (May), 1942. III. Prevention with Special Reference to the Rh and Cross-Matching Test. Ibid. *12*:302–311 (June), 1942.

CHAPTER IV

CLINICAL CHEMISTRY

THE application of chemistry to examinations of blood, tissues, and body fluids has progressed to such an extent that clinical laboratories must now conduct, as part of their daily routine, chemical examinations which formerly were thought to be the duties of analytic chemists in research laboratories. While these various tests usually are carried out on whole blood, serum, or plasma, the same method may often, with slight modifications, be applied to examinations of urine or other fluids. Accordingly, the name "clinical chemistry," rather than "blood chemistry," has been chosen for this chapter, although most of the chemical procedures that are described are carried out in that portion of a clinical laboratory organization which is known as the "blood chemistry laboratory." It is assumed that the student, technician, or practitioner, who undertakes the tests which are described, has had sufficient fundamental training in quantitative analysis to enable him to find readily, in the various standard textbooks on quantitative methods, the information that he must have for the preparation of the various normal and molecular solutions which will be mentioned. Many of the tests which are described may be performed with many modifications. The methods which have been chosen may not necessarily be the best method in every instance, but they have all been used for routine work. It is my desire to impress the student with the importance of this rapidly growing branch of clinical pathology.

Colorimetric Methods.—These combine comparative simplicity and great accuracy and are steadily growing in popularity.

In general, they consist in treating the fluid under examination with such reagents as will produce a soluble colored compound with the substance to be estimated, and in comparing this color with that of a similar solution of known strength, upon the principle that the depth of color is directly proportionate to the amount of the substance present. Some preliminary treatment is usually necessary to remove interfering substances. Any device which will show the quantitative relationship between the colors is called a colorimeter.

The chief hindrances to the wide adoption of colorimetric methods for clinical purposes are the cost of the colorimeter and the difficulties in the way of preparing standard color solutions. Relatively stable standard solutions for many of the methods can now be purchased ready prepared.

The **Duboscq type** of colorimeter, including the original Duboscq, the Kober, and certain others manufactured in this country, represents the highest type of colorimeter available for clinical work.

The general construction is well shown in Fig. 160. The solutions to be compared are placed in glass-bottomed cups, which can be raised by means of rack and pinion until the lower ends of the clear glass plungers are immersed in the fluid, the excess of fluid rising between the plungers and the walls of the cups. By raising or lowering the cups the layer of fluid between the lower ends of the plungers and the bottom of the cups may be made of any desired thickness, and the thickness of each is indicated by a scale placed in a convenient position. Beneath the cups is a mirror which

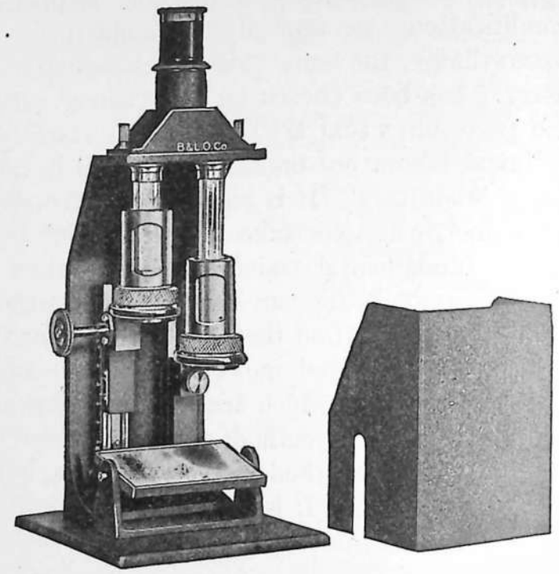


Fig. 176.—The biologic colorimeter, a small colorimeter of the Duboscq type, which is very satisfactory for clinical laboratory work. The light-shield has been removed to show the cups.

reflects light up through the cups and the long axis of the plungers into a series of prisms. These reflect the light from the two cups into a single field which is viewed by an eye-lens. Each lateral half of the field receives its light through one of the cups. The raising or lowering of the cups, by diminishing or increasing the thickness of the layer of fluid through which the light passes, diminishes or increases the depth of color of the corresponding half of the field.

If identical fluids are poured into both cups and the cups placed at the same height, then the two halves of the field should have exactly the same depth of color. If they do not exactly match, the scale, which is movable upon most instruments, must be brought to accurate adjustment.

To use the colorimeter, focus the eyepiece and arrange the reflector so

that the two halves of the field are equally illuminated. Fill one of the cups half full of the standard color solution and raise this cup until the layer of fluid between the bottom of the cup and the lower end of the plunger is of a convenient thickness. This will usually be 10 or 20 mm., as indicated by the scale. Place the unknown solution in the other cup and move this cup up and down until the two halves of the field viewed by the eyepiece exactly match in color. Either daylight or artificial light which is filtered through daylight glass may be used. With daylight, readings are most accurate when the colorimeter is placed in front of a window, but far enough from it—6 to 10 feet—to avoid any strong light entering the eye. Artificial light is best used in a darkened room or a dark corner of the laboratory. Note the reading on the scale.

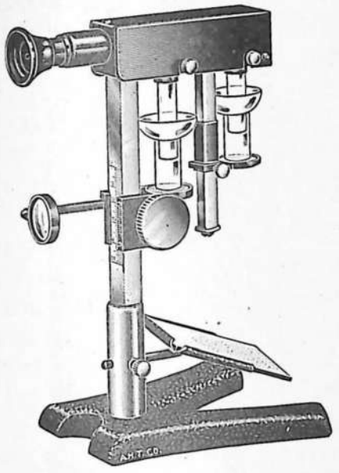


Fig. 177.—Bock-Benedict colorimeter.

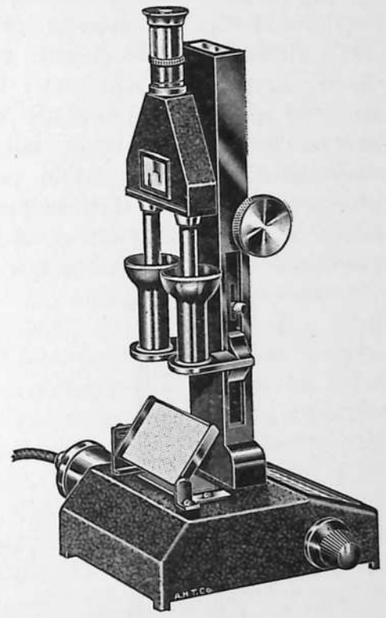


Fig. 178.—Klett bio-colorimeter.

The concentrations of the two solutions are inversely proportional to the respective readings when the colors match. This may be expressed in the formula:

$$\text{Strength of unknown} = \frac{\text{Reading of standard} \times \text{Strength of standard}}{\text{Reading of unknown}}$$

If, for example, in the phenolsulfonephthalein test the 50 per cent standard be used, while the cup containing it is placed at 10 mm., and the unknown stands at 15 mm. when the colors match, then

$$\text{Strength of unknown} = \frac{10 \times 50}{15} = 33.3 \text{ per cent}$$

Results are always most accurate when the unknown and the standard have nearly the same depth of color.

The **Bock-Benedict colorimeter** (Fig. 177) employs the same general principle as the Duboscq except that the prisms are replaced by two mirrors which are protected by a housing. A reading lens is provided for the unknown scale.

The **Klett bio-colorimeter** (Fig. 178) presents several unique features, such as a built-in substage lamp and a revolving calculation table which contains a hemoglobin scale for the Newcomer method, which gives the value of hemoglobin in grams per 100 c.c. of whole blood. The principle of operation is essentially the same as that of the Duboscq colorimeter.

The **Hellige colorimeter** (Fig. 179), devised by Autenrieth and Koenigsberger, but sold under the name of the manufacturer, is less accurate than the Duboscq, but is very satisfactory for certain purposes, particularly the phenolsulfonephthalein test. The solution under examination is placed in the box or trough, while the standard solution is placed in the wedge-shaped bottle, which can be moved up or down beside the trough. The front is slipped into place, and the two solutions are viewed through the window behind which is a double prism to bring the two colors close together. The wedge is moved up and down by means of the knurled head, until a point is reached where the two colors match. The figure on the scale which then stands opposite the pointer indicates the relation between the strengths of the two solutions. If the pointer stands at 40, then the unknown solution is 40 per cent as strong as the known standard; if at 70, it is 70 per cent as strong. Hermetically sealed standard wedges for most of the tests can be purchased with the instrument.

Fig. 179.—Hellige colorimeter.

Fig. 180.—The Peebles-Lewis colorimeter, laboratory model. (Courtesy, Denver Fire Clay Company, Denver, Colorado.)

Before the instrument is used its accuracy should be tested. The following plan is simple: Place the same colored solution—for example, a phenolsulfonephthalein solution—in both the wedge and the glass box, and rack the wedge up and down until the colors match. The scale should then read 100. Now dilute the solution in the glass box exactly 1 in 10 (1 part of solution, 9 parts of water). This should give a reading of 10 when the colors match. Test the intermediate graduations of the scale in the same way. If the scale proves to be inaccurate, make a record of the figures to be added or subtracted. An improved model of this colorimeter, which has a metal case, is illustrated in Fig. 179.

The **Denison laboratory colorimeter**[1] (Fig. 180) is one of the simplest, most convenient, and least expensive yet devised, and is sufficiently accurate for most clinical purposes, including ordinary blood-chemical determinations.

To use the instrument the unknown solution is pipeted into one tube exactly to the 10-c.c. mark, the reading being taken at the bottom of the meniscus, and the standard solution is placed in the other, a little at a time by means of a capillary pipet, until the colors in the two tubes just match when viewed above from over a sheet of white paper or a small mirror, so placed that it reflects the light from a window. A small reflector can be placed in the bottom of the box at an angle of 45° if desired. When the two colors match, the height of the standard color solution expressed in tenths of a cubic centimeter will indicate in percentage the relation between the strengths of the two solutions. If, for example, the top of the standard solution stands at the 7.5-c.c. mark, then the unknown solution is 75 per cent as strong as the known standard. When desired the instrument may be used in the same manner as the Duboscq, placing the standard solution to a desired height, usually to the 10-c.c. mark, and varying the amount of the unknown until the colors match. In this case all the formulae given for calculation with the Duboscq may be used. Readings are most accurate when the unknown solution and the standard have nearly the same depth of color and are best made in front of a window and at some distance—5 to 10 feet—from it. Lewis[2] has reported on two improved designs of this clinical colorimeter. One is a student model with a wooden box, and the other, a laboratory model, is constructed with a metal box (Fig. 180).

Photo-electric Colorimeters.—There are now available several types of colorimeters using photo-electric cells. These are superior in

[1] Designed by the late A. R. Peebles, while director of the Denison Research Laboratory, University of Colorado.

[2] Lewis, R. C.: Two Improved Designs of the Peebles-Lewis Clinical Colorimeter. Jour. Lab. and Clin. Med., *16*:914–917 (June), 1931. The instruments may be purchased from Denver Fire Clay Company, Denver, Colorado, P. O. Box 1107.

every way to the old type of colorimeters, as the subjective errors incident to attempts to match colors visually are eliminated. The photelometer described on page 215 was the first instrument of this sort on the market. Though the early investigations made by Sheard and Sanford were for the estimation of hemoglobin, it was pointed out that the photelometer could be used for a number of clinical chemical procedures (Fig. 181). The field of photelometric clinical

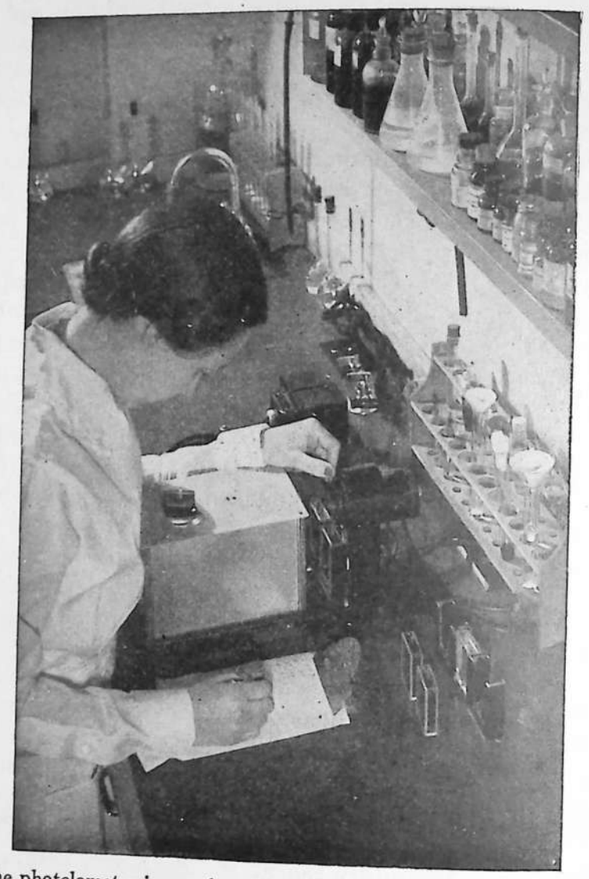


Fig. 181.—The photelometer in use in a biochemical laboratory. (Central Scientific Co.)

chemistry has been well covered by Hoffman.[1] He described the following methods in detail. Determinations of dextrose, total nitrogen, nonprotein nitrogen, albumin and globulin nitrogen, urea by direct nesslerization, urea clearance, uric acid, creatinine and creatine, cholesterol, chloride, inorganic phosphate, serum phosphatase, calcium,

[1] Hoffman, W. S.: Photelometric Clinical Chemistry. New York, Wm. Morrow and Company, 1941, 254 pp.

magnesium, sodium, potassium, lactic acid, ascorbic acid, sulfanilamide and its derivatives, ethyl alcohol, bilirubin in serum and inorganic sulfate in serum. In addition to the biochemical applications of the photelometer, there are scores of methods in industrial chemistry for which this apparatus has proved useful.

I. TESTS FOR THE RECOGNITION OF BLOOD

The microscopic recognition of erythrocytes is the surest and simplest means of detecting the presence of blood. In most pathologic material, however, the corpuscles are too much disintegrated for recognition with the microscope, and one has to rely upon a test for hemoglobin or its derivatives. Of such tests, those given in this section are probably the best. Each is reliable within its own sphere, but each has its limitations. The guaiac, benzidine, and similar tests are reliable only when negative. When, however, proper care is taken to exclude fallacies, they are the most useful and reliable tests for clinical purposes, although they could not be accepted medicolegally. The hemin test is reliable only when *positive*. The spectroscope offers perhaps the most simple and dependable means of identifying blood pigment, but, except under favorable conditions, it is not adapted to the detection of traces. Its particular field lies in distinguishing between the various hemoglobin derivatives.

The only reliable test for human blood as distinguished from that of animals is the precipitin test described on page 662.

1. Guaiac Test.—The technic of this test has been given (p. 115). It may be applied directly to a suspected fluid, but in order to avoid other substances which might cause the reaction the following procedure is advised: Remove fat if present (for example, in feces) by shaking with an equal volume of ether and discarding the ether. It is necessary to make sure that the original fluid is not strongly acid in reaction, otherwise the blood pigment may go into solution in the ether used for the fat extraction and be unwittingly discarded. Add 3 or 4 c.c. of glacial acetic acid to about 10 c.c. of the fat-free fluid; shake thoroughly with an equal volume of ether; decant, and apply the test to the ether. Should the ether not separate well, add an equal volume of alcohol and mix gently. It should then separate nicely. When the amount of blood is very small the ether may be concentrated by evaporation, or it may be completely evaporated and the residue taken up in a few drops of water, which is then tested. In case of dried stains upon cloth, wood, or other material dissolve the stain in distilled water and test the water, or press a piece of moist blotting paper against the stain and touch the paper with drops of

the guaiac and the turpentine successively. The test may be applied to microscopic particles by running the reagents under the cover glass.

The **benzidine test** (p. 115) is similar to the guaiac test and has the same fallacies, but is distinctly more sensitive.

2. Teichmann's Test.—This depends upon the production of characteristic crystals of *hemin*. It is not sufficiently delicate to detect the minute quantities of blood with which we frequently have to deal in the clinical laboratory, but, when positive, it is absolute proof of the presence of blood. A number of substances—lime, fine sand, iron rust—interfere with production of the crystals; hence negative results

Fig. 182.—Hemin crystals obtained in two tests for blood by Teichmann's method. Note the great difference in size of crystals formed under slightly different conditions (photographs, × 250).

are not always conclusive. Dissolve the suspected stain in a few drops of physiologic salt solution upon a slide. If a liquid is to be tested, evaporate some of it upon a slide and dissolve the residue in a few drops of the salt solution. Let dry, apply a cover glass, and run glacial acetic acid underneath it. Heat *very gently* until bubbles begin to form, replacing the acid as it evaporates. Allow to cool slowly. When cool, replace the acid with water, and examine for hemin crystals with 16-mm. and 4-mm. objectives. The crystals are dark brown rhombic plates, lying singly or in crosses, and easily recognized (Fig. 182). Failure to obtain them may be due to too much salt, too

great heat, or too rapid cooling. If not obtained at first, let the slide stand in a warm place, as upon a hot-water radiator, for an hour, replacing the acid as it evaporates.

3. Spectroscopic Method.—Spectrum analysis depends upon the fact that solutions of many substances, when held so as to intercept the light entering the spectroscope, will absorb certain colors, thus causing dark bands to appear at definite locations in the spectrum. A small direct-vision instrument meets all ordinary requirements and may be recommended as a useful addition to the regular laboratory equipment. The form with a side mirror and reflecting prism (Fig. 183) which gives two spectra side by side is most convenient. Before use, the width of the slit should be so adjusted and the eyepiece so focused that Fraunhofer's lines (Fig. 184, B, C, D, E; b, F) are clearly seen, since it is by means of these lines that the absorption bands are located. The examination is best made by daylight. With artificial light the Fraunhofer lines do not appear. The solution under exam-

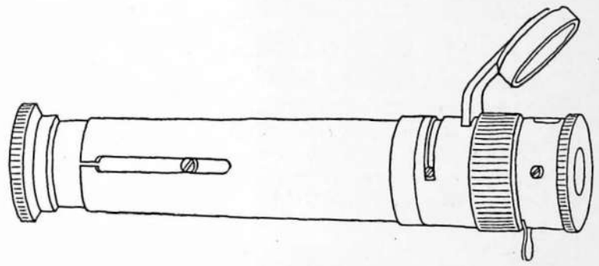


Fig. 183.—Small direct-vision spectroscope with side mirror. (About natural size.)

ination may be held in a test tube or small beaker. If a test tube be used, only 1 to 3 c.c. will be required. *The solution must be made absolutely clear* by filtration, or, in case the quantity is very small, by centrifugation.

The **treatment of the suspected material** will depend upon its condition and the purpose of the examination:

1. When *fresh blood* is studied for oxyhemoglobin or methemoglobin a large drop from a skin puncture is received in 1 or 2 c.c. of water in a test tube and cautiously diluted to the point where the bands become distinct. The optimum dilution is much less for methemoglobin than for oxyhemoglobin.

2. *Urine* and *other fluids* suspected to contain blood may be cleared by filtration and examined directly. When this proves unsatisfactory, as is often the case owing to persistent cloudiness, to the presence of other pigments which darken the whole spectrum, or to the small amount of blood present, the blood pigment in 200 to 500 c.c. of the unfiltered fluid should

be extracted as follows: Add a little white of egg if the fluid is not already sufficiently albuminous, boil, acidify, centrifugalize, remove supernatant fluid, and treat the sediment as described for feces in the following paragraph.

3. *Feces, gastric contents,* and other material should be treated with glacial acetic acid and extracted with ether as described under the guaiac

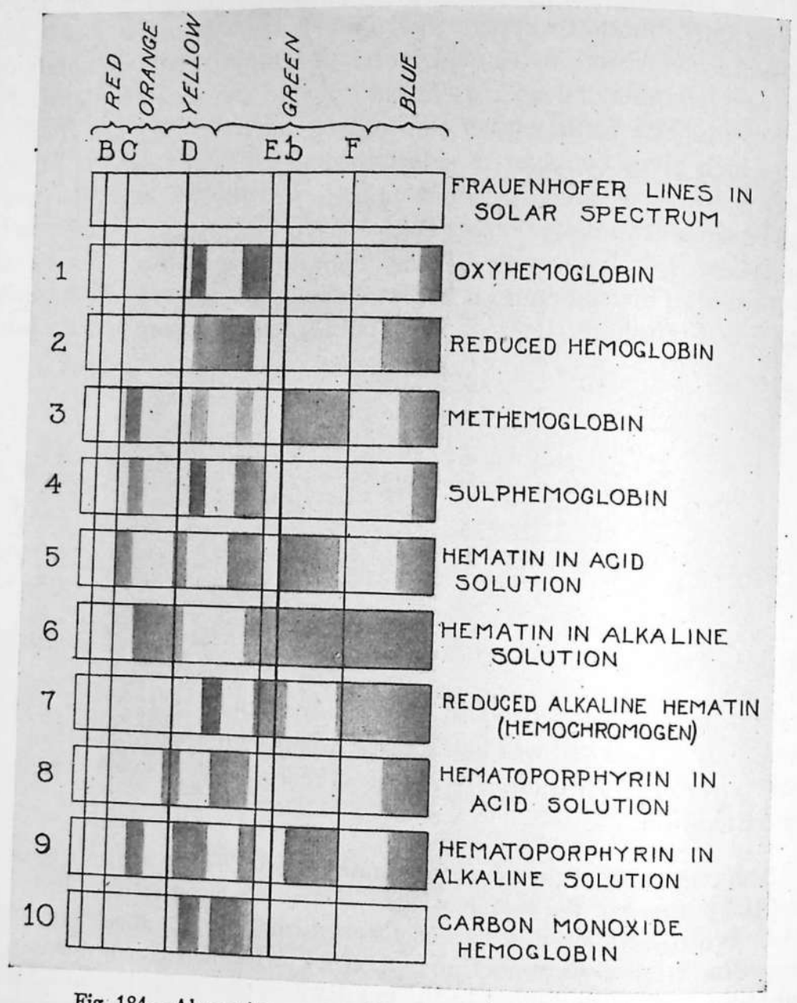


Fig. 184.—Absorption spectra of hemoglobin and its derivatives.

test (p. 353). Blood pigment is thus changed to acid hematin, which is taken up by the acidified ether, giving a clear solution suitable for spectroscopic examination. If the ether does not take up the blood pigment well, a little more acetic acid should be added. In order that the solution may not be too dilute to show the bands, a less amount of ether than is recommended for the guaiac test may be used, or the ether extract may be concentrated by evaporation.

When the result is in doubt the acid hematin may be transformed into the more easily identified hemochromogen as follows: Render the ethereal extract alkaline with strong ammonia, cooling if necessary, mix well, and let stand until the fluids separate. The ammonia will contain alkali hematin. By means of a pipet transfer it to another test tube and add a few drops of fresh yellow ammonium sulfide or Stokes' reagent.[1] Any precipitate may be removed by centrifugalization. The bands of hemochromogen should appear at once.

4. *Stains of blood dried on clothing,* and so forth, should be dissolved in 1 or 2 c.c. of 10 per cent caustic soda solution, heated to a point just short of boiling, cooled, and treated with a few drops of ammonium sulfide or Stokes' reagent. The solution is then examined for the characteristic bands of hemochromogen.

5. In *very old blood stains* the hemoglobin may have been transformed to the iron-free pigment hematoporphyrin, which is very resistant to solution. It will usually dissolve in strong sulfuric acid. It has been advised to place a few small bits of the dry stain on a slide in a drop of concentrated sulfuric acid, to apply a cover, and rub the bits of blood between slide and cover. Enough may go into solution to admit of spectroscopic examination. Particles of wood, cloth, or other organic material which might blacken the acid should be avoided.

For accurate spectral analysis it is necessary to use a high-grade, wide dispersion spectroscope. The **characteristic absorption spectra** of the more important hemoglobin derivatives are as follows:

1. **Oxyhemoglobin** is present only in comparatively fresh blood. It gives two dark bands between the lines D and E, the one nearer D being the stronger. In concentrated solution these unite to form a single broad band. Upon addition of a few drops of fresh ammonium sulfide, or, much better, Stokes' reagent, the spectrum changes to that of reduced hemoglobin.

2. **Hemoglobin** (also called *reduced hemoglobin*) gives a single broad band between D and E. By shaking with air it is changed to oxyhemoglobin, whose bands in the same dilution are more distinct.

3. **Methemoglobin** occurs in the circulating blood under the conditions which have been described (p. 205). It may also be found in urine and in hemorrhagic cyst fluids. In neutral or faintly acid solution its most characteristic band is situated between the lines C and D. Two less distinct bands lie between D and E, possibly due to accompanying oxyhemoglobin and a broad one beyond E; but these are usually not clearly seen. The blood must be diluted cautiously, as it is easy to pass the point where the

[1] Stokes' reagent consists of ferrous sulfate, 2 Gm.; tartaric acid, 3 Gm.; water, 100 c.c. When needed for use take a few cubic centimeters in a test tube and add strong ammonia drop by drop until the precipitate which forms at first has entirely dissolved.

characteristic band is most distinct. Upon addition of a few drops of fresh ammonium sulfide or Stokes' reagent, methemoglobin is changed to reduced hemoglobin with its single broad band. This will serve to distinguish it from acid hematin.

Methemoglobin can be prepared for purposes of comparison by diluting 2 drops of blood with 20 drops of water, adding 1 or 2 drops of strong potassium ferricyanide solution, and shaking. The solution turns chocolate brown, and may then be diluted until the characteristic band is distinct.

4. **Sulfhemoglobin.**—The substance is found most often in cases of intestinal stasis, or in so-called "enterogenous cyanosis." Sulfhemoglobinemia is probably more common than methemoglobinemia, with which it was formerly confused. The absorption spectrum (Fig. 184) is very similar to that of methemoglobin, except that there are only three bands, there being none between E and F. The reaction of sulfhemoglobin to a reducing substance is far different than is that of methemoglobin. On the addition of ammonium sulfide, or of Stokes' reagent, the bands are not changed to those of reduced hemoglobin. This is especially noticeable by observing the bright band near C.

5. **Hematin** may be formed through the action of acids or alkalis, as in gastric and intestinal bleeding. It is sometimes found in old extravasates, in the urine, and elsewhere. It is insoluble in water or weak acids, readily soluble in acidified ether and weak alkalis.

As seen in Fig. 184, the absorption bands of hematin in *acid solution* ("acid hematin") are somewhat similar to those of methemoglobin. That between C and D is most definite and characteristic; the others may not be clearly seen. In contrast to methemoglobin, the addition of ammonium sulfide or Stokes' reagent does not produce the spectrum of reduced hemoglobin, but rather (if the solution has been sufficiently alkalinized to produce alkali hematin) that of hemochromogen.

6. **Hematin** in *alkaline solution* ("alkali hematin") gives a rather indefinite broad band between C and D. Its presence may be confirmed by adding a few drops of ammonium sulfide or Stokes' reagent. The solution becomes brighter red in color, and the spectrum changes to the more easily identified one of hemochromogen.

7. **Hemochromogen,** also called *reduced alkali hematin*, gives a narrow, very distinct band between D and E, and if not in too dilute solution, a fainter band between E and b. This is one of the most definite and characteristic of the blood pigment spectra.

8. **Hematoporphyrin** is an iron-free, hemoglobin derivative, artificially produced. Although it was formerly thought to be present in urine in pathologic conditions, it is known now that the naturally formed coproporphyrin is the substance most usually found in urine. The similarity to the absorption bands of coproporphyrin and hematoporphyrin is shown in Fig. 185.

9. **Hematoporphyrin** in *alkaline solution* produces the absorption bands in the spectrum shown in Fig. 184.

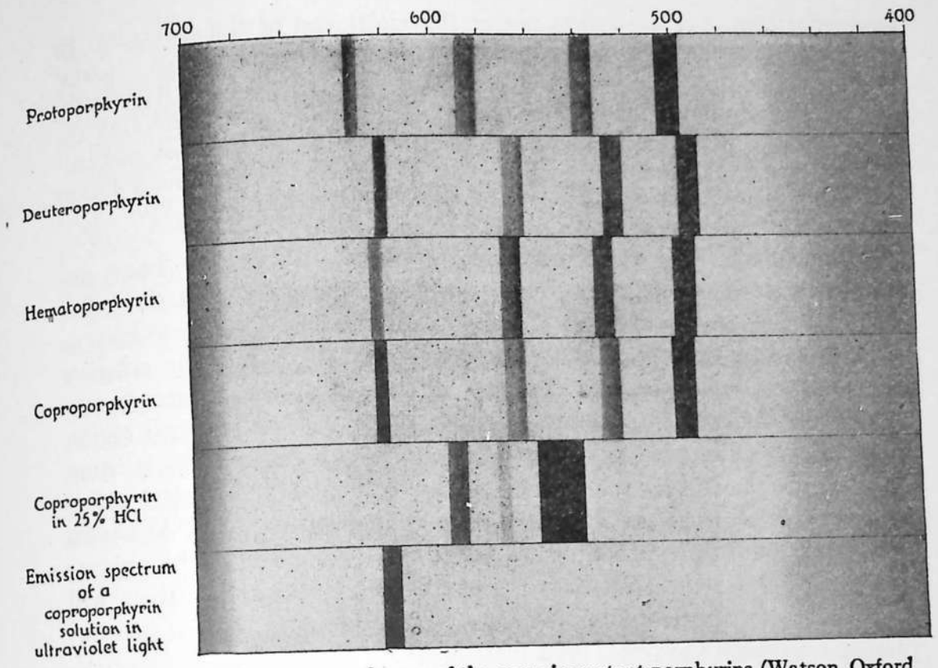


Fig. 185.—Absorption spectra of some of the more important porphyrins (Watson, Oxford Medicine).

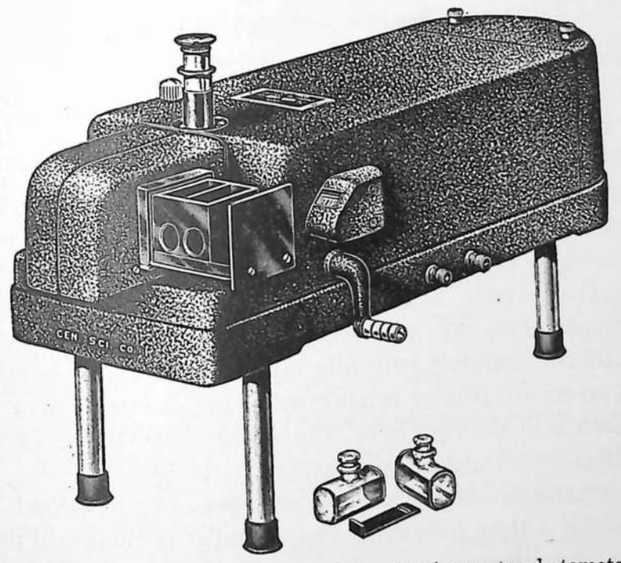


Fig. 186. Spectrophotelometer; a photo-electric spectrophotometer.

10. **Carbon monoxide hemoglobin,** which appears in the circulating blood in carbon monoxide poisoning, gives two bands very like those of

oxyhemoglobin, but somewhat nearer the violet end of the spectrum. In contrast to oxyhemoglobin, addition of ammonium sulfide or Stokes' reagent leaves these bands unchanged. Owing to the small quantity usually present in poisoning, the chemical test is preferable for its detection (p. 205). Carbon monoxide hemoglobin can be prepared for purpose of comparison by causing illuminating gas to bubble through an aqueous solution in a test tube.

Photo-electric Spectrophotometer.—Sheard and States[1] have developed a new, compact instrument which utilizes some of the principles of the Cenco-Sheard-Sanford photelometer. This new instrument is called the spectrophotelometer (see Fig. 186). The relative intensity of illumination in any selected region of the spectrum can quickly be measured by means of this apparatus. The spectral region in which the measurement is to be made is quickly selected by turning a small crank and watching an indicator. The transmission curve may be plotted from the galvanometric deflection readings. Although this instrument will probably be used chiefly for research, anyone wishing to do spectral analysis of hemoglobin or its derivatives will find that the spectrophotelometer is far simpler than is spectrophotometry with the older type instruments.

II. CHEMICAL EXAMINATIONS

Obtaining Blood for Chemical Examination

Blood for chemical examination is obtained from a vein (p. 193). If the examination is to be made on serum, place the blood, usually about 10 c.c., in a chemically clean 15 c.c. centrifuge tube, allow the blood to clot, and centrifugalize to obtain clear serum. If plasma is desired the receiving tube must contain a small amount, about 20 mg., of potassium oxalate to prevent coagulation. The oxalate is best introduced by placing in the tube 2 drops of a 20 per cent solution of chemically pure neutral potassium oxalate and drying in a hot-air sterilizer. For uric acid determinations Folin advises use of 10 mg. of lithium oxalate.[2] Immediately after the blood is added it is well shaken to insure proper mixing with the oxalate. The amount of blood required will depend upon the estimations to be carried out. From 6 to 10 c.c. will usually suffice.

The concentration of various constituents of the blood is materially altered for a time following meals; and it is therefore necessary, for the sake of uniform results, to obtain the blood after a twelve-

[1] Sheard, Charles and States, M. D.: A Concave Grating Photoelectric Spectrophotometer. Jour. Optical Soc., America. *31:*64–69 (Jan)., 1941.

[2] For the preparation of lithium oxalate, see reagent (*d*), Folin's new uric acid method, page 373.

hour fast—that is, before food is taken in the morning. Unless a preservative is used the examination should be started on the same day, preferably within an hour, and the blood should be kept on ice in the interval.

Sander[1] has recommended as a preservative a mixture of 0.01 Gm. of sodium fluoride and 0.001 Gm. of thymol for each cubic centimeter of blood. The nonprotein nitrogen content remains constant with this mixture for six days, and the urea, uric acid, creatinine, creatine, and sugar are preserved for as long as fourteen days. The mixture is prepared by powdering 1 part of thymol with 10 parts of sodium fluoride in a mortar, and passing several times through a 100-mesh sieve. A weighed amount, sufficient for the quantity of blood that is to be taken (0.011 Gm. for each cubic centimeter), is then put in each bottle. The sodium fluoride should be free from ammonia; 1 Gm. should give no color when treated with 5 c.c. of Nessler's solution. This preservative also serves as an anticoagulant, obviating the use of oxalate.

Lewis and Mills[2] confirm the findings of Rose and Schattner on the value of monochlorbenzene-fluoride as a blood preservative in determinations of sugar, urea, uric acid, and creatinine, when it is necessary to delay such determinations for three or four days. They recommend a combination of 0.275 Gm. of potassium fluoride and 0.2 Gm. of monochlorbenzene for each 20 c.c. of blood. This preservative is active at room temperature, or in the refrigerator, at 6° C. However, if the blood becomes heated to 37° C. for twenty-four hours or longer, as it might in the mails, the blood is not preserved, and such a condition will be indicated by the appearance of small clots. The absence of such clots is an indication of proper preservation.

Removal of Blood Proteins.—1. By means of a pipet place a measured amount, usually 5 to 10 c.c., of the oxalated blood (p. 360) in a 200-c.c. flask, add seven times its volume of distilled water, and mix well.

2. Add an amount of 10 per cent solution of sodium tungstate[3] equal to the volume of oxalated blood used, and mix.

[1] Sander, F. V.: The Preservation of Blood for Chemical Analysis, Jour. Biol. Chem., *58:*1–15 (Nov.), 1923.

[2] Lewis, R. C., and Mills, G. E.: The Comparative Value of Monochlorbenzene and Thymol When Used with Fluoride as Preservatives of Blood for Chemical Analysis, Am. Jour. Clin. Path., *3:*17–28 (Jan.), 1933.

[3] Folin has pointed out that the sodium tungstate upon the market is not uniform. Some lots are alkaline and some acid. A satisfactory sodium tungstate gives a solution which is neutral or faintly alkaline to phenolphthalein. The sample should be discarded if it is so alkaline that more than 0.4 c.c. of decinormal acid are required to neutralize 10 c.c. of the 10 per cent solution with phenolphthalein as indicator. Acid-reacting tungstate may be used, provided the 10 per cent solution be brought to neutrality or very faint alkalinity with sodium hydroxide.

3. Add very slowly drop by drop, with constant shaking, an amount of two thirds normal sulfuric acid equal to the volume of oxalated blood used.

4. Insert a rubber stopper and give a few vigorous shakes. A dark-brown coagulum should form. Should it fail to do so, coagulation is incomplete owing, probably, to use of too much oxalate. In such cases add a few drops of twice normal sulfuric acid, shake vigorously, and allow the mixture to stand for five minutes for the coagulum to change from bright-red to dark-brown, before filtering.

5. Filter through paper and collect the clear filtrate. Should the first that comes through be cloudy, return it to the funnel. The filtrate should show no acid when tested with Congo red paper. When uric acid is to be determined, Benedict recommends that the mixture be allowed to stand ten to twenty minutes after the sulfuric acid is added and before filtering.

Each cubic centimeter of the clear filtrate represents 0.1 c.c. of blood. It will serve for determinations of nonprotein nitrogen, urea, uric acid, creatinine, blood sugar, and other substances, and may be kept without deterioration for two days or longer if covered with a few drops of toluene or xylene, and kept on ice.

Haden's Modification.—Haden[1] has simplified the previous method of making a protein-free blood filtrate. Make a twelfth normal solution of sulfuric acid by adding 2.5 c.c. of concentrated sulfuric acid to 1 liter of distilled water. Twenty c.c. of twelfth normal sulfuric acid should require 16.7 c.c. of decinormal sodium hydroxide for neutralization. The improved method consists of laking one volume of blood with 8 volumes of twelfth normal sulfuric acid. Then, add one volume of 10 per cent solution of sodium tungstate, shake the mixture well, and filter.

Osterberg[2] has further modified the Haden method of making protein-free blood filtrate. He mixes one part of a 10 per cent solution of sodium tungstate with eight parts of twelfth normal sulfuric acid. One part of oxalated blood is added directly to nine parts of this mixture. This gives a water-clear, protein-free filtrate without any laking of the blood. In this respect the method is similar to Folin's new method for protein precipitation which follows.

Folin's New Method for Protein Precipitation.—Folin[3] published a modified method for removing proteins from blood samples which gives a water-clear solution, with no laking of erythrocytes. It is claimed for this extract that more accurate determinations can be made for blood sugar and for uric acid, and that urea determinations are identical with those obtained with the older method.

1. Transfer to a small flask 40 c.c. (8 volumes) of a solution containing 15 Gm. of anhydrous sodium sulfate and 6 Gm. of sodium tungstate per liter.

[1] Haden, R. L.: A Modification of the Folin-Wu Method for Making Protein-free Blood Filtrates, Jour. Biol. Chem., *56*:469–471 (June), 1923.

[2] Osterberg, A. E.: Personal communication.

[3] Folin, O.: Unlaked Blood as a Basis for Blood Analysis, Jour. Biol. Chem., *86*:173–178 (Mar.), 1930.

2. Add 5 c.c. of oxalated blood. Mix by occasionally shaking very gently.

3. After five minutes or longer add slowly from a pipet with constant, but gentle mixing, 5 c.c. (1 volume) of N/3 sulfuric acid.

4. Transfer the mixture to 15-c.c. centrifuge tubes, and centrifugate for ten minutes at moderate speed. The supernatant fluid should be water clear.

A. Nonprotein Nitrogen. Urea. Creatinine. Uric Acid

As was stated in connection with the tests of renal function, it is customary to divide the nitrogen-containing constituents of the blood into two groups, one including the proteins (albumins and globulins) and the other including the various nonprotein nitrogenous substances (unutilized food derivatives, waste metabolic products, and so forth).

Because of the light which they throw on the problems of metabolism and excretion it is the nonprotein group, and particularly the waste products, urea, uric acid, and creatinine, which are of chief interest from the clinical point of view. The amounts present in the blood in health are as follows:

	Per 100 c.c. of blood.
Total nonprotein nitrogen	25–30 mg.
Urea nitrogen	12–15 "
Uric acid	2– 4 "
Creatinine	1– 2 "

Lower figures are infrequent and have no definite clinical import. Higher figures are usually referable to accumulation of the respective substance in the blood because of defective elimination. The subject is discussed in the sections dealing with the functional capacity of the kidneys and with laboratory findings in nephritis. Since blood urea has apparently the same significance as regards kidney function as has the total nonprotein nitrogen, and since estimation of urea is much the simpler, the method for nonprotein nitrogen is much less used than is that for urea. Studies by Behre and Benedict throw doubt upon the existence of creatinine in the blood, but the facts which have been gathered regarding the clinical significance of the determinations still stand.

The methods which follow are, for the most part, based upon the system of blood analysis devised by Folin and Wu.[1]

Determination of Total Nonprotein Nitrogen

Method of Folin and Wu.—*Reagents.*—(*a*) Digestion mixture. To 50 c.c. of 5 per cent copper sulfate solution add 300 c.c. of 85 per cent phos-

Folin, O., and Wu, H.: A System of Blood Analysis, Jour. Biol. Chem., *38*:81–110 (May), 1919.

phoric acid and mix. Add 100 c.c. of concentrated sulfuric acid and mix. For use dilute 100 c.c. of the acid mixture with 100 c.c. of water. Cover well.

(*b*) Nessler's solution (1). Stock solution of mercuric potassium iodide. Transfer 150 Gm. of potassium iodide and 100 Gm. of iodine to a 500-c.c. Florence flask; add 100 c.c. of water and 140 to 150 Gm. of metallic mercury. Shake the flask continuously and vigorously for from seven to fifteen minutes, or until the dissolved iodine has nearly all disappeared. The solution becomes hot. When the red iodine solution has begun to become visibly very pale, cool in running water, and continue the shaking until the red color of the iodine has been replaced by the green color of the double iodide. Do not cool too soon. Separate the solution from the surplus mercury by decantation and washing with liberal quantities of distilled water. Dilute the solution and washings to a volume of 2 liters.

(2) Final solution. Prepare a solution of 55 Gm. sodium hydroxide for each 100 c.c. distilled water. Decant the clear supernatant liquid and dilute to a concentration of 10 per cent as determined by titration, an error of not over 5 per cent being allowed. It is important, however, to have enough sodium hydroxide. Transfer to a bottle 1400 c.c. of this 10 per cent sodium hydroxide. Add 300 c.c. of the stock mercuric potassium iodide solution and 300 c.c. distilled water, making 2 liters of Nessler's reagent.

(*c*) Standard ammonium sulfate (0.47165 Gm. ammonium sulfate in 1 liter of water); 10 c.c. of this standard contains 1 mg. of nitrogen.

Method.—1. Transfer 5 c.c. protein-free blood filtrate (p. 362) to a dry pyrex tube graduated at 35 and 50 c.c.

2. Add 1 c.c. of the diluted acid digestion mixture and a quartz pebble. Boil vigorously over a microburner until the characteristic dense fumes nearly fill the tube (from three to seven minutes).

3. Reduce flame sharply, so the boiling is barely perceptible, cover the mouth of the test tube with a watch glass, and continue heating for two minutes or until the solution is nearly colorless.

4. Remove the flame and allow mixture to cool for from seventy to ninety seconds.

5. Add from 15 to 25 c.c. of boiling distilled water. If the mixture has solidified, stir well with a glass rod.

6. Cool to room temperature, and add water to the 35 c.c. mark.

7. Add 15 c.c. Nessler's reagent; insert clean rubber stopper, and mix. If the solution is turbid, centrifugalize a portion very slowly and for a short time before comparing with the standard.

8. Prepare the standard (usually 0.3 mg. of nitrogen required); place 3 c.c. of standard ammonium sulfate (*c*) and 2 c.c. of acid digestion mixture, in a 100 c.c. volumetric flask; add 60 c.c. water, 30 c.c. Nessler's reagent, and make up to 100 c.c. Nesslerize practically simultaneously with the unknown.

9. Compare the unknown with the standard in a colorimeter. If a Duboscq type colorimeter is used, set the standard at 20 mm.

Calculation:

$$\frac{\text{Reading of Standard (20)}}{\text{Reading of Unknown}} \times 30 = \text{mg. of nonprotein nitrogen in 100 cc.. blood.}$$

Photelometric Determination of Nonprotein Nitrogen.[1]—The method has been adapted to the photelometer using either blood or urine and making the calculations from a curve that has been prepared from photelometric readings made with standard solutions that have been analyzed according to the method described.

Determination of Blood Urea

Method of Folin and Wu with Aeration.—*Reagents Required.*—All of these may be purchased ready prepared.

(*a*) Urease solution:[2] Place about 3 Gm. of permutit powder in a 200-c.c. flask. Wash by shaking with 2 per cent acetic acid, allowing to settle, and pouring off the supernatant fluid. Wash twice with distilled water in a similar manner. Add to the moist permutit 100 c.c. of 30 per cent alcohol (35 c.c. of 95 per cent alcohol and 70 c.c. of water). Add 5 Gm. jack-bean meal and shake for ten minutes. Filter and store in small bottles. The solution remains good for about a month if kept on ice. Each estimation of blood urea will require 0.5 to 1 c.c.

(*b*) Pyrophosphate solution: Dissolve 14 Gm. of sodium pyrophosphate, U. S. P., and 2 Gm. of glacial phosphoric acid in 100 c.c. distilled water. Two drops are used in each estimation to accelerate the action of urease.

(*c*) Sodium hydroxide, 10 per cent solution. Each estimation requires 1 or 2 c.c.

(*d*) Antifoam liquid. Pure caprylic alcohol is best and requires the use of only 4 or 5 drops. Pure amyl alcohol may be substituted, but 1 or 2 c.c. must be used.

(*e*) Standard ammonium sulfate solution. (See Nonprotein Nitrogen Method, Reagent *c*.)

(*f*) Nessler's reagent (Folin). (See Nonprotein Nitrogen Method, Reagent *b*.)

(*g*) Decinormal hydrochloric acid solution. One c.c., diluted with water, is used in the determination to catch the ammonia carried over by aeration.

[1] Hoffman, W. S.: Photelometric Clinical Chemistry, New York, Wm. Morris and Company, 1941, pp. 77–89.

Hoffman, W. S., and Osgood, Bess: The Photoelectric Microdetermination of Nitrogenous Constituents of Blood and Urine by Direct Nesslerization. Jour. Lab. & Clin. Med. *25*:856–866 (May), 1940.

[2] Instead of Folin's urease and pyrophosphate solutions here described one may use a 5 per cent solution of the urease powder prepared by E. R. Squibb & Sons, and the Arlington Chemical Co., or the 0.025 Gm. tablets prepared by Hynson, Westcott and Dunning. Two tablets broken up in 1 c.c. of water make a 5 per cent solution. All of these have the activating phosphate already added.

Method: 1. Pipet 5 c.c. of the protein-free filtrate[1] (p. 362), representing 0.5 c.c. of blood, into a test tube of such size that it will readily slip into cylinder A of the aeration apparatus of Myers (Fig. 187).

2. Add 0.5 to 1 c.c. of the urease solution and 2 drops of the pyrophosphate solution. The pyrophosphate is to be omitted if the urease solution is prepared as described in the footnote on page 365.

3. Let stand at room temperature for fifteen or twenty minutes, or place in a water bath at 50° to 55° C. for five minutes. The urea is converted into ammonium carbonate.

4. Add 1 or 2 c.c. of 10 per cent sodium hydroxide solution, mix and add 4 or 5 drops of caprylic alcohol or 1 or 2 c.c. of pure amyl alcohol. Immediately place the test tube in cylinder A (Fig. 187) and adjust the

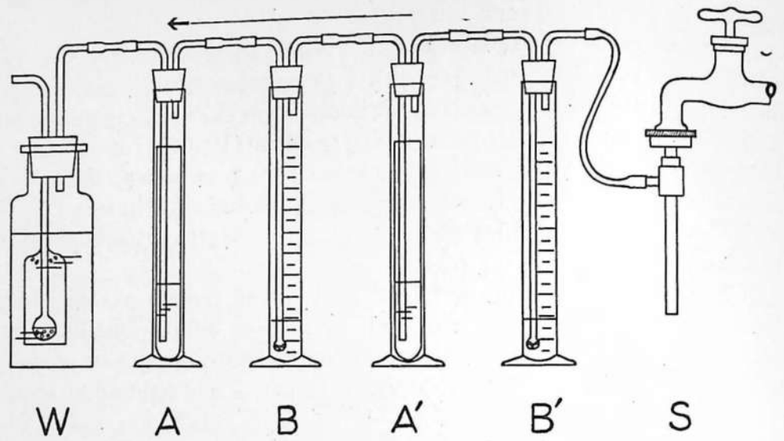


Fig. 187.—Aeration apparatus for estimation of urea and ammonia: W, Bottle containing dilute sulfuric acid to remove ammonia from the air which passes through the apparatus, A, A′, cylinders in which the test fluids are placed; B, B′, cylinders with decinormal acid to receive the ammonia; S, suction.

stopper, taking care that the end of the delivery tube is well below the surface of the fluid.

5. In graduated cylinder B place 1 c.c. of decinormal hydrochloric acid and 15 or 20 c.c. of water. Adjust the stopper with the end of the delivery tube reaching nearly to the bottom of the cylinder.

6. Start the suction, allowing the air to pass only very gently for the first few minutes, later as actively as the apparatus will stand. Continue aeration for thirty to forty-five minutes.

7. When aeration is completed add 2.5 c.c. of Nessler's solution to the acid in cylinder B, fill to the 25 c.c. mark with distilled water, and mix well.

[1] Precipitation of proteins is not necessary and is done only when other substances are to be determined in the same sample of blood. When only urea is to be determined it is more convenient to use the whole blood as follows: Take 1 c.c. of the oxalated blood, add 9 c.c. of water, mix well, and use 5 c.c. in Step 1 above.

8. Prepare the standard color solution containing 0.3 mg. of nitrogen as follows: In a 100-c.c. volumetric flask take exactly 3 c.c. of the standard ammonium sulfate solution, about 70 c.c. of water, and 10 c.c. Nessler's reagent. Make up to the 100-c.c. mark with water, and mix well. *The standard and the unknown should be Nesslerized as nearly simultaneously as practicable.*

9. Compare the unknown with the standard in a colorimeter, calculate the amount of the urea nitrogen in the 0.5 c.c. of blood used, and from this the amount of urea nitrogen in 100 c.c. of the blood.[1] The details of the calculation vary with the colorimeters used, but depend upon the fact that 25 c.c. of the unknown represent 0.5 c.c. of blood, while 100 c.c. of the color standard contain 0.3 mg. nitrogen. With the Duboscq type of colorimeter the following formula may be used:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 15 = \text{mg. urea nitrogen in 100 c.c. blood.}$$

While the method as given above is well suited to the needs of most clinical laboratories a variation which is given preference by Folin and Wu is widely used. This consists in carrying the ammonia from the blood filtrate into the acid by means of distillation instead of aeration. The tube containing the blood filtrate and urease is connected to a receiving tube in a manner similar to that shown in Fig. 43 (p. 107). The method is exactly the same as that given above, except that Steps 4 to 7 inclusive are changed to read as follows:

4. To the digested blood filtrate add 2 or 3 drops of paraffin oil and 1 c.c. of 10 per cent sodium hydroxide, and insert the stopper.

5. Quickly place 1 c.c. of decinormal hydrochloric acid and about 1 c.c. of distilled water in the receiving test tube and connect with the delivery tube. The end of the delivery tube must extend below the surface of the acid. The receiving test tube should have a 25 c.c. mark.

6. Boil moderately over a microburner for four minutes. At the end of this time lower the receiving test tube so that the tip of the delivery tube touches its wall near the top, and boil vigorously for another minute. Rinse the end of the delivery tube with distilled water into the receiving tube.

7. Cool the acid, which now contains the ammonia, dilute to about 20 c.c. with distilled water, add 2.5 c.c. Nessler's reagent, make up to exactly 25 c.c. with distilled water, and mix.

Photelometric Determination of Urea.[2]—The method has been adapted to the photelometer by using either blood or urine and making the calculations from a curve that has been prepared with nesslerized standard solutions.

Van Slyke and Cullen Modification of Marshall Urease Method.—(Also p. 81.)

[1] Urea and urea-nitrogen figures should not be confused. To find the amount of urea multiply the urea-nitrogen figures by 2.14.

[2] Hoffman, W. S.: Photelometric Clinical Chemistry. New York, Wm. Morris and Company, 1941, pp. 90–99.

Reagents.—(*a*) Urease solution, 10 per cent urease powder in distilled water.

(*b*) Antifoam solutions.

1. Rosin, 20 Gm.; turpentine 80 c.c. To be used in the tubes containing blood.

2. Amyl alcohol, 30 c.c.; kerosene, 70 c.c. To be used in the tubes containing acid.

(*c*) Potassium carbonate, 100 Gm. in distilled water, 100 c.c.

(*d*) Indicator, 1 per cent solution alizarin red S (sodium alizarin sulfonate), in distilled water.*

(*e*) Volumetric solutions, fiftieth normal sulfuric, or hydrochloric acid, and fiftieth normal alkali.*

Method.—1. Pipet 3 c.c. whole oxalated blood into a 100-c.c. pyrex test tube.

2. Add 1 c.c. urease solution. (*a*) Place tube in water bath at 50° to 55° C. for fifteen minutes.

3. Add 1 to 2 c.c. rosin antifoam solution (*b*, 1).

4. Place 10 c.c. fiftieth normal acid in acid tube.

5. Add 1 c.c. amyl alcohol antifoam solution (*b*, 2) to this tube.

6. Connect apparatus with suction pump and start aeration of blood tubes into acid tubes (see Fig. 188).

7. Add 10 c.c. saturated solution potassium carbonate (*c*) to blood tube, stopper quickly and tightly, continue aeration for forty-five minutes, driving off all ammonia.

8. Determine the excess of acid by titrating the contents of the acid tube with fiftieth normal sodium hydroxide, using 1 drop of alizarin red indicator (*d*).

9. Blank tubes, without blood, are set up, aerated and titrated, to determine the amount of ammonia in the reagents.

Calculation.—Number cubic centimeters of fiftieth normal sodium hydroxide used to titrate blank acid tube − number cubic centimeters fiftieth normal hydroxide used to titrate blood acid tube = number cubic centimeters of fiftieth normal acid neutralized by ammonia from the blood.

* Instead of the indicator given above, the method is much simplified by using the following two-dye indicator. Dissolve 300 mg. methyl red by grinding in 11.1 c.c. of tenth normal sodium hydroxide. Make up to one liter with ethyl alcohol. Make a 0.1 per cent solution of methylene blue in water and mix 15 c.c. of this methylene blue solution with 100 c.c. of the methyl red solution. Add 1 c.c. of this mixture to every 60 c.c. of a 2 per cent solution of boric acid. Use 15 c.c. of this mixture in step 4 of the method instead of fiftieth normal solution of sulfuric acid. The color of the indicator is bright purple. After aeration the ammonia changes the indicator to green. Titrate the mixture with fiftieth normal solution of sulfuric acid until the bright purple color returns. The formula for a simple calculation is $\frac{60}{\text{c.c. of blood}} \times (\text{titration} - \text{titration of the control}) = \text{mg. of urea}$ per 100 c.c. By using this method it is not necessary to use fiftieth normal solution of alkali for back titration.

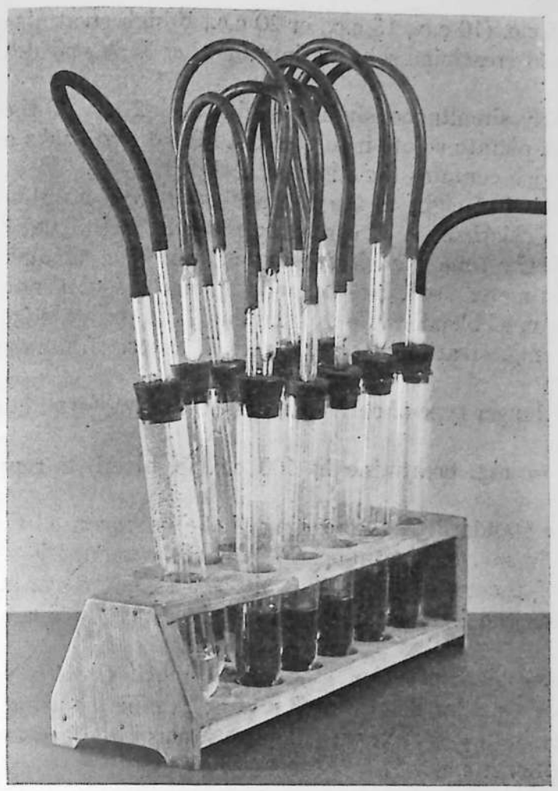


Fig. 188.—Aeration block for determination of urea.

Amount of acid neutralized by ammonia from the blood × 20 × 0.467 = mg. urea nitrogen for each 100 c.c. blood.

Determination of Blood Creatinine

Reagents Required.—(*a*) Alkaline picrate solution: Saturated solution purified picric acid, 25 c.c.; sodium hydroxide, 10 per cent solution, 5 c.c. This solution must be freshly mixed for each test. The picric acid and the sodium hydroxide solutions keep for a long time.

(*b*) Standard creatinine solution: Dissolve 0.1 Gm. creatinine, or 0.161 Gm. of creatinine zinc chloride, in 80 c.c. decinormal hydrochloric acid and make up to 100 c.c. This constitutes a stock solution from which the standard creatinine solution is made as follows: In a liter volumetric flask place 6 c.c. of the stock solution, fill to the 1000 c.c. mark with decinormal hydrochloric acid, and mix well. Preserve by adding a few drops of toluene or xylene; 5 c.c. of this standard contain 0.03 mg. creatinine.

Method.—1. Pipet 10 c.c. of the protein-free blood filtrate (p. 362) into a small flask.

2. Pipet 5 c.c. (10 c.c., 15 c.c., or 20 c.c., if high creatinine is expected) of the standard creatinine solution into another flask and dilute to 20 c.c. with water.

3. As nearly simultaneously as possible add 5 c.c. of the freshly prepared alkaline picrate solution to the first of the two flasks and 10 c.c. to the second which contains the standard. Mix well.

4. At the end of eight to ten minutes compare the unknown with the standard in a colorimeter. The reading must be completed within fifteen minutes from the time the picrate was added. The calculation will vary with the colorimeter used, but depends upon the fact that the unknown represents 1 c.c. of blood, while the standard, diluted to twice the volume, contains 0.03 mg. creatinine (or 0.06 mg. if 10 c.c. of standard creatinine were used).

With the plunger type of colorimeter the following formula may be used:

$$\frac{S \times C \times 50}{R} = \text{mg. creatinine in 100 c.c. of blood,}$$

S representing the reading of the standard, R the reading of the unknown, C the strength of the standard in mg. of creatinine. Thus if the standard is set at 20, and the reading of the unknown is 10 while the standard contains 0.03 mg. creatinine, then the formula gives a creatinine value of 3 mg. per 100 c.c. of blood.

Photelometric Determination of Creatinine and Creatin.[1]—The photelometer may be used for determination of creatinine by using a calibration curve that has been prepared with the colorimetric method and by using a Cenco green filter number 1.

Determination of Blood Uric Acid

Benedict's Method.—*Reagents Required.*—(*a*) Standard uric acid solution (Benedict and Hitchcock). In about 500 c.c. of hot distilled water dissolve 9 Gm. pure crystalline hydrogen disodium phosphate and 1 Gm. dihydrogen sodium phosphate. Filter if not perfectly clear. In a 1000-c.c. volumetric flask place exactly 200 mg. uric acid suspended in a few cubic centimeters of distilled water. Pour the hot phosphate solution into the flask. Agitate until the uric acid is completely dissolved and add 1.4 c.c. glacial acetic acid. Cool to room temperature, make up to 1000 c.c. with distilled water, and mix well. Add 5 c.c. of chloroform as preservative; 5 c.c. of this solution contain 1 mg. uric acid. It remains good for at least two months.

From the above stock of uric acid solution prepare two standards for use in uric acid determinations:

No. 1. Stronger standard. Measure 25 c.c. of the above stock solution into a 500-c.c. volumetric flask, add about 250 c.c. of water and 25 c.c. diluted hydrochloric acid (concentrated hydrochloric acid 1 part, water 9 parts). Dilute to 500 c.c. and mix well. This solution contains 0.05 mg. uric acid in 5 c.c.

[1] Sanford, A. H., Sheard, Charles, and Osterberg, A. E.: The Photelometer and Its Use in the Clinical Laboratory. Amer. Jour. Clin. Path. *3*:405–420 (Nov.), 1933.
Hoffman, W. S.: Photelometric Clinical Chemistry, New York, Wm. Morris and Company, 1941, pp. 111–118.

No. 2. Weaker standard. Proceed in exactly the same way as for the stronger standard, but use 10 c.c. of the stock solution instead of 25 c.c. This standard contains 0.02 mg. uric acid in 5 c.c. and is the one most frequently required. It remains good for two weeks.

(*b*) Sodium cyanide, 5 per cent. Dissolve 25 Gm. sodium cyanide in 450 c.c. distilled water, add 1 c.c. concentrated ammonia, make up to 500 c.c., and mix. This solution must be freshly prepared once in two months.

(*c*) Uric acid reagent (Benedict). Place 100 Gm. of sodium tungstate (Merck or Baker, c. p.) in a liter pyrex flask and dissolve in about 600 c.c. of distilled water. Add 50 Gm. of pure arsenic acid (As_2O_5), 25 c.c. of 85 per cent phosphoric acid, and 20 c.c. of concentrated hydrochloric acid. Boil for about twenty minutes, cool, and dilute to 1000 c.c. This reagent remains good indefinitely.

Method.—1. In a test tube of about 18 to 20 mm. diameter place 5 c.c. of the clear, protein-free blood filtrate (p. 362) representing 0.5 c.c. of blood, and add 5 c.c. of distilled water. Mark the tube U.

2. In a test tube of the same diameter, place 5 c.c. of the stronger uric acid standard solution described above, and add 5 c.c. of water. Mark this tube S 1. It contains 0.05 mg. uric acid.

3. In a third test tube place 5 c.c. of the weaker standard, and add 5 c.c. of water. Mark this tube S 2. It contains 0.02 mg. uric acid.

4. To each tube add 4 c.c. of 5 per cent sodium cyanide solution from a buret.

5. To each tube add 1 c.c. of the uric acid reagent. Mix by one inversion and immediately place in boiling water for three minutes after immersion of the last tube. The time elapsing between immersion of the first and last tubes must not exceed one minute.

6. Remove the tubes, cool for three minutes in a beaker of cold water, and compare the unknown in a colorimeter with the standard which it more nearly matches. To avoid precipitates the comparison should be made within five minutes after removal from the cold water.

The calculation is based upon the fact that the unknown represents 0.5 c.c. of blood, while No. 1 and No. 2 standards contain 0.05 and 0.02 mg. of uric acid respectively. With the Duboscq or Denison Laboratory colorimeter and the No. 2 standard the following formula may be used:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 4 = \text{mg. of uric acid in 100 c.c. of blood.}$$

Folin's Method.—*Reagents Required.*—(*a*) Standard stock solution of uric acid. Place 0.45 to 0.5 Gm. lithium carbonate in a 300-c.c. beaker. Add 150 c.c. water and heat to 60° C. Stir until dissolved. Transfer exactly 1 Gm. of uric acid to a funnel on a 300-c.c. flask. Rinse this into the flask with the hot lithium carbonate solution and shake. When the uric acid has

dissolved, cool under running water and transfer to a 1 liter volumetric flask. Dilute to about 500 c.c. Add 25 c.c. of 40 per cent formalin, shake, and acidify with 3 c.c. glacial acetic acid. Shake to remove most of the CO_2, and add distilled water exactly to the liter mark, and mix by shaking. This stock standard solution will keep for several months, but should be placed in small, tightly stoppered bottles, and kept in a dark place. Each cubic centimeter contains 1 mg. uric acid.

(*b*) Diluted uric acid standard. Transfer, with an Ostwald pipet, 1 c.c. of the stock standard to a 250-c.c. volumetric flask. Fill the flask half full with distilled water, and add 10 c.c. of two thirds normal H_2SO_4, and 1 c.c. of 40 per cent formalin. Dilute to 250 c.c. volume and mix. The diluted standard should keep for a month; 5 c.c. contains 0.02 mg. uric acid.

(*c*) Uric acid reagent. Transfer 50 c.c. of 85 per cent phosphoric acid, and 160 c.c. of distilled water to a 500-c.c. pyrex flask. Heat nearly to boiling and add 100 Gm. of sodium tungstate. The mixture begins to boil from the heat of the reaction. Boil gently and continuously over a microburner for one hour. Close the tube with a funnel covered with a 200-c.c. Florence flask filled with water. Transfer 25 Gm. of lithium carbonate to a liter beaker, add 50 c.c. of 85 per cent phosphoric acid, and 200 c.c. of water. Boil off the CO_2, and cool. Mix the two solutions, and dilute to 1 liter.

(*d*) Sodium cyanide solution, 15 per cent, in tenth-normal sodium hydroxide. Let stand two weeks before using.

Method.—1. Transfer 5 c.c. of protein-free blood filtrate (p. 362) to a test tube graduated at the 25-c.c. mark. Folin advises the use of lithium oxalate (p. 373) as an anticoagulant.

2. Transfer 5 c.c. of the diluted uric acid standard (*b*) to a similar tube.

3. Add to each test tube 2 c.c. of water, 2 c.c. of the cyanide solution (from a buret), and exactly 1 c.c. of uric-acid reagent. If a cloudy precipitate forms add 5 c.c. of water to each tube instead of 2 c.c. Mix and let stand for exactly two minutes.

4. Heat in a boiling-water bath for only eighty seconds. Cool and dilute to 25 c.c.

5. The standard should first be read against itself in the colorimeter, and then the standard set at 20 mm., and the unknown compared with it. Readings between 40 and 10 mm. (2 to 8 mg. uric acid) are dependable.

Calculation:

$$\frac{\text{Reading of the Standard (20 mm.)}}{\text{Reading of the Unknown}} \times 4 = \text{mg. uric acid for each 100 c.c. blood.}$$

Folin's Modified Method.[1]—*Reagents.*—(*a*) Standard stock solution of uric acid. Dissolve exactly 1 Gm. of uric acid in 150 c.c. of lithium carbonate solution as described in the old method, using 0.6 Gm. of lithium carbonate instead of 0.45 to 0.5 Gm. Instead of 25 c.c. of 40 per cent formalin add only 20 c.c. of 40 per cent formalin. Add a few drops of methyl orange solution, and add slowly from a pipet 25 c.c. of normal sulfuric acid. The solution should turn pink just before the last 2 or 3 c.c. of acid are added. Dilute to exactly 1 liter. Mix thoroughly, tightly stopper, and keep stored away from the light.

[1] Folin, O.: An Improved Method for the Determination of Uric Acid in the Blood, Jour. Biol. Chem., *86*:179–187 (Mar.), 1930.

(*b*) Diluted uric acid standard. Dilute exactly 1 c.c. with water only to exactly 250 c.c.

(*c*) Uric acid reagent (phenol free) (older method, see also new improved method below). Dissolve 100 Gm. of sodium tungstate in 200 c.c. of water in a 500-c.c. pyrex flask. Add slowly, with shaking, and thorough cooling, 20 c.c. of 85 per cent phosphoric acid. Pass a slow hydrogen sulfide current through the solution for twenty minutes, and during the process (at the end of three or four minutes) add another 10 c.c. of 85 per cent phosphoric acid. Filter through a good grade of quantitative filter paper (Whatman No. 41); collect the first 40 c.c. of filtrate separately, and pour back on the filter. Transfer the filtrate to a separatory funnel of 1 liter capacity and shake for two minutes with 300 c.c. of alcohol. Transfer the lower layer to a weighed 500-c.c. flask. Discard the upper layer. Make the mixture by weight up to 300 Gm. by adding water. Boil the solution a few minutes to remove the hydrogen sulfide. Remove the flame, add 20 c.c. of 85 per cent phosphoric acid, and boil slowly for one hour, using a condenser to prevent concentration. At the end of an hour remove the flame and decolorize with a few drops of bromine. Boil off the excess of bromine and cool. Transfer 12 Gm. of lithium carbonate to a 500-c.c. beaker, add first 25 c.c. of phosphoric acid, and then slowly 150 c.c. of water. Boil to remove the carbon dioxide. See that the carbonate is completely dissolved. Cool the lithium phosphate solution. Mix with the concentrated uric acid reagent, and dilute to 1 liter. Keep in well-stoppered brown bottles.

New, Simplified Uric Acid Reagent.—It is possible to obtain sodium tungstate which is completely free from molybdate.[1] The following formula is then much simpler than is that which was described previously.

Place 100 Gm. of sodium tungstate (molybdate free) in a 500 c.c. Florence flask. Dissolve 32 to 33 c.c. of 85 per cent phosphoric acid in 150 c.c. of water. Add the diluted phosphoric acid to the sodium tungstate and shake the flask. Add a few pebbles and boil gently for fifty to sixty minutes, using a 10-cm. funnel to hold a 200-c.c. flask of cold water as a condenser. Decolorize with a little bromine water, boil off the surplus bromine, cool, and dilute to 500 c.c. with distilled water.

Test Uric Acid Reagent.—Place 5 c.c. water in a test tube. Add 4 c.c. of uric acid reagent and 10 c.c. urea cyanide solution. Mix and let stand for fifteen minutes. If a blue color forms, the most probable cause is the use of too much phosphoric acid. It may be possible to adjust the reagent by adding not more than 5 Gm. of sodium tungstate, and boiling again for ten or fifteen minutes. Decolorize with bromine water if necessary; cool, and dilute to volume.

(*d*) Lithium oxalate. Transfer 50 Gm. of lithium carbonate and 85 Gm. of oxalic acid to a 3-liter beaker. Pour on the mixture about 1 liter of hot

[1] Mallincrodt Chemical Company, St. Louis, Missouri, Folin's Laboratory Manual of Biological Chemistry, Ed. 5, 1934, p. 293.

water (70° C.). Stir cautiously to avoid loss by foaming until the evolution of carbon dioxide ceases. Add 1 liter of alcohol and filter with a Büchner funnel.

(*e*) Urea-cyanide solution. Transfer 75 Gm. of Merck's reagent sodium cyanide to a 2-liter beaker, add 700 c.c. of water, and dissolve by stirring. Add 300 Gm. of urea and stir. Add 4 to 5 Gm. of calcium oxide and stir for ten minutes. Filter, preferably on the following day. Add about 2 Gm. of powdered lithium oxalate (*d*), shake occasionally for ten to fifteen minutes and filter for use.

Method.—(Revised in 1934 Folin Manual.) 1. Transfer 5 c.c. of unlaked blood filtrate to a 25-c.c. graduated test tube.

2. Transfer 5 c.c. of the standard uric acid solution (*b*) to a similar tube.

3. Add 10 c.c. of urea-cyanide solution (*e*) to each tube, and mix by whirling the test tubes.

4. Add 4 c.c. of concentrated uric acid reagent (*c*), keeping the tubes in a vertical position. Let stand for twenty minutes. Dilute to volume and make the color comparison in a colorimeter, with the standard set at 20 mm.

Calculation:

$$\frac{20}{\text{Reading of Unknown}} \times 4 = \text{mg. uric acid per 100 c.c. of blood.}$$

Photelometric Determination of Uric Acid.—Hoffman[1] has developed a good photelometric method for determination of uric acid by using Folin standard stock solution. He preferred, however, the Newton[2] acid reagent. This reagent contains a highly chromogenic arsenotungstate.

B. Amino-acids, Cysteine, and Cystine

Determination of Amino-acid Nitrogen in Blood.—Danielson[3] has published a method for determination of amino-acid nitrogen in blood. It is best performed with Folin's unlaked blood filtrate, as described on page 362. Danielson felt that by using laked blood nondiffusible amino-nitrogen is also added to the filtrate, and that it is undesirable to confuse the study of one fraction by including in the analysis all the amino-nitrogen of the second fraction.

Reagents Required.—(*a*) Stock standard amino-acid solutions.

(1) Dissolve 53.6 mg. glycine in 100 c.c. of seven-hundredth normal solution of hydrochloric acid which contains 0.2 per cent sodium benzoate. This represents 0.1 mg. of amino-nitrogen per cubic centimeter.

(2) Dissolve 105.1 mg. glutamic acid in 100 c.c. of seven-hundredth normal solution of hydrochloric acid which contains 0.2 per cent sodium benzoate. This represents 0.1 mg. of amino-nitrogen per cubic centimeter.

[1] Hoffman, W. S.: Photelometric Clinical Chemistry. New York, Wm. Morris and Company, 1941, pp. 101–110.

[2] Newton, Eleanor C.: A Chromogenic Tungstate and Its Use in the Determination of Uric Acid of Blood. Jour. Biol. Chem. *120*:315–329 (Aug.), 1937.

[3] Danielson, I. S.: Amino-acid Nitrogen in Blood and Its Determination, Jour. Biol. Chem., *101*:505–522 (July), 1933.

(*b*) Standard amino-acid solutions for tests. Place 15 c.c. of (1) and (2) of the above stock standards in a 100-c.c. volumetric flask and make up the contents to the 100-c.c. mark with seven-hundredth normal solution of hydrochloric acid which contains 0.2 per cent sodium benzoate. This makes a 0.03 mg. per cubic centimeter standard for use with unlaked human blood filtrate. If a stronger standard is desired for use with animal blood, or with laked human blood filtrate, take 25 c.c. each of (1) and (2) and make up to 100 c.c. with the diluent. This will make a standard containing 0.05 mg. per cubic centimeter.

(*c*) Borax solution. Dissolve 1.5 Gm. borax in 100 c.c. of distilled water.

(*d*) Bleaching reagents. (1) Prepare a tenth molar solution of sodium thiosulfate, which need not be standardized.

(2) Dilute 11.3 c.c. of ordinary 40 per cent formaldehyde solution to 1000 c.c. with distilled water. To 4 volumes of this dilute formaldehyde solution (fifteen-hundredth molar) add 3 volumes of one and a half normal solution of hydrochloric acid and 1 volume of glacial acetic acid.

(*e*) Sulfate-tungstate solution should be added to standard solution if unlaked blood filtrate is used. This is made as follows: Dissolve 15 Gm. of anhydrous sodium sulfate (Na_2SO_4) and 1.5 Gm. of sodium tungstate in 1125 c.c. of distilled water.

(*f*) Beta-naphthoquinone sulfonic acid solution. Make a fresh 0.5 per cent solution in water.

Method.—The determination may be made with either 5 or 10 c.c. of filtrate. When 10 c.c. of filtrate are used, proceed as follows:

1. Transfer 10 c.c. of filtrate into a test tube which is graduated at the 25-c.c. mark. Add 2 c.c. of borax solution (*c*) and 2 c.c. of freshly prepared solution (*f*). Mix thoroughly.

2. Place 1 c.c. of standard solution (*b*) in a similar tube. Add 9 c.c. of sulfate-tungstate solution (*e*) if *unlaked blood filtrate* is used; use 9 c.c. of distilled water if *laked blood filtrate* is used. Add 2 c.c. of (*c*) and 2 c.c. of (*f*), and mix thoroughly.

3. Set both the standard and unknown mixtures in a dark closet for eighteen to twenty-four hours.

4. Add 2 c.c. of acid formaldehyde solution (*d*, 2), and 2 c.c. of sodium thiosulfate solution (*d*, 1).

5. Dilute the contents of each tube to a volume of 25 c.c. with distilled water and mix thoroughly.

6. Complete bleaching of the excess quinone reagent will have taken place after four or five minutes. Compare the color of the unknown with the standard in colorimeter, while the standard is set at 20 mm.

Calculation.—This is very simple. If the standard contains 0.03 mg. per cubic centimeter, the factor is 3 for mg. per 100 c.c., and if the standard used contains 0.05 mg., the factor is then 5. The formula depending on the standard used is either $\frac{20}{R} \times 3 =$ mg. per cent, or $\frac{20}{R} \times 5 =$ mg. per cent.

Cysteine and Cystine.—These amino-acids are produced by the digestion or acid hydrolysis of proteins. Cysteine is alpha-amino-beta-thiolactic acid, and is easily oxidized to cystine, which is dicysteine. In the tests which are described for these substances, the reaction is for cysteine, and cystine is reduced to the simpler form with sodium cyanide. Cystine crystals may be found in the urine (see p. 134). Quantitative cystine determinations are of interest in studying sulfur metabolism.

Sullivan's Method for Cysteine.[1]—*Reagents Required.*—(*a*) One per cent sodium cyanide in an eight-tenth normal solution of sodium hydroxide. Dissolve 1 Gm. of sodium cyanide in 80 c.c. of a normal solution of sodium hydroxide and 20 c.c. distilled water.

(*b*) Five-tenths per cent solution of sodium beta-naphthoquinone-4-sulfonate in water.

(*c*) Fifteen per cent solution of sodium sulfite (Na_2SO_3) in half normal solution of sodium hydroxide.

(*d*) Two per cent solution of sodium hydrosulfite ($Na_2S_2O_4$) in half normal solution of sodium hydroxide.

Method.—1. To 5 c.c. of urine add 50 per cent hydrochloric acid until the urine is acid to Congo red paper.

2. Add 1 or 2 c.c. of freshly prepared 1 per cent sodium cyanide solution (*a*).

3. Add 1 c.c. freshly prepared solution (*b*). Mix.

4. Add 5 c.c. of solution (*c*). Mix and let stand thirty minutes in the dark.

5. If a red color forms, add 1 c.c. of solution (*d*).

If the red color persists it indicates the presence of cysteine.

Cystine Determination.—The qualitative test is exactly the same as the test for cysteine, except that a 5 per cent solution of sodium cyanide in water must be used to reduce the cystine to cysteine. After acidifying 5 c.c. of urine, add 2 c.c. of a freshly prepared 5 per cent sodium cyanide solution. Mix and let reduction proceed for ten minutes at 20° to 25° C. Then carry out the test as described for cysteine.

Quantitative Test.—Standard Solutions.—(*a*) Dissolve 100 mg. of cystine in 10 c.c. of decinormal hydrochloric acid with heat, and make up to 100 c.c.

(*b*) Use fresh, clear, normal urine for a control, and prepare seven tubes as follows:

Tube 1.—5 c.c. urine (control).
Tube 2.—4.95 c.c. urine + 0.05 c.c. (*a*) = 0.05 mg. cystine in 5 c.c.
Tube 3.—4.9 c.c. urine + 0.1 c.c. (*a*) = 0.1 mg. cystine in 5 c.c.
Tube 4.—4.8 c.c. urine + 0.2 c.c. (*a*) = 0.2 mg. cystine in 5 c.c.
Tube 5.—4.7 c.c. urine + 0.3 c.c. (*a*) = 0.3 mg. cystine in 5 c.c.
Tube 6.—4.6 c.c. urine + 0.4 c.c. (*a*) = 0.4 mg. cystine in 5 c.c.
Tube 7.—4.5 c.c. urine + 0.5 c.c. (*a*) = 0.5 mg. cystine in 5 c.c.

[1] Sullivan, M. X.: Studies on the Biochemistry of Sulphur. II. Further Studies on the Distinctive Reaction for Cysteine and Cystine. U. S. Public Health Reports, *44*:1421–1428 (June 14), 1929. The details of the procedure as outlined were furnished by Dr. Osterberg of The Mayo Clinic, as he has adapted it for clinical laboratory use.

Method.—Treat the seven standards in the same manner as the unknown urine in the qualitative cysteine test given previously. Put the standards and the unknown in tubes of the same diameter and compare the unknown with that standard which has the nearest depth of color.

Multiply the number of milligrams in 5 c.c. of urine by 20 to obtain the milligrams per 100 c.c.

C. Serum Protein, Albumin-globulin Ratio, and Fibrinogen

Total protein in serum is determined by estimating the nitrogen content by the Kjeldahl method. The digestion is carried out in a Kjeldahl flask and the distillation of the ammonia on a regular Kjeldahl rack. For the details of these well-known chemical procedures, of which there are various modifications, the student should consult any recent textbook of quantitative analysis. The nitrogen also may be determined by the micro-Kjeldahl method for nonprotein nitrogen, which is described on page 363.

Total Serum-protein Determination.—*Method.*—First determine the total nitrogen.

1. Place exactly 1 c.c. of serum in an 800 c.c. Kjeldahl flask. Add 5 Gm. of potassium sulfate, a crystal of copper sulfate, and 15 c.c. of concentrated sulfuric acid. Digest until the solution is light green in color.

2. When the flask cools, fill it about half full with ammonia-free water, add 80 to 100 c.c. of 40 per cent sodium hydroxide.

3. Distill completely the ammonia that is formed in 20 c.c. of decinormal solution of sulfuric acid which contains a few drops of Congo red, or alizarin indicator.*

4. Titrate the partly neutralized decinormal sulfuric acid solution with decinormal solution of sodium hydroxide.*

Calculation.—One c.c. of a decinormal solution of sulfuric acid is equivalent to 0.0014 Gm. of nitrogen (20 − number cubic centimeters of decinormal alkali used) × 1.4 × 100 = mg. total nitrogen in 100 c.c. of serum. (It is more accurate to carry out a blank determination, making the back titration, and using the figure obtained in place of 20 in the above formula. For example, if 19.8 c.c. of alkali are used, then use this figure instead of 20.) The next procedure is to determine the nonprotein nitrogen.

Method for Nonprotein Nitrogen.—This is described on page 363.

The total protein equals (*total nitrogen* − *nonprotein nitrogen*) × 6.25.

Albumin-globulin Ratio—Albumin-nitrogen Determination.—(Kingsley[1]

* If in step 3, in place of the decinormal solution of sulfuric acid, a 2 per cent solution of boric acid is used to which has been added a double-dye indicator given in the footnote reference on page 368 in the proportion of 3 c.c. to every 60 c.c. of 2 per cent solution of boric acid, the titration may be carried out with decinormal sulfuric acid instead of decinormal solution of sodium hydroxide. The calculation then is titration × 140 = mg. of total nitrogen per 100 c.c.

[1] Kingsley, G. R.: A Rapid Method for the Separation of Serum Albumin and Globulin. Jour. Biol. Chem. *133*:731–735 (May), 1940.

method). *Method.*—1. Place 15 c.c. of 23 per cent solution of anhydrous sodium sulfate (stored in an incubator at 37° C.) in a 50 c.c. centrifuge tube. Add exactly 1 c.c. of serum.

2. Add 6 c.c. of ethyl ether (U. S. P.) and stir the mixture vigorously with a glass rod for thirty seconds. Cap the tube to avoid loss of ether and centrifuge at 2200 revolutions per minute for ten to fifteen minutes.

3. Slant the tube so that the tightly packed globulin layer floating on the sodium sulfate solution is separated from the walls of the tube. Insert a pipet through the ether layer along the lower wall of the tube and remove 10 c.c. of the centrifugate.

4. Carry out the digestion, distillation, and back titration as for serum protein nitrogen determination.*

Calculation.—(20 − back titration) × 1.4 × 16/10 × 100 − nonprotein nitrogen = (20 − back titration) × 224 − nonprotein nitrogen = mg. albumin nitrogen per 100 c.c.

Protein nitrogen − albumin nitrogen = globulin nitrogen.

Albumin nitrogen per 100 c.c. × 6.25 = albumin per 100 c.c. of serum.

Globulin nitrogen per 100 c.c. × 6.25 = globulin per 100 c.c. of serum.

$$\text{The albumin-globulin ratio (A/G)} = \frac{\text{Albumin per 100 c.c.}}{\text{Globulin per 100 c.c.}}$$

The normal total protein is 6.5 to 8 Gm. per 100 c.c. of serum. The normal A/G ratio is 1.7/1 *to* 3/1.

Fibrinogen.—Fibrinogen in plasma may be determined by the Kjeldahl method. Estimate the total protein in the plasma, making the appropriate correction for nonprotein nitrogen. The fibrinogen is estimated from the difference between total protein and the protein content of the filtrate that is obtained after precipitation of fibrinogen by calcium chloride. Fibrinogen is increased in pregnancy, pneumonia, and infections which are accompanied by leukocytosis. It is decreased in typhoid fever, in chloroform and phosphorus poisoning, and in acute yellow atrophy of the liver.

Fibrinogen Determination.—1. Place 1 c.c. of plasma in a 50-c.c. graduated cylinder. Add 48 c.c. of 0.9 per cent sodium chloride solution, and 1 c.c. of 2.5 per cent solution of calcium chloride. Mix and let stand for twenty minutes.

2. Break up the clot that forms with a glass rod, and filter. The clot may be removed by wrapping it around the glass rod by rotating it in the cylinder.

3. Determine the protein content of the filtrate by first determining the nitrogen content by the Kjeldahl method.

Calculation.—Total protein in plasma − protein in fibrinogen-free filtrate = fibrinogen. The normal amount of fibrinogen in plasma is 0.3 to 0.6 per cent.

Falling Drop Method of Determining Total Protein.—The total protein in serum or plasma may be determined by measuring first the specific gravity by the falling drop method as described by Kagan.[1] There is a linear relationship between the specific gravity and the protein content. Fig. 189 illustrates the use of an apparatus for determining the specific gravity by this method.

Scudder[2] described this method of estimating the amount of total protein in the treatment of shock.

* See footnote reference (*) for total serum protein determination. The calculation would then be titration × 224 − the nonprotein nitrogen = mg. of albumin nitrogen.

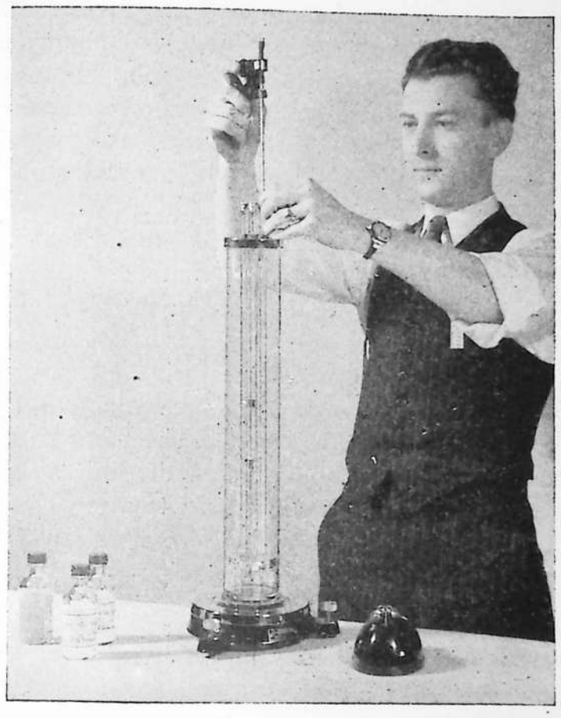


Fig. 189.—The "falling drop" method of determining total protein.

D. Lipids, Cholesterol, Cholesterol Esters, and Lecithin

In certain diseases the study of fat metabolism has assumed considerable importance.

Estimations of total lipids, or more often, of cholesterol, cholesterol esters, or lecithin, have become clinical laboratory procedures. Increased cholesterol values are found in chronic and acute nephritis,

[1] Kagan, B. M.: A Simple Method for the Estimation of Total Protein Content of Plasma and Serum. I. A Falling Drop Method for the Determination of Specific Gravity. II. The Estimation of Total Protein Content of Human Plasma and Serum by the Use of the Falling Drop Method. Jour. Clin. Invest. *17*:369–376 (July), 1938.

[2] Scudder, John: Shock, Blood Studies as a Guide to Therapy. Philadelphia, J. B. Lippincott Company, 1940, 315 pp.

and are of diagnostic significance in chronic nephrosis which is accompanied by a decrease in the blood urea. In diabetes, there may be an increase of cholesterol in the blood, which is reduced with the use of insulin. In lipemia, the values for the lipids are very high. Other conditions which are accompanied by hypercholesterolemia are pregnancy, cholelithiasis, and hypothyroidism. On the other hand, hyperthyroidism is characterized by low cholesterol findings. In studies on blood lipoids, Epstein and Lande[1] demonstrated that the blood cholesterol is high when the basal metabolic rate is low and that it is low when the basal metabolic rate is high. Mason, Hunt, and Hurxthal[2] concluded that there is no definite correlation between cholesterol and the basal metabolic rate, but that the changes in cholesterol content were very significant. There is a markedly elevated blood cholesterol in true myxedema, which is reduced with administration of thyroid extract. Greene, Hotz and Leahy[3] found that patients with evident hepatic damage had decreased combined cholesterol in the blood. At times there was also a decrease in the total cholesterol but the ratio between cholesterol and cholesterol esters is not as diagnostic as the total amount of esters. Progressive decrease in cholesterol esters in hepatic disease is a poor prognostic sign.

Determination of Blood Cholesterol.—The method is that which has been described by Bloor,[4] and which is dependent on the Lieberman-Burchard color reaction produced by treating cholesterol with acetic anhydride and sulfuric acid. The normal amount is 165 to 200 mg. per 100 c.c. of plasma.

Reagents Required.—(*a*) Alcohol-ether. Mix 3 parts of pure grain alcohol (95 per cent) with 1 part of anesthesia ether (redistilled).

(*b*) Chloroform (Mallinckrodt, redistilled).

(*c*) Concentrated sulfuric acid.

(*d*) Acetic anhydride.

(*e*) Standard solutions:

(1) Stock standard. Dissolve exactly 200 mg. of pure cholesterol in exactly 200 c.c. chloroform (*b*); 1 c.c. = 1 mg. cholesterol.

[1] Epstein, A. A., and Lande, Hermann: Studies on Blood Lipoids. I. The Relation of Cholesterol and Protein Deficiency to Basal Metabolism, Arch. Int. Med., *30*:563–577 (Nov.), 1922.

[2] Mason, R. L., Hunt, H. M., and Hurxthal, L. M.: Blood Cholesterol Values in Hyperthyroidism and Hypothyroidism—Their Significance, New England Med. Jour., *203*:1273–1278 (Dec. 25), 1930.

[3] Greene, C. H., Hotz, Richard, and Leahy, Evelyn: Clinical Value of Determination of Cholesterol Esters of Blood in Hepatic Disease. Arch. Int. Med. *65*:1130–1143 (June), 1940.

[4] Bloor, W. R.: The Determination of Cholesterol in Blood, Jour. Biol. Chem., *24*:227–231, 1916.

(2) Dilute standard. Take exactly 10 c.c. of stock standard (1) and make up to 100 c.c. with pure chloroform (*b*); 5 c.c. = 0.5 mg. cholesterol.

Method.—1. Place 75 c.c. of reagent (*a*) in a 100-c.c. volumetric flask. Add slowly, from a volumetric pipet, exactly 3 c.c. of plasma or serum. Keep the contents of the flask in constant motion to avoid clumping of the precipitate.

2. Raise the temperature of the contents of the flask to boiling by placing in a water bath; constantly shake.

3. Cool to room temperature, and make up to 100 c.c. with alcohol-ether solution (*a*). Filter, and place the filtrate in a tightly stoppered bottle, if it is not convenient to carry out the next step at once.

4. Measure 10 c.c. of the filtrate into a small flat-bottomed beaker, and evaporate *just to dryness* on a water bath or electric stove.

5. Extract the cholesterol from the dry residue by boiling three or four times with successive small portions of chloroform (*b*), and decanting the extract into a calibrated 10-c.c. glass-stoppered, graduated cylinder. Make the combined extracts up to 5 c.c. with chloroform (*b*).

6. Measure into a similar 10-c.c. cylinder exactly 5 c.c. of standard cholesterol solution (*e*, 2).

7. Add to each of the solutions 2 c.c. of acetic anhydride (*d*), and 0.1 c.c. of concentrated sulfuric acid (*c*). Mix by inverting the stoppered cylinders several times, and place in a dark closet for ten minutes. Read the unknown in a colorimeter against the standard which is set at 10 mm.

Calculation:

$$\frac{10}{\text{Reading of Unknown}} \times 0.5 \times \frac{100}{3 \times 10} \times 100 = \text{mg. per 100 c.c.}$$

Determination of Cholesterol Esters.—The method of Bloor and Knudson[1] depends on the determination of cholesterol by the Bloor II method, which has been described previously, and on the precipitation of the free cholesterol with digitonin, and on the extracting of the esters with petroleum ether.

Reagents Required.—The reagents are the same as those which are used for the determination of cholesterol (*a*), (*b*), (*c*), (*d*), (*e*).

(*f*) One per cent alcoholic solution of digitonin.

(*g*) Petroleum ether.

Method.—1. The total cholesterol is determined by the method described.

2. Place 20 c.c. of alcohol-ether extract (see step 3 in previous test), in a small flat-bottomed Erlenmeyer flask. Add 1 c.c. of digitonin solution (*f*). (Digitonin cholesteride, which is insoluble in petroleum ether, is formed with the free cholesterol.)

3. Evaporate *just to dryness*.

4. Extract the dried residue with successive small amounts of petroleum ether (boiling below 60° C.). It is best to start with about 15 c.c. of solvent,

[1] Bloor, W. R., and Knudson, Arthur: The Separate Determination of Cholesterol and Cholesterol Esters in Small Amounts of Blood, Jour. Biol. Chem., *27*:107–112, 1916.

cover the flask with a watch glass, and boil gently until about half the liquid is gone. The succeeding extractions are made in a similar manner, using 7 to 8 c.c. of petroleum ether.

5. Evaporate the combined extracts, which contain the cholesterol esters, *just to dryness*, and follow the same procedures as in steps 5, 6, and 7 in the preceding method.

Calculation:

$$\frac{10}{\text{Reading of Unknown}} \times 0.5 \times \frac{100}{3 \times 20} \times 100 = \text{mg. cholesterol esters per 100 c.c. of plasma or blood.}$$

Total Lipid in Blood Plasma.—It is sometimes desirable in certain diseases, such as arthritis or diabetes, to determine the value for total lipids and the cholesterol by the method described and to calculate the value for the total fatty acids, which is usually about twice that of the cholesterol. For example, if the value for cholesterol is 175 mg. per 100 c.c. of plasma, the value for the total fatty acids would normally be about 350 mg. per 100 c.c., and that for the total lipids would be about 525 mg. per 100 c.c. of plasma.

The method for the determination of the total lipids is somewhat more tedious than those methods which have been described for the determination of cholesterol and lecithin, although the principle can be readily understood by carefully following the steps in the method which has been devised by Bloor.[1]

Apparatus.—Water bath that can be maintained at 88° to 90° C. or an electrically heated hot air oven that can be maintained at that temperature, and a glass stoppered, 125 to 150 c.c. digestion flask.

Reagents.—See reagents required for cholesterol and cholesterol esters, (*a*), (*b*), (*c*), (*d*) and (*e*). ([*f*] is not used in this test.)

(*g*) Petroleum ether. Boil below 60° C., wash with concentrated sulfuric acid and redistill.

(*h*) Sodium ethylate, approximately normal. Dissolve 2 to 3 Gm. of clean metallic sodium in 100 c.c. of absolute alcohol. Keep the solution cool during its preparation and store in a cool, dark place. Discard when discolored.

(*i*) Dilute sulfuric acid. Take one part of concentrated sulfuric acid (*c*) and three parts of distilled water.

(*j*) Sulfuric acid reagent. Dissolve 5 Gm. of silver nitrate in 25 c.c. of water in a 100 c.c. centrifuge tube. Add 5 Gm. of potassium dichromate dissolved in about 50 c.c. of water. Remove the precipitated silver dichromate by centrifugation. Wash twice with water, then centrifuge to remove the nitric acid. Dissolve the precipitated cake of silver dichromate without drying in 500 c.c. of concentrated sulfuric acid (*c*).

(*k*) Normal solution of potassium dichromate.

(*l*) Twenty per cent solution of potassium iodide.

[1] Bloor, W. R.: The Determination of Small Amounts of Lipid in Blood Plasma. Jour. Biol. Chem. 77:53–73 (Apr.), 1928.

(*m*) Tenth normal solution of sodium thiosulfate.

(*n*) One per cent solution of starch.

Method.—1. Make an ether alcohol extract as described for determination of cholesterol on page 381, using 3 c.c. of blood plasma.

2. Measure 15 c.c. of the alcohol ether extract into a 100 c.c. Erlenmeyer flask. Add 2 c.c. of sodium ethylate (*h*) and evaporate on the water bath until there is no odor of alcohol. Sweep the traces of alcohol vapor from the flask by a gentle current of air, leaving the residue pasty but not dry.

3. Add 1 c.c. of dilute sulfuric acid (*i*). Heat on the water bath for one minute and add to the hot mixture 10 c.c. of petroleum ether (*g*). Rotate the flask gently on the water bath for two or three minutes. Pour off the solvent completely from the watery residue into a 25 c.c. volumetric flask. Repeat the extraction two or three times, using 5 c.c. portions of petroleum ether, washing down the sides of the flask and pouring off the solvent completely until the volumetric flask is nearly full. Cool to room temperature and fill the flask to the 25 c.c. mark.

4. Measure 10 c.c. of petroleum ether extract into a 125 c.c. glass stoppered digestion flask. Evaporate the solvent and blow out the last traces with a gentle stream of air. Add 5 c.c. of the sulfuric acid reagent (*j*). Add 3 c.c. of normal potassium dichromate (*k*) rotating the flask. Prepare a control, containing all of the reagents except the extracted fatty material, and run along with the samples under exactly the same conditions. Loosely stopper the flasks and set on the steam bath or in the hot air oven at 88° to 90° C. After five minutes, remove and rotate the flasks to mix the contents. Then tightly insert the stoppers and replace the flasks in the oven for a total of sixty minutes. Remove the flasks and without cooling add 75 c.c. of distilled water.

5. Add 5 c.c. of 20 per cent solution of potassium iodide (*l*) and without stirring run in tenth normal solution of sodium thiosulfate (*m*) while rotating the flask gently. There will be a white precipitate formed by the silver, but this does not interfere with the end point of the titration. When the titration is nearly complete, add a few drops of 1 per cent solution of starch (*n*). The solution will turn a muddy, dark blue. Continue the titration until the starch indicator suddenly turns a clear light blue (aqua color). Then carry out the titration on the blank in the same manner. The difference between the titration of the blank and the sample will be the amount of tenth normal solution of dichromate used by the fatty material in the oxidation.

6. Determine the amount of cholesterol in the alcohol ether extract in the usual manner.

Calculation.—Titration of the blank minus the titration of the sample is the equivalent to 9.55 c.c. of tenth normal solution of potassium dichromate. As 1 mg. of cholesterol requires 3.92 c.c. of tenth normal solution of dichromate for complete oxidation, multiply the number of milligrams in a 10 c.c. aliquot (or 0.36 c.c. of plasma) by 3.92. Subtract the cholesterol equivalent in cubic centimeters of dichromate solution from the number of cubic centimeters of dichromate equivalent required for complete oxidation.

The result equals the equivalent for the total fatty acids. Divide this number by 3.6 and the result will equal the weight of the total fatty acids in the 10 c.c. aliquot. The final aliquot represents 0.36 c.c. of plasma (3 c.c. of plasma dissolved in 50 c.c.; 15 c.c. taken equals $3/50 \times 15 = 0.9$ c.c. of plasma; 0.9 c.c. of plasma in 25 c.c. petroleum ether, 10 c.c. taken equals $10/25 \times 0.9 = 0.36$ c.c. of plasma).

Formula. $\frac{100 \times \text{weight of fatty acid in aliquot}}{0.36} = \text{total fatty acids}$ (milligrams per cent). Total fatty acids (milligrams per cent) + cholesterol (milligrams per cent) = total lipid (milligrams per cent).

Determination of Phospholipids.—The method for the determination of lecithin (lipoid phosphorus) in blood that is now preferred is that of Youngburg and Youngburg.[1] Lecithin contains about 4 per cent of phosphorus. The normal amount of lipoid phosphorus in whole blood is 12 to 14 mg., or 300 to 350 mg. of lecithin for each 100 c.c. About half of this is in the corpuscles.

Reagents Required.—All solutions must be made with phosphate-free water. If a blank determination gives a blue color, the contaminated reagent must be detected and eliminated.

(*a*) Alcohol-ether mixture. Mix 3 parts of pure ethyl alcohol (95 per cent) with 1 part of anesthesia ether (redistilled).

(*b*) Sulfuric acid, 10 N. Add 450 c.c. of concentrated sulfuric acid to 1200 c.c. of water. Titrate this solution with normal sodium hydroxide and dilute to make a 10 N solution of sulfuric acid.

(*c*) Thirty per cent hydrogen peroxide. Merck's superoxol, blue label. Purchase in 50-Gm. containers and keep in the refrigerator.

(*d*) Molybdate-sulfuric acid mixtures. Make a 7.5 per cent solution of sodium molybdate (Baker's P-free).

(A) Mix 50 c.c. of 7.5 per cent sodium molybdate with 50 c.c. of 10 N sulfuric acid (*b*).

(B) Mix 50 c.c. of 7.5 per cent sodium molybdate with 25 c.c. of water and 25 c.c. of 10 N sulfuric acid (*b*).

(*e*) Stock stannous chloride solution. Dissolve 15 Gm. of stannous chloride, c.p., in 25 c.c. concentrated chemically pure hydrochloric acid. Store in glass-stoppered brown bottles. Prepare fresh solutions each month.

(*f*) Dilute stannous chloride solution. Dilute 1 c.c. of stock solution (*e*) to 200 c.c. with water, mix well. Discard any unused solution.

(*g*) Stock standard phosphate solution. Dissolve 0.4389 Gm. of pure dried monopotassium phosphate (Baker's analyzed) in distilled water, enough to make 100 c.c. (1 c.c. = 1 mg. of phosphorus). To prevent the formation of mold add a few drops of chloroform.

[1] Youngburg, G. E. and Youngburg, Mamie V.: Phosphorus Metabolism. I. A System of Blood Phosphorus Analysis, Jour. Lab. and Clin. Med., *16*:158–166 (Oct.), 1930. The slight modifications in technic are those developed by Elizabeth Maclay, Section of Clinical Chemistry, Mayo Clinic.

(*h*) Dilute standard phosphate solution. For use in the test, dilute 2 c.c. of stock standard solution (*g*) to 100 c.c. (1 c.c. = 0.02 mg. of phosphorus). Make fresh dilute standard every three or four days.

Method.—1. Place 35 c.c. of alcohol-ether mixture (*a*) in a 50-c.c. volumetric flask. Add slowly from a volumetric pipet, exactly 3 c.c. of plasma of serum. Keep the contents of the flask in constant motion to avoid clumping of the precipitate.

2. Raise the temperature of the contents of the flask to boiling by placing in a water bath; shake constantly.

3. Cool to room temperature, and make up to 50 c.c. with alcohol-ether mixture (*a*). Filter.

4. Transfer 5 c.c. of alcohol-ether extract to a pyrex test tube (180 × 18 mm.) graduated at 15 c.c. Add one or two glass beads and evaporate the extract to dryness by placing the tubes in a boiling electric water bath.

5. Add to the residue 0.5 c.c. of 10 N sulfuric acid (*b*) and digest over a micro-burner, with the tube in a slanted position, until the organic matter is carbonized. Cool. Add two drops of perhydrol (*c*), heat on micro-burner again. Cool. If not clear add one or two drops of perhydrol and heat again. Sweep out the last remaining fumes of perhydrol by passing the tube through an open flame two or three times to heat it all the way to the top. Cool.

6. Add 2 c.c. of water. Heat to boiling to convert the metaphosphate or pyrophosphate to orthophosphate. Add 4 c.c. of distilled water and 2 c.c. of molybdate-sulfuric acid reagent (*d*, B).

7. To another tube transfer 1 c.c. of standard phosphate solution (*h*). Add 4 c.c. of water and 2 c.c. of molybdate-sulfuric acid reagent (*d*, A).

8. To each tube add 1 c.c. of dilute stannous chloride solution (*f*). Dilute to the 15-c.c. mark, mix quickly and after 1 minute compare in the colorimeter with the standard set at 15 mm.

Calculation:

$$\frac{15}{\text{Reading of Unknown}} \times 0.02 \times 333 = \text{mg. phosphorus per 100 c.c.} \times 25 = \text{mg. lecithin per 100 c.c.}$$

E. Blood Chlorides

The normal amount of sodium chloride in the whole blood is 0.45 to 0.50 per cent. The plasma, however, contains 0.55 to 0.65 per cent. In nephritis, eclampsia, anemia, and at times in malignant conditions and cardiac disease, the chloride content of the blood is above normal. In cases with fever, in diabetes, in pneumonia, and in severe toxemia with obstructions of the upper gastro-intestinal tract the blood chlorides are low. In the treatment of this last condition the administration of sodium chloride, along with dextrose and water, has been found of value.

Many methods have been devised for the estimation of blood chlorides. The two methods described have proved very satisfactory.

Method of Wilson and Ball.[1]—*Reagents Required.*—(*a*) Standard silver nitrate solution (fifteen hundredths normal).

(*b*) Concentrated nitric acid.

(*c*) Five per cent ferric alum solution.

(*d*) Fiftieth normal solution of ammonium thiocyanate.

Method.—1. Place 1 c.c. of serum or whole blood in an Erlenmeyer flask. Add 1 c.c. of standard silver nitrate solution (*a*), and 3 c.c. of nitric acid (*b*).

2. Digest for from fifteen to forty minutes in a boiling water bath.

3. Add 6 c.c. of (*c*), and cool to room temperature, or below; the colder the solution, the sharper the end-point.

4. Titrate with (*d*). Carry titration until 1 drop causes a color change which persists for about one minute at room temperature. The end-point is sharp.

Calculation.—0.02 to 0.04 c.c. is subtracted from the titration to correct for the amount of thiocyanate required to give a suitable end-point in the presence of the silver chloride and thiocyanate, the nitric acid, and the water. (Blank titration [7.5] − back titration) × 0.711 × 100 = mg. chlorine per 100 c.c. (7.5−back titration) × 1.17 × 100 = mg. sodium chloride per 100 c.c.

Plasma Chlorides. Method of Osterberg and Schmidt.[2]—*Reagents Required.*—(*a*) Silver nitrate solution. Dissolve 4.791 Gm. of chemically pure silver nitrate in distilled water, and dilute to 1 liter in a volumetric flask (1 c.c. is equivalent to 1 mg. of chlorine). Preserve in a brown bottle.

(*b*) Potassium sulfocyanate solution. Dissolve about 3 Gm. of potassium sulfocyanate (KCNS) in 1 liter of water. Standardize this solution against the silver nitrate solution so that 5 c.c. is exactly equivalent to 5 c.c. of the silver nitrate solution.

(*c*) Ferric ammonium sulfate, 20 per cent solution.

(*d*) Nitric acid solution: One part nitric acid added to 3 parts of water.

Method.—1. Place exactly 1 c.c. of plasma in a 125-c.c. Erlenmeyer flask. Add slowly 10 c.c. of (*d*) from a buret, agitating the flask to form a white, flocculent precipitate.

2. Add 5 c.c. of silver nitrate solution (*a*), and 1 c.c. of ferric ammonium sulfate (*c*).

3. Back titrate the excess silver with potassium sulfocyanate (*b*).

Calculation.—Five − number of cubic centimeters of (*b*) used in titration × 100 = mg. of chlorine for each 100 c.c. of plasma. Multiply by 1.65 to obtain mg. of sodium chloride per 100 c.c. of plasma.

[1] Wilson, D. W., and Ball, E. G.: A Study of the Estimation of Chloride in Blood and Serum, Jour. Biol. Chem., *79:*221–227 (Sept.), 1928.

[2] Osterberg, A. E., and Schmidt, Edna V.: The Estimation of Plasma Chlorides, Jour. Lab. and Clin. Med., *13:*172–175 (Oct.), 1927.

Photelometric Determination of Chlorides.—Hoffman[1] has modified the Sendroy[2] method for determination of chloride. He stated that this method has been one of the most accurate and satisfactory methods.

F. Blood Sugar

In health, the blood contains about 100 mg. of a reducing substance calculated as dextrose in each 100 c.c. By the Folin and Wu method, 90 to 120 mg. has been widely accepted as the upper and lower limits of normal, respectively. Higher values constitute *hyperglycemia.* These figures are based upon blood taken in the morning before breakfast. For a short time following meals rich in carbohydrates there is a slight rise, "normal alimentary hyperglycemia."

Under ordinary conditions the kidneys allow only negligible traces of sugar—too small for detection by the ordinary clinical tests—to pass out into the urine. When, however, from any cause the blood sugar progressively rises, a point is finally reached when the kidneys no longer hold the sugar back, but begin to excrete it actively, and it then appears in the urine in notable quantities. The point at which the "renal barrier" is overcome is called the "renal threshold for sugar," although the nature and significance of the phenomenon are not well understood. The threshold varies considerably in different persons and under various conditions—is lower, for example, when diuresis exists—but is ordinarily about 170 to 180 mg. blood sugar for each 100 c.c. of blood; it may be as low as 140 mg. in apparently healthy individuals. Obviously, then, the appearance of sugar in the urine in any given case is the resultant of two factors: (*a*) The blood-sugar concentration and (*b*) the renal threshold.

Ingestion of approximately 100 Gm. of glucose causes a sharp rise of blood sugar to about 150, or even 160 mg., in each 100 c.c. in one-half to one hour, the exact time depending somewhat upon the rate of absorption, with a gradual return nearly or quite to the original level by the end of the second hour. Sugar does not appear in the urine. A higher rise or a slower return to the normal, sometimes with the appearance of sugar in the urine, occurs in a number of pathologic conditions to be mentioned later. This is known as the "sugar tolerance test," but the name is somewhat confusing, since low tolerance results in a high blood-sugar curve and high tolerance in a low flat curve. The rate of absorption influences the height of the curve and the time at which this is reached, but does not usually materially

[1] Hoffman, W. S.: Photelometric Clinical Chemistry. New York, Wm. Morris and Company, 1941, pp. 129–137.

[2] Sendroy, Julius, Jr.: Photoelectric Microdetermination of Chloride and Biological Fluids, and of Iodate in Protein-free Solutions. Jour. Biol. Chem. *130:*605–623 (Oct.), 1939.

affect the type of curve. Carbohydrate restriction of the diet will cause a marked delay in the rate of utilization by a normal individual. The antecedent diet should always contain a normal amount of carbohydrate before undertaking a sugar tolerance test.

The **sugar tolerance test** is carried out as follows:[1]

1. Direct the patient to take no food after 7 P. M.

2. On the following morning obtain blood for determination of the fasting blood sugar, and give the patient 1.75 Gm. dextrose in solution for each kilogram (2.2 pounds) of his body weight. Many workers give 100 Gm. of dextrose to all patients. Some patients find it impossible to retain so much. The solution is best taken cold. It is prepared by dissolving pure dextrose in water in the proportion of 10 c.c. of water for each 4 Gm. of dextrose and adding the juice of a lemon for flavoring.

3. Secure blood for sugar determinations at the end of the first half hour, and the first, second, and third hour. In routine work it will generally suffice to take blood at the end of the second and third hours only. It may be possible to enter the vein several times through the same puncture. Estimate blood sugar and plot a curve.

4. Collect a twenty-four-hour sample of urine, beginning at the time the glucose is given, and make qualitative and quantitative tests for glucose.

Exton and Rose[2] have proposed a modification of the sugar tolerance test, and definite criteria for interpretation. This method is based on the law that when more sugar is administered to normal individuals, more sugar will be utilized, while the reverse is true in diabetes. The test is as follows: Dissolve 100 Gm. of dextrose in 650 c.c. of water. Flavor with lemon and divide into two portions.

1. Collect the first blood and urine samples, and give the first dose of dextrose (50 Gm.).

2. Thirty minutes later collect the second blood sample, and give the second portion of dextrose (50 Gm.).

3. Thirty minutes later collect the third blood sample, and the second urine sample.

4. The patient is given a container for a third sample of urine which is the next urine voided, if considered desirable.

With this method, the typical criteria of normal response are: (*a*) A fasting blood sugar within normal limits; (*b*) an increase in blood sugar of not more than 75 mg. in the thirty-minute blood sample; (*c*) a further rise of not more than 5 mg. in the sixty-minute blood sample, and there may be even a fall; (*d*) all of the samples of urine should not reveal sugar with Benedict's test. The chief criterion for diabetes mellitus is in the consideration of the sixty-minute blood sample. If there is a steep rise of not

[1] Janney, N. W., and Isaacson, V. I.: A Blood Sugar Tolerance Test, Jour. Am. Med. Assn., *70*:1131–1134 (Apr. 20), 1918.

[2] Exton, M. G., and Rose, A. R.: One Hour Two-dose Dextrose Tolerance Test, Am. Jour. Clin. Path., *4*:381–399 (Sept.), 1934.

less than 10 mg. above that which occurs in the thirty-minute sample, and if sugar also appears in the urine, the test is diagnostic of this disease. In renal glycosuria, blood sugar follows the normal curve, though sugar appears in both the first and second samples of urine, while in alimentary glycosuria the blood sugar is normal, and sugar appears only in the last sample of urine. This test avoids one venipuncture.

Gould, Altshuler and Mellen[1] modified the technic of Exton and Rose slightly but administered the glucose in two doses. They found that the following criteria for the diagnosis of diabetes mellitus were more satisfactory than the criteria advanced by Exton and Rose: (1) the value for the fasting blood sugar must exceed 120 mg. per 100 c.c., (2) the value for the sugar in the specimen of blood obtained thirty minutes after the ingestion of the last dose of glucose must exceed the value for the fasting blood sugar by 50 mg., or more, per 100 c.c. and (3) the value for the sugar in the specimen of blood obtained at the end of one hour must exceed by 30 mg., or more, per 100 c.c. the value for the sugar in the specimen obtained at the end of thirty minutes.

The study of blood sugar has clinical value in diabetes mellitus, in renal glycosuria, and in certain other conditions.

1. **Diabetes Mellitus.**—The important consideration here is not so much the glycosuria as the hyperglycemia, which the glycosuria is an attempt to relieve. The degree of hyperglycemia depends upon the severity of the disease, upon the diet, and upon the renal threshold. In mild cases blood sugar may be 150 to 250 mg. in each 100 c.c., and with the lower figure the urine is free from sugar; in severe cases, 300 to 400 mg. or higher. The renal threshold is inconstant: In early cases generally unchanged; in long-continued cases sometimes low, sometimes high. It is particularly high in cases with coexisting nephritis, in which sugar may fail to appear in the urine when blood sugar rises to 300 mg. or above.

In the tolerance test the maximum blood-sugar concentration is generally reached in about two hours, and it often does not fall to the normal level before the end of the third or fourth hour. The test has great value in the diagnosis of very early diabetes and even of the "prediabetic state." Here curves similar to that given in Fig. 190 for hyperthyroidism, although often lower, may be expected.

2. **Renal Glycosuria.**—This has been discussed on page 187. The essential feature is the occurrence of sugar in the urine without increase of sugar in the blood, owing to lowered renal threshold.

3. **Other Conditions with Altered Blood Sugar.**—In general it

[1] Gould, S. E., Altshuler, S. S., and Mellen, H. S.: The One-hour Two-dose Glucose Tolerance Test in the Diagnosis of Diabetes Mellitus, Am. Jour. Med. Sci., *193*:611–617 (May), 1937.

appears that overfunction of the ductless glands is accompanied by hyperglycemia and underfunction by hypoglycemia, but the change in fasting blood sugar is so uncertain as to lack diagnostic value. Of much greater significance is the blood-sugar curve after ingestion of glucose. Most conditions of hyperfunction of these glands, particularly hyperthyroidism and hyperpituitarism, cause an accentuation of the curve with more or less pronounced prolongation. The curve is generally much flattened in hypothyroidism, hypopituitarism, and Addison's disease. Typical curves are shown in Fig. 190.

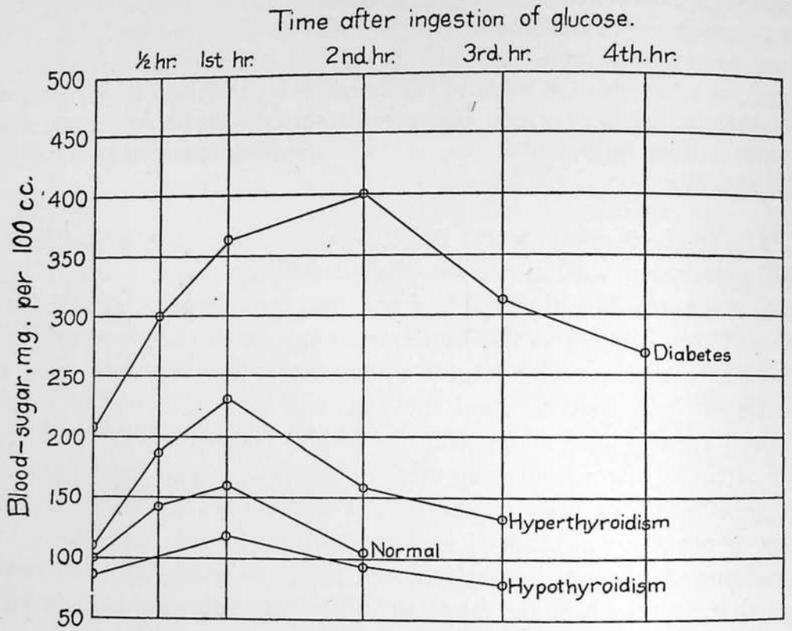


Fig. 190.—Chart showing blood-sugar curves obtained in the tolerance test.

In chronic nephritis the renal threshold for sugar is generally raised, although sometimes lowered, and the alimentary hyperglycemia curve is generally accentuated and prolonged. Slight or moderate alterations in blood sugar occur in many other diseases, but are probably of no diagnostic importance. A diminished sugar tolerance may be due to diminished ability of the liver to store glycogen.

The methods described below are those that have been found useful in most laboratories. However, both Folin[1] and Benedict[2]

[1] Folin, Otto: The Determination of Sugar in Blood and Normal Urine, Jour. Biol. Chem., *67*:357–370 (Feb.), 1926.

[2] Benedict, S. R.: The Estimation of Sugar in Blood and Normal Urine, Jour. Biol. Chem., *68*:759–767 (June), 1926.

devised new methods, and later Folin[1] offered still further revisions. The tendency now is to use reagents and methods that yield lower normal values.

Method of Folin and Wu.—*Reagents Required.*—(*a*) Standard sugar solutions. Prepare a stock solution containing 10 Gm. pure anhydrous dextrose in 1000 c.c. distilled water, and preserve with a little xylene. From this prepare the two standard solutions used for blood-sugar determinations as follows:

Standard No. 1. Place 5 c.c. of the stock solution in a 500-c.c. volumetric flask, add distilled water to the mark, and mix. Two c.c. of this standard solution contain 0.2 mg. dextrose.

Standard No. 2. Place 5 c.c. of the stock solution in a 250-c.c. volumetric flask, add distilled water to the mark, and mix. Two c.c. of this standard solution contain 0.4 mg. dextrose.

(*b*) Alkaline copper sulfate solution.[2] Dissolve 40 Gm. pure anhydrous sodium carbonate in about 400 c.c. of distilled water, and place in a 1000-c.c. volumetric flask. In this dissolve first 7.5 Gm. of tartaric acid, and then 4.5 Gm. crystalline copper sulfate. Make up to 1000 c.c. with distilled water, and mix. Should a precipitate of cuprous oxide form, after a week or two, owing to use of impure chemicals, it may be removed by filtering through a good quality paper.

(*c*) Molybdate phosphate solution. In a large beaker place 35 Gm. of molybdic acid, 5 Gm. of sodium tungstate, 200 c.c. of 10 per cent sodium hydroxide, and 200 c.c. of water. Boil vigorously for twenty to forty minutes. Cool, dilute to about 350 c.c., and add 125 c.c. of 85 per cent phosphoric acid. Make up to 500 c.c. with distilled water, and mix.

Fig. 191.—Folin's tube for use in blood-sugar estimations. The narrow portion is 4 cm. long and 8 mm. in diameter. The bulb must be of such size that 4 c.c. of fluid will reach into the lower third of the constricted portion, and not above it.

Method.—1. Transfer 2 c.c. of the protein-free blood filtrate, representing 0.2 c.c. blood (p. 362), to a Folin blood-sugar tube (Fig. 191).

Into a second blood-sugar tube place 2 c.c. of standard sugar solution

[1] Folin, O.: Two Revised Copper Methods for Blood Sugar Determination, Jour. Biol. Chem., *82*:83–93 (Apr.), 1929.

[2] Folin's 1929 revised copper solution. Stock solution (*a*) contains 35 Gm. anhydrous sodium carbonate, dissolved in 200 c.c. distilled water, 13 Gm. sodium tartrate, and 11 Gm. sodium bicarbonate dissolved in the order named in distilled water and made up to 1 liter. Stock solution (*b*) contains 5 per cent copper sulfate acidified with a trace of sulfuric acid. The working reagent is prepared by mixing 25 c.c. of the alkaline tartrate solution (*a*) with 5 c.c. of the copper solution (*b*) and making the total up to 50 c.c. Keep only a few days and only in the refrigerator when not in use.

No. 1, containing 0.2 mg. dextrose. (A standard containing 0.15 mg. dextrose may be used preferably.)

Into a third tube place 2 c.c. of standard sugar solution No. 2, containing 0.4 mg. dextrose.

2. To each tube add 2 c.c. of the alkaline copper sulfate solution, and mix. The surface of the fluid must reach the constricted portion of the tubes, but must not go above it.

3. Immerse the tubes in boiling water for six minutes.

4. Place in cold water, without shaking, for two to three minutes.

5. To each tube add 2 c.c. of the molybdate phosphate solution.

6. When the precipitate of cuprous oxide has dissolved, which should be within two minutes, dilute with water to the 25-c.c. mark, insert a rubber stopper, and mix thoroughly.

7. Compare the unknown in a colorimeter with the standard which it more nearly matches. Calculate the amount of sugar in 100 c.c. of blood upon the basis that the unknown represents 0.2 c.c. of blood and the two standards 0.2 and 0.4 mg. of dextrose, respectively. With the Duboscq type of colorimeter the following formula is applicable, D representing the number of milligrams of dextrose in the standard:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times D \times 500 = \text{mg. sugar in 100 c.c. of blood.}$$

Improved Folin-Wu Method.[1]—*Reagents Required.*—(*a*) Standard sugar solutions. The best preservative for a number of different substances which are likely to be decomposed by molds is a nearly saturated solution of benzoic acid. Dissolve 2.5 Gm. of benzoic acid in 1 liter of boiling water, and cool. Transfer to a bottle; the solution will keep indefinitely.

Stock standard solution. Dissolve 1 Gm. of pure anhydrous dextrose in 50 c.c. of the benzoic acid solution. (The most reliable dextrose for such standard solutions is that which is obtainable from the Bureau of Standards, Washington, D. C.) Transfer the solution to a 500-c.c. volumetric flask and dilute to volume with the saturated solution of benzoic acid. Transfer to a bottle, label, and preserve. The solution will keep indefinitely.

Working standard solution (1). Transfer 5 c.c. of this stock solution, containing 10 mg. of glucose, to a 100-c.c. volumetric flask and dilute to volume with water. This solution will keep for at least a week without any additional preservative, but if it is to be kept for a long time it is best to add a few drops of toluene.

Working standard solution (2). For work with diabetic blood it is necessary to have on hand another working standard which is twice as strong; take 10 c.c. of stock standard solution and dilute to 100 c.c., this solution accordingly contains 0.2 mg. per cubic centimeter or 0.4 mg. in the 2 c.c. actually used for each analysis.

[1] Folin, Otto: Two Revised Copper Methods for Blood Sugar Determinations, Jour. Biol. Chem., *82*:83–93 (Apr.), 1929. Laboratory Manual of Biological Chemistry, Ed. 5, New York, D. Appleton-Century Co., 1934, pp. 301–313.

(*b*) A 5 per cent solution of crystallized copper sulfate. Dissolve 25 Gm. of the salt in a little water, transfer to a 500-c.c. volumetric flask, add 5 or 6 drops of concentrated sulfuric acid, dilute to volume, and preserve in a clean bottle.

(*c*) Alkaline tartrate solution. Transfer 35 Gm. of anhydrous sodium carbonate to a volumetric liter flask, add 175 to 200 c.c. of water and shake for a few moments to bring the sodium carbonate into solution. Then add 13 Gm. of sodium tartrate (Merck's highest purity) and 11 Gm. of sodium bicarbonate. Add water to a volume of about 800 c.c., and shake until a clear solution is obtained. Dilute to volume and mix.

(*d*) Alkaline copper tartrate solution. This solution should be prepared fresh each day, because it may deteriorate by autoreduction if kept overnight. The reagent is simply a mixture of 9 volumes of the alkaline tartrate solution and 1 volume of copper sulfate solution.

(*e*) Acid molybdate reagent. The active ingredient of this reagent is probably some phosphomolybdate of unknown constitution. The reagent was originally devised as a part of the Folin-Wu method, and it is still probably the most dependable reagent for the colorimetric estimation of cuprous oxide.

For temporary use (not more than a week), the reagent can be quickly prepared as follows:

Dissolve 40 Gm. of sodium molybdate in 100 c.c. of distilled water in a 500-c.c. beaker. To the turbid solution add, while stirring, 55 c.c. of 85 per cent phosphoric acid, 40 c.c. of cool sulfuric acid (25 per cent; 1 volume of sulfuric acid to 3 volumes water), and finally 20 c.c. of 99 per cent acetic acid. The resulting mixture is ready for use.

For the preparation of a permanent reagent, which will not turn blue on standing, it is convenient to keep on hand a brominized 30 per cent solution of sodium molybdate.

By means of a funnel and glass rod, transfer 300 Gm. of sodium molybdate to a volumetric liter flask. Add about 800 c.c. of water and shake until solution is complete except for the turbidity. Dilute to volume, mix, and transfer this stock solution to a bottle. Add 0.2 to 0.3 c.c. of bromine. Shake and set aside until wanted.

Transfer 500 c.c. of the clear supernatant solution to a 1500-c.c. Florence flask. Add, while stirring, 225 c.c. of 85 per cent phosphoric acid. Some bromine is liberated and imparts a yellow color to the solution. Next add 150 c.c. of cool 25 per cent sulfuric acid, and let stand overnight. Remove the remaining bromine by means of an air current. Then add 75 c.c. of 99 per cent acetic acid, mix, and dilute to a volume of 1 liter. If kept protected from organic matter, this reagent will remain colorless for years.

The special "blood-sugar tube," which is illustrated in Fig. 191, is indispensable, because in open tubes reoxidation of cuprous oxide always occurs.

Method.—1. Transfer 2 c.c. of blood filtrate to a Folin-Wu sugar tube, or 1 c.c. plus 1 c.c. of water, if very high blood-sugar values are expected.

2. Transfer 2 c.c. of the sugar standard (*a*) (0.1 mg. of dextrose per cubic centimeter) to another similar tube.

3. Add 2 c.c. of freshly mixed copper tartrate reagent (*d*) to each tube and heat for eight minutes in a beaker of rapidly boiling water. Cool in running water.

4. Add 4 c.c. of the acid molybdate reagent (*e*) and, after waiting for about one minute, dilute to volume with water, or preferably with a solution containing 1 volume of the molybdate reagent (*e*) to 4 volumes of water. Mix, and make the color comparison.

Calculation:

$$\frac{20}{\text{Reading of Unknown}} \times 100 \text{ (or 200 if stronger standard used)} = \text{mg. per 100 c.c. of blood.}$$

Multiply by 2 if only 1 c.c. of blood filtrate is used.

Folin Micromethod for the Determination of Blood Sugar.[1]—*Reagents Required.*—(*a*) Sulfate-tungstate solution. Transfer to a 500-c.c. volumetric flask 10 Gm. of chemically pure anhydrous sodium sulfate and 15 c.c. of a 10 per cent solution of sodium tungstate. Half fill the flask with distilled water and shake until the sulfate has dissolved. Dilute to volume and mix.

(*b*) Sulfuric acid. Transfer 12 c.c. of a two thirds normal solution of sulfuric acid and 2 Gm. of anhydrous sodium sulfate to a 100-c.c. volumetric flask. Shake till the sodium sulfate has dissolved; then dilute to volume, and mix.

(*c*) Potassium ferricyanide solution. Dissolve 1 Gm. of chemically pure potassium ferricyanide in distilled water and dilute to a volume of 250 c.c. The major part of this solution should be kept in a brown bottle and in a dark closet. The part in daily use should also be kept in a brown bottle.

(*d*) Sodium cyanide-carbonate solution. Transfer 8 Gm. of anhydrous sodium carbonate to a 500-c.c. volumetric flask. Add 40 to 50 c.c. of water, and shake to promote rapid solution. With a cylinder, add 150 c.c. of freshly prepared 1 per cent solution of sodium cyanide; dilute to volume; and mix. It is easiest and best to prepare more sodium cyanide solution than is needed, and to throw away the unused portion.

(*e*) Ferric iron gum ghatti solution. Fill a liter cylinder with water. Suspend 20 Gm. of gum ghatti on a wire screen of copper or galvanized iron (just below the surface of the liquid) and leave overnight. Remove the screen, and strain the liquid through a double layer of a clean laboratory towel. Acacia may be substituted if more of this material is used. Transfer 30 Gm. of gum acacia to a liter Florence flask and add 600 c.c. of distilled water. Heat on a water bath, shaking the flask until the gum has all been dissolved.

Place 5 Gm. of anhydrous ferric sulfate and 75 c.c. of 85 per cent phosphoric acid in a 250-c.c. beaker which contains 100 c.c. of water; dissolve by the aid of heat. Cool this solution and add it, while stirring, to the

[1] Folin, Otto: Two Revised Copper Methods for Blood Sugar Determinations, Jour. Biol. Chem., *82*:83–93 (Apr.), 1929. Laboratory Manual of Biological Chemistry, Ed. 5, New York, D. Appleton-Century Co., 1934, pp. 301–313.

strained gum ghatti solution. Finally, add about 15 c.c. of a 1 per cent solution of potassium permanganate, a few cubic centimeters at a time. The purpose of the permanganate solution is to oxidize certain reducing impurities which are present in the gum ghatti. The slight turbidity of the solution will disappear completely if the solution is kept in a warm place (37° C.) for a few days. This reagent keeps indefinitely.

(*f*) Standard stock solution of dextrose. Dissolve 1 Gm. of benzoic acid in about 300 c.c. of hot distilled water. Weigh out exactly 980 mg. of chemically pure anhydrous dextrose on a watch glass. Rinse the dextrose through a funnel into a volumetric liter flask with the help of the warm benzoic acid solution; add about 400 c.c. of distilled water; then cool to room temperature; and finally, make up to volume, and mix. Transfer to a clean, dry, glass-stoppered bottle.

This stock solution will keep indefinitely if preserved with 0.1 per cent of benzoic acid. It is made to contain 0.98 mg. of dextrose per cubic centimeter instead of 1 mg. in order to make the calculations easier.

The dilute working standard may be obtained by diluting 1 c.c. of this stock solution to 100 c.c. By incorporating a little benzoic acid (no more than 25 mg.) into this solution, it will keep for a long time.

Method.—1. Transfer 4 c.c. of the sulfate-tungstate solution (*a*) to a clean dry centrifuge tube.

2. Prick a finger with the lancet, and collect exactly 0.1 c.c. of blood, by means of the micro-blood pipet. Transfer at once (before clotting has had time to occur) to the solution in the centrifuge tube. Rinse the pipet two or three times (by suction) and stir a little with the pipet. Let stand for about fifteen minutes, or longer if convenient.

3. Add 1 c.c. of the acid sulfate solution (*b*) and stir with the micro-pipet in the centrifuge tube, or with a fine glass rod. Centrifugalize for five minutes.

4. Transfer 2 c.c. of the water-clear supernatant solution in the centrifuge tube and also 2 c.c. of water to a 25-c.c. test tube which is graduated at 25 c.c.

5. To another similar test tube add 4 c.c. of the standard dilute dextrose solution (*f*).

6. To each tube add 1 c.c. (or 2 c.c.) of the 0.4 per cent solution of potassium ferricyanide (*c*) and then add 1 c.c. of the cyanide-carbonate solution (*d*) to each tube.

7. Heat both test tubes together in a beaker of boiling water for eight minutes, cool, and to each tube add 5 c.c. of the ferric iron gum ghatti solution (*e*).

8. Dilute the contents of the tubes to 25 c.c. and mix. Make the color comparison in the usual manner, with the standard set at 20 mm.

Calculation:

$$\frac{20}{\text{Reading of Unknown}} \times 100 = \text{mg. per 100 c.c.}$$

One detail in the determination has been left to the experience of the

worker. He has been given the choice of using 1 or 2 c.c. of the potassium ferricyanide solution. If 1 c.c. is used, the results are a little more accurate, particularly if the blood happens to contain a small amount of sugar. On the other hand, if only 1 c.c. is used, the determination is dependable only up to a little over 300 mg. per cent, and if more than this amount is found, the determination should be repeated with 1 c.c. of the blood extract plus 3 c.c. of water. When 2 c.c. of potassium ferricyanide are used, one single determination will cover the range from far less than 100 to more than 500 mg. per cent, but the highest values so obtained will tend to be a shade too low, and the lowest values a trifle too high. The reason for this is that the blank (autoreduction of the ferricyanide) is appreciably stronger when 2 c.c. of this solution are used.

Those who are easily puzzled by calculations may find it a little difficult to understand why the dilute standard dextrose solution is made to contain 0.0098 mg. of dextrose per cubic centimeter instead of 0.01 mg. The reason for this is that 0.1 c.c. of blood is diluted to a volume of 5.1 c.c. instead of to 5 c.c. Since 5.1 is 2 per cent greater than 5, the standard dextrose solution has been made 2 per cent weaker than 0.01 mg. The calculation can then be made as if the standard contained 0.01 mg. per cubic centimeter, and as if 0.1 c.c. of blood had been diluted to 5 c.c.

The blood-sugar values obtained by this micromethod are very close to the true dextrose values of the blood.

Another micromethod has been described by Byrd.[1] This method requires special glassware and has a unique tube in which 0.1 c.c. of blood may be measured and the blood laked and diluted in a bulb above the capillary portion. The reagents used are those of the regular Folin-Wu method, so that with these reagents alone, and the necessary apparatus, the laboratory has a choice of a macromethod or a micromethod.

Photelometric Method (Hoffman[2]).—The reduction of potassium ferricyanide to ferrocyanide by dextrose has been used successfully by Hagedorn and Jensen for the determination of blood sugar. Hoffman adapted this method.

Reagents.—Alkaline Ferricyanide Reagents.—(*a*) Dissolve 1.8 Gm. of pure potassium ferricyanide and 30 Gm. of anhydrous sodium carbonate in distilled water and make up to a liter.

(*b*) Dilute 65 c.c. of solution (*a*) to 100 c.c. Store both solutions in dark bottles away from sunlight.

Method for Blood.—Place exactly 2 c.c. of Folin-Wu filtrate in a large test tube marked for 16 c.c. Add exactly 2 c.c. of the diluted ferricyanide reagent (*b*). Place the tube in actively boiling water for five minutes. Cool

[1] Byrd, T. L.: A Micro-Folin-Wu Method of Quantitative Blood-sugar Estimation Using 0.1 c.c. of Blood, Jour. Lab. and Clin. Med., *11*:67–75 (Oct.), 1925. The pipets for this test can be obtained from the Central Scientific Co., Chicago.

[2] Hoffman, W. S.: Photelometric Clinical Chemistry. New York, Wm. Morris and Company, 1941, pp. 63–76.

Hoffman, W. S.: A Rapid Photoelectric Method for the Determination of Glucose n Blood and Urine. Jour. Biol. Chem. *120*:51–55 (Aug.), 1937.

in tap water and dilute to the 16 c.c. mark and mix by inversion using a clean rubber stopper. Read in the photolometer using the Cenco blue glass filter number 1.

Method for Urine.—Add 10 c.c. of N/20 oxalic acid to 10 c.c. of urine. Mix. Add 1.5 Gm. of Lloyd's reagent. Shake for two minutes. Filter through a small, dry filter. Dilute 5 c.c. of this filtrate to 25–50–100 or even 500 c.c., depending upon the concentration of dextrose expected from the qualitative test or from the specific gravity of the urine. To 2 c.c. of this dilute urine add 4 c.c. of solution (*b*) in a tube marked for 16 c.c. and proceed as directed above for blood filtrate.

Calculations.—Refer to curve or table of values prepared from standard solutions of dextrose made by diluting respectively 10–8–6–4–2 and 0 c.c. of a stock solution of dextrose containing 2 mg. per cubic centimeter to 100 c.c. These solutions are equivalent to Folin-Wu filtrates from blood specimens containing respectively 200–160–120–80–40 and 0 mg. per 100 c.c. These solutions are treated as described above for blood filtrates and the photelometer readings are charted on semi-logarithmic paper. The curve differs from the usual curve in that the concentration of glucose increases with higher galvanometric readings. If the ferricyanide solution is decomposed, it is necessary to repeat the analysis with a freshly prepared solution (*b*). This can be most easily determined by running a blank with each series of analyses using water alone with the ferricyanide.

G. Carbon Dioxide Combining Power of Blood Plasma

As is shown in the section on Acidosis, page 648, the determination of the capacity of the blood plasma to bind carbon dioxide offers what is probably the most reliable means of detecting acidosis and of measuring its degree. The normal range for adults is 53 to 75 c.c. of carbon dioxide in each 100 c.c. of blood plasma; infants, 40 to 55 per cent. Figures below 50 per cent in adults indicate acidosis; below 30 per cent, severe acidosis. Determinations are especially useful as a guide in alkali treatment of acidosis, during which the carbon dioxide combining power steadily rises. Palmer and Van Slyke estimate that as a rule 0.5 Gm. of sodium bicarbonate for each 42 pounds of body weight will raise the plasma carbon dioxide volume 1 per cent. Mild alkalosis without symptoms is indicated by figures of 80 to 90 per cent. Above 90 per cent there is evidence of severe alkalosis associated with symptoms of tetany.

Method of Van Slyke and Cullen.—I. *Obtaining the Blood.*—The patient should avoid muscular exercise for an hour or two before the blood is taken. About 6 to 8 c.c. of blood are required. Withdraw this from a vein with no more constriction than is necessary, and with the least possible exposure to the air; and immediately place it in a tube with enough dry potassium

oxalate to make about 0.5 per cent. Mix by gentle stirring. Separate the plasma by thoroughly centrifugalizing within half an hour.

A better plan is to secure the blood under oil in a device similar to that shown in Fig. 94, but with the entrance tube reaching to the bottom in which has been placed 2 or 3 c.c. of paraffin oil and the dry potassium oxalate. When this plan is followed it is essential that the arm constriction be loosened before the blood is withdrawn.

II. *Saturation with Carbon Dioxide.*—1. Transfer about 3 c.c. of the plasma to a large separation funnel arranged as shown in Fig. 192.

2. Without inspiring more deeply than is normal, blow air from the lungs into the funnel through the bottle of beads, which is used to remove excessive moisture from the air. Expiration should be rather rapid and as complete as possible. Insert the stopper just before expiration is finished and close the stopcock.

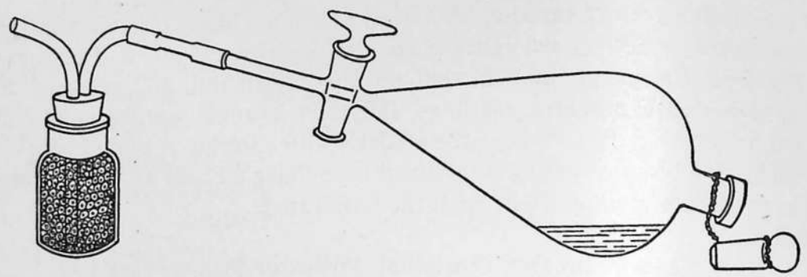


Fig. 192.—Apparatus used for saturating blood plasma with carbon dioxide. Air is blown from the lungs into the tube at the left, the stopper is inserted and the separatory funnel rotated. The air passes among the glass beads in the bottle which remove its moisture. (After Van Slyke.)

3. Rotate the funnel for two minutes, during which time the plasma should be distributed as completely as possible over the inner surface of the funnel. Carry out the analysis without delay.

III. *The Analysis.*—A special apparatus devised by Van Slyke is required (Fig. 193). The directions appear complicated, but the manipulation of the apparatus is not difficult.

1. Set up the apparatus upon a ring stand with mercury bulb held at position 1. The instrument must be firmly held by a clamp whose jaws are protected by heavy rubber and a support should be placed below the lower stopcock. Completely fill the apparatus with mercury by pouring it into the bulb. About 3 pounds will be required. Make sure that both openings in the lower and upper stopcocks are filled, and also the two capillaries above the upper stopcock. The stopcocks should be well anointed with vaselin, and should be held securely in place by means of heavy rubber bands.

2. With the upper stopcock closed and the lower one open, make sure that there is no leak by lowering the mercury bulb to position 2. This should produce a vacuum in the upper part of the apparatus; and when

the bulb is returned to position 1, the mercury should fill it completely and strike the upper stopcock with a click. If air has entered, it forms a cushion and the click is not heard.

3. Rinse out the cup at the top of the apparatus with distilled water. Leave 1 c.c. of the water in the cup.

4. Transfer to the cup 1 c.c. of the plasma which has been freshly saturated with carbon dioxide, keeping the tip of the pipet below the surface of the fluid. Add 1 small drop of pure caprylic alcohol.

5. Place the mercury bulb in position 2, and by carefully turning the upper stopcock allow the plasma and water to flow down into the main stem of the apparatus, leaving the capillary portion of the cup filled with the fluid.

6. Place 0.5 c.c. of 10 per cent lactic acid in the cup and admit enough of the acid into the main stem of the apparatus to bring the level of the fluid down exactly to the 2.5 mark.

7. Place a drop of mercury in the cup and let it run down in the narrow portion to seal the stopcock.

8. Lower the mercury bulb to a position about 80 cm. below the lower stopcock, where it may be supported by a ring or hook on the side of the work table.

9. When the level of the mercury in the apparatus has fallen to the 50-c.c. mark close the lower stopcock.

10. Remove the apparatus from its clamp, without disconnecting the rubber tubing, and turn it upside down at least fifteen times in order to thoroughly agitate the contents.

11. Replace the apparatus in its clamp, and turn the lower stopcock so that the fluid is drained as completely as possible into the bulb beneath. To avoid letting any of the gas escape with the fluid the last drop or two of fluid may be allowed to remain.

12. Raise the levelling bulb very slowly with the left hand, and with the right turn the lower stopcock so that the mercury rises gradually into

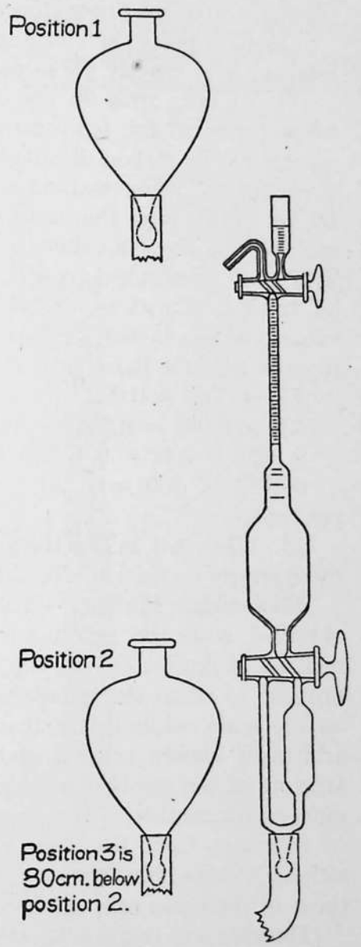


Fig. 193.—Van Slyke's apparatus for carbon dioxide of blood, showing the three positions of the mercury bulb. The bulb is connected to the apparatus by a heavy rubber tube which is merely indicated in the drawing. The apparatus is set up on an iron ring stand.

the apparatus through the left-hand tube below the stopcock. The mercury will fill the body of the apparatus and as much of the graduated stem at the top as is not occupied by the carbon dioxide liberated from the blood plasma. Leave the lower stopcock open.

13. Now place the levelling bulb at such height that the surface of the mercury is at exactly the same level as that in the apparatus.

14. At once read off the volume of gas in the graduated stem. This, when corrected for temperature, barometric pressure, and other factors, represents the carbon dioxide bound by 1 c.c. of blood plasma. For clinical work the following method will suffice if the room temperature is near 18° or 20° C. Find the barometric pressure in millimeters, divide by 760, multiply by the gas-volume reading on the apparatus, and subtract 0.12. The result, multiplied by 100, gives the amount of carbon dioxide bound by 100 c.c. of plasma, or the "volume per cent." *Example:* Suppose the volume of gas in the graduated stem of the apparatus was 0.6 c.c., and suppose that the barometric pressure at the time was 754 mm.

$754 \div 760 = 0.992$.

$0.6 \times 0.992 = 0.5952$ = reading corrected for pressure.

$0.5952 - 0.12 = 0.4752$ = fully corrected volume CO_2 in 1 c.c. plasma.

$0.4752 \times 100 = 47.52$ = volume CO_2 in 100 c.c. plasma "volume per cent."

15. When not in use the apparatus should be filled with water. Clean the mercury occasionally by filtering through chamois skin.

Manometric Method.—When a higher degree of accuracy than can be obtained with the volumetric apparatus is necessary, the manometric method is preferable.[1] In the volumetric method the pressure is always brought to equal the atmospheric pressure and the volume of gas is read on the scale, while in the manometric method, the gas is brought to an arbitrarily chosen volume, and from the pressure on the manometer, the amount of the gas is determined. The percentage of error is less with the manometric method. The apparatus is more complicated and more expensive to purchase than that which is needed for volumetric determinations, although some features of the manometric apparatus are simpler than those of the volumetric method (Fig. 194).

Haskins and Osgood Titration Method.—Van Slyke's method for titrating the bicarbonate content of plasma was modified by Haskins and Osgood.[2] The principle of this method is very simple, as the bicarbonate of 2 c.c. of plasma is decomposed by 5 c.c. of a fiftieth normal solution of hydrochloric acid. The excess of acid is titrated with fiftieth normal solution of sodium hydroxide at a *p*H of 7.4. The number of cubic centimeters of acid combined with the carbonate (5 c.c. of alkali used for titration) multiplied by 22.4 gives the alkali reserve figure. This method is much simpler than the gasometric method and is much preferred in many laboratories. The alkali reserve figure is the cubic centimeters of dry carbon dioxide (measured at 0° C. and 760 mm.) which can be held in chemical combination, excluding dissolved carbon dioxide, by 100 c.c. of plasma after exposure to an atmosphere containing 5.5 per cent of carbon dioxide (alveolar air) at 20° C.

[1] Van Slyke, D. D., and Neill, J. M.: The Determination of Gasses in Blood and Other Solutions by Vacuum Extraction and Manometric Measurement. Jour. Biol. Chem. *61*:523–584 (Sept.), 1924.

[2] Haskins, H. D., and Osgood, E. E.: Modifications of Van Slyke's Titration Method for Estimating the Alkali Reserve of Blood. Jour. Lab. & Clin. Med. *6*:37–41 (Oct.), 1920.

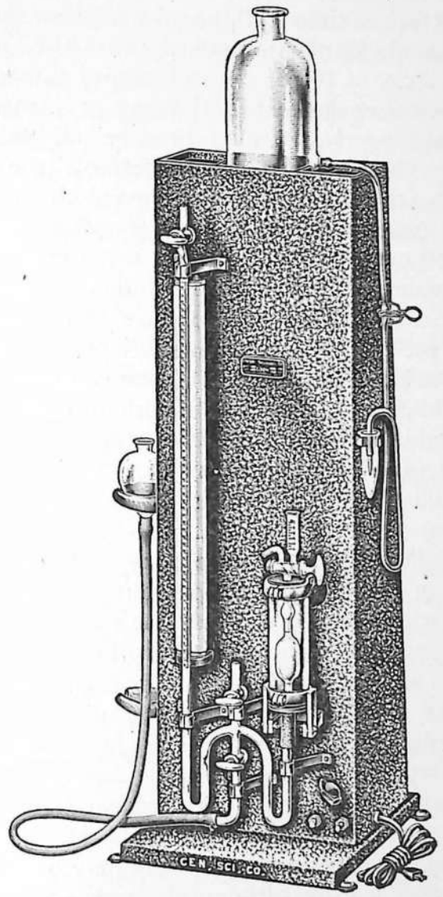


Fig. 194.—Manometric blood-gas apparatus.

Reagents.—(*a*) Neutral red indicator. Dissolve 65 mg. neutral red in 100 c.c. of 50 per cent alcohol. (*b*) Permanent standards.* (1) Prepare *buffer phosphate solution of p H 6.8* by taking equal parts of primary and secondary phosphate solution (see p. 836). (2) Also prepare *buffer phosphate solution of p H 7.4* by adding 21 c.c. of primary phosphate solution to 79 c.c. of sec-

* Permanent standards may be purchased from the Shaw Surgical Company, Portland, Oregon.

ondary phosphate solution. (3) *Paranitrophenol Solution.*—Dissolve 20 mg. of paranitrophenol in 10 c.c. of 95 per cent alcohol and dilute to 100 c.c. with distilled water. (4) *Amaranth Solution.*—Dissolve 8 mg. of amaranth in 100 c.c. of distilled water. Place 60 c.c. of buffer solution of *p*H 7.4 (*b* 2) in a 120 c.c. pyrex Erlenmeyer flask and add 0.6 c.c. of neutral red indicator (*a*). Place 60 c.c. of buffer phosphate of *p*H 6.8 (*b* 1) in a similar flask and add 5.2 c.c. of the paranitrophenol solution (*b* 3) and then add the amaranth solution (*b* 4) a little at a time until the color matches that in the first flask. Put 30 c.c. of this standard into each of two 120 c.c. pyrex Erlenmeyer flasks and add 0.2 c.c. of 10 per cent solution of thymol in chloroform to each. Keep one as a *clear standard*. Add 20 mg. of cornstarch powder to the other flask to make a *turbid standard*. Stopper and seal with paraffin and store in the dark. These are permanent standards; the turbid standard is used for the test. (*c*) Fiftieth normal hydrochloric acid. This should be prepared once in two months by diluting 50 c.c. of tenth normal hydrochloric acid to 250 c.c. Add chloroform as a preservative. Determine the titration value against fiftieth normal solution of sodium hydroxide as follows: Mix 20 c.c. of distilled water, 0.3 c.c. of neutral red indicator (*a*) and 1 c.c. of 0.05 per cent solution of disodium phosphate. Add fiftieth normal hydrochloric acid, a small drop at a time until the color matches the *clear standard*. Add 5 c.c. of fiftieth normal hydrochloric acid and titrate. This titration should require 4.95 to 5.05 c.c. of fiftieth normal solution of sodium hydroxide. (*d*) Fiftieth normal sodium hydroxide, free of carbonate. Boil a liter of distilled water for two minutes, cork loosely and cool rapidly. Add 1.2 c.c. of clear 65 per cent solution of sodium hydroxide and mix well. Keep the flask tightly corked. Titrate 5 c.c. portions of fiftieth normal hydrochloric acid as directed for titration value of hydrochloric acid. Keep the buret covered with a test tube. Dilute the solution of sodium hydroxide with the proper amount of recently boiled distilled water to make the titration just 5 c.c. Mix and recheck. Put 25 c.c. portions into a large number of 50 c.c. pyrex flasks. Cork each flask at once and seal the corks with hot paraffin. Use the solution of sodium hydroxide in one flask for only one day. Fiftieth normal solution of sodium hydroxide may also be stored in a large pyrex flask fitted with a soda lime tube to prevent ingress of carbon dioxide and a siphon tube of pyrex glass for filling the buret. (*e*) Caprylic alcohol.

Method.—Either blood or plasma or serum may be used. Centrifuge the blood as soon as possible after withdrawal. Draw off the plasma or serum and place it in the icebox in a tightly corked test tube unless the estimation is to be made immediately. It may also be kept under a layer of mineral oil if the determination is not to be done at once.

1. Measure exactly 2 c.c. of plasma or serum into a Florence flask, not the titration flask. Add exactly 5 c.c. of fiftieth normal hydrochloric acid and 1 drop of caprylic alcohol (*e*). Then rotate the flask for one or two minutes so as to spread the mixture in a thin film on the wall. Pour the liquid into a 120 c.c. Erlenmeyer flask and wash the residue into the flask, using three rinsings totaling 20 c.c. of distilled water.

2. Add 0.3 c.c. of indicator (*a*) and titrate with carbonate-free fiftieth normal solution of sodium hydroxide (*d*) until the color matches the *turbid standard*, adding at the last single 0.02 c.c. drops from a fine tip. Make the color comparison by reflected light with both flasks standing on a white surface. When an exact match is secured, read the buret. If the right end point has been reached, the addition of another drop will make the mixture slightly too yellowish.

Calculation.—5 c.c. of alkali used in the titration × 22.4 = the alkali reserve figure ("volume per cent"). See p. 397 for the normal range and figures indicating acidosis or alkalosis.

The alkali may be checked once a day by adding another 5 c.c. of fiftieth normal hydrochloric acid to the titrated mixture and titrating back to the same end point with fiftieth normal solution of sodium hydroxide. Always keep a test tube over the top of the buret to exclude carbon dioxide. The flask should be kept free of the deposit of neutral red by washing with ammonia water.

H. Calcium

In parathyroid tetany the calcium content of the blood serum falls below the normal amount of 9 and 11 mg. for each 100 c.c. The condition is relieved by administering calcium. The determination of calcium is an exact chemical procedure somewhat difficult for the clinical laboratory. However, there is an increasing demand for this test and the technic is therefore given in detail.

Clark-Collip Method for Calcium (*Modification of Tisdall's Method*).[1]—All glassware for this test must be cleaned in cleaning solution and rinsed in calcium-free distilled water. The syringes and needles used for venipuncture must be cleaned only with distilled water and are sterilized preferably by baking in the hot-air oven.

Reagents.—(*a*) Ammonium oxalate, 4 per cent.

(*b*) Dilute ammonia; 2 c.c. concentrated ammonia, 98 c.c. distilled water. Place in a wash bottle.

(*c*) Approximately normal sulfuric acid solution; dilute 28 c.c. concentrated sulfuric acid to 1 liter.

(*d*) Stock solution 0.1 normal potassium permanganate. Dissolve in a Florence flask 3.5 Gm. pure potassium permanganate in 1 liter of redistilled water. Use a funnel covered with a watch glass as a condenser and heat for several hours to nearly boiling. Cool, and let stand overnight. Filter through a 3-inch Büchner funnel lined with ignited asbestos using gentle suction. Standardize against 0.1 normal sodium oxalate solution. Store in a clean glass-stoppered bottle in a dark place.

(*e*) Potassium permanganate solution, 0.01 normal. Immediately before

[1] Clark, E. P., and Collip, J. B.: A Study of the Tisdall Method for the Determination of Blood Serum Calcium with a Suggested Modification, Jour. Biol. Chem., *63*:461–464 (Mar.), 1925.

use dilute a suitable portion of 0.1 normal permanganate solution (*d*) to make an exact 0.01 normal solution and titrate against absolutely correct 0.01 normal sodium oxalate solution (Sörenson quality).

Method.—1. Draw about 10 c.c. of blood by venipuncture in a carefully cleaned syringe and place in a 15-c.c. centrifuge tube which has also been carefully cleaned. Separate the clot and centrifugate.

2. Place exactly 2 c.c. of clear serum in a clean 15-c.c. graduated centrifuge tube. Add 2 c.c. of calcium-free distilled water and 1 c.c. of 4 per cent ammonium oxalate solution (*a*). Mix thoroughly. Allow the mixture to stand four hours or over night, and then centrifugate thoroughly. Drain off the supernatant fluid carefully and thoroughly.

3. Stir up the precipitate and wash down the sides of the tube with 3 c.c. of dilute ammonia (*b*). Centrifugate again and drain off the supernatant fluid thoroughly.

4. Add exactly 2 c.c. of normal sulfuric acid (*c*). Blow the acid upon the precipitate to break up and thoroughly dissolve the mass. Place the tube in boiling water for one minute.

5. A tube containing everything except the serum should be set up as a blank, and treated in the same manner as the unknown. It is often well to set up more than one such blank.

6. Titrate the unknown and the blanks with 0.01 normal potassium permanganate (*e*) to a pink color that persists for one minute, using a microburet graduated in 0.02 c.c. Keep the solution at 70° to 75° C. in a warm bath. If the permanganate solution is not exactly 0.01 normal a correction factor must be used in the subsequent calculation.

Calculation.—One c.c. of exactly 0.01 normal potassium permanganate is equivalent to 0.2 mg. of calcium. Subtract the number of cubic centimeters of the permanganate solution used in titrating the blank from the number of cubic centimeters used in titrating the unknown solution of calcium. Let x represent this difference and substitute for x in the following formula:

$$\frac{x \times 0.2 \times 100}{2} = \text{mg. of calcium in 100 c.c. of serum.}$$

I. Phosphorus

The amount of inorganic phosphorus in the blood plasma or serum of the normal adult is usually about 3.7 mg. per 100 c.c. In the plasma or serum of children, the level is slightly higher, or about 5 mg. In severe nephritis, the inorganic phosphorus may rise to 15 to 20 mg. per 100 c.c. of serum or plasma. There is usually an elevation in phosphorus in the serum or plasma of adults who have major fractures. This high level may be maintained as long as the fracture remains ununited. Perhaps a more significant finding is the low phosphorus content of the serum or plasma of children who are suffering from rickets. In this disease, the value for the phosphorus

may be as low as 2 mg. per 100 c.c. There is also a seasonal fluctuation; the highest values occur during the summer months, and the lowest values during the winter. The method[1] which is given here has proved very convenient and it is described in detail as it is used also for phosphatase determinations. Phosphatase is an enzyme which splits the phosphoric acid esters of carbohydrates. It must be remembered that Bodansky phosphatase units do not correspond to values used in other methods.

Bodansky's Method for Determination of Serum Inorganic Phosphate and of Serum Phosphatase.[2]—**Stock Solutions.**—(*a*) *Substrate.*—Place successively into a 100 c.c. volumetric flask about 3 c.c. of petroleum ether (J. T. Baker Analyzed, special), about 80 c.c. of distilled water, 0.5 Gm. of sodium beta-glycerophosphate (Eastman Kodak Research Laboratories), 0.424 Gm. of sodium diethylbarbiturate (Merck, U. S. P., Ed. 10), and water to volume; empty into a 100 c.c. glass-stoppered pyrex bottle containing about an inch of petroleum ether; keep in the refrigerator. When multiples of 100 c.c. are prepared, it is advisable to distribute the substrate into small bottles.

(*b*) *Trichloracetic Acid, 10 Per Cent Solution.*—(J. T. Baker, analyzed, reagent).

(*c*) *Sodium Molybdate Solution, 7.5 Per Cent.*—Dissolve 90 Gm. of molybdic acid ("Sodium" and "phosphorus" free, Mallinckrodt reagent quality) in 250 c.c. of 5 normal solution of sodium hydroxide in a 2 liter volumetric flask. Make up to volume. Keep in a glass-stoppered bottle.

(*d*) *Sulfuric Acid, 10 Normal.*—Dilute about 290 c.c. of concentrated sulfuric acid (Specific gravity about 1.83, Mallinckrodt reagent) to 1 liter; standardize and dilute if necessary; *keep in refrigerator.*

(*e*) *Stannous Chloride Solution, 60 Per Cent.*—To 15 Gm. of stannous chloride (J. T. Baker analyzed) add concentrated hydrochloric acid (Mallinckrodt analytical reagent) to a volume of 25 c.c.; *keep in refrigerator.*

(*f*) *Phosphate Stock Solution.*—Two hundred fifty c.c. is to contain 110 mg. of potassium acid phosphate (LaMotte buffer quality), and 1 c.c. of concentrated sulfuric acid; 1 c.c. contains 0.1 mg. of phosphorus.

(*g*) *Phosphate Standard Solution.*—Dilute 10 c.c. of stock solution to 300 c.c.; add a drop of toluene; 6 c.c. contains 0.02 mg. of phosphorus.

"Check solutions" of known but arbitrarily varying concentration (a half to three or four times that of the standard solution) may be made by suitable dilution of the stock solution of phosphate; preserve with a drop of toluene.

Reagents Prepared Daily.—(1) *Acid-molybdate Solution.*—To the cold 10 N solution of sulfuric acid (*d*) in a flask add, while mixing, an equal

[1] Bodansky's method is an adaptation of the Kuttner-Lichtenstein method for the estimation of phosphorus.

[2] Bodansky, Aaron: Notes on Determination of Serum Inorganic Phosphate and of Serum Phosphatase Activity, Am. Jour. Clin. Path., Tech. Suppl., 7:51–59 (Sept.), 1937.

volume of 7.5 per cent solution of sodium molybdate (*c*); when the mixture is properly prepared it is free of even the slightest tinge of yellow.

(2) *Dilute Stannous Chloride Solution.*—Dilute 1 c.c. of the 60 per cent solution (*e*) to 400 c.c. with water; keep in refrigerator between analyses.

Analytical Procedures.—*Collection of Serum.*—Collect about 5 c.c. of whole blood into a centrifuge tube, allow to clot at room temperature, centrifuge, remove serum and centrifuge it. Analysis may be delayed for several hours if the separated serum is kept in ice water or in the refrigerator. If a longer delay is necessary, add a small drop of toluene. After twenty-four to forty-eight hours in the refrigerator, serum phosphatase activity is about 10 to 15 per cent higher than originally. At room temperature (as in mailed specimens) the results are subject to an error of ±20 per cent. Clinically valid interpretations may, however, be based upon the analyses.

Preparation of Filtrates.—(1) "Serum inorganic phosphate" filtrate: To 1 c.c. of serum in a test tube add 9 c.c. of 10 per cent trichloracetic acid, mix well and filter after a few minutes through 9 cm. filter paper (Whatman number 44, or other "ashless" grade). (2) "Total inorganic phosphate" filtrate (serum inorganic phosphate *plus* inorganic phosphate liberated from the substrate by the serum phosphatase). Measure 10 c.c. of substrate, preferably into a glass-stoppered test tube;[1] place in a water bath at 37° C. for a few minutes, add 1 c.c. of serum, with the tip of the pipet about 1 cm. above the surface of the liquid, invert once or tap to impart rotary motion, mixing the contents well (but without undue aeration); replace in water bath; remove after exactly one hour; cool in ice water for about two minutes, add 9 c.c. of 10 per cent trichloracetic acid; mix; let stand for a few minutes, and filter as before.

When a high phosphatase activity is expected a high dilution is therefore desired. This is accomplished by the addition of large volumes of 10 per cent trichloracetic acid. Half-hour or quarter-hour periods of incubation may be used when high serum phosphatase activity is expected.

When half quantities are used, about 1 c.c. of petroleum ether precedes the addition of 5 c.c. of substrate and 0.5 c.c. of serum; 4.5 c.c. of 10 per cent trichloracetic acid is added for a dilution of 20, 9.5 c.c. for a dilution of 30 and so forth.

Reagent Blanks.—Add 2 c.c. of acid molybdate solution (1) and 2 c.c. of a dilute solution of stannous chloride (2), mixing after each addition, to each of two tubes—one containing 6 c.c. of water and the other 6 c.c. of 10 per cent trichloracetic acid. The blanks should be colorless or at most tinged faintly green or blue.

Preparation of Aliquots for Analysis.[2]—The comparison standards (2 or

[1] The glass-stoppered test tubes were made by Scientific Glass Apparatus Co., Bloomfield, N. J. (interchangeable stoppers), and by Eimer and Amend, New York, N. Y. Ordinary test tubes and *clean* rubber stoppers may be employed.

[2] When colorimeter cups of internal diameter of about 12 mm. or less are employed, aliquots of half the stated volumes may be used with half quantities of the reagents (see Table 1).

more in each set) contain 0.02 mg. of phosphorus. In accordance with the concentration expected transfer 2, 4, and 6 c.c. of filtrate, and make up with water, if necessary to a total volume of 6 c.c. Include in each set some known "check solutions" to verify the accuracy of the determination.

Color Development and Comparison.—To each tube, in sequence, add 2 c.c. of acid molybdate solution (1); mix by tapping, in the same sequence; add 2 c.c. of a dilute solution of stannous chloride (2) to each tube, mixing *during* the addition. The color of the mixture develops rapidly. Colorimetric comparison may be made at convenience—immediately after addition of stannous chloride or at any time within about two hours.

Calculations.—After colorimetric comparison and calculation in the usual manner from the recorded readings, corrections must be made for the deviation from Beer's law, and for the effects of trichloracetic acid and glycerophosphate (about 1.5 per cent for each cubic centimeter of aliquot used). More rapid as well as more accurate calculation of the results is obtained by the use of tables containing corrected values corresponding to the given readings: The values of the "check solutions," which contain neither trichloracetic acid nor glycerophosphate, may be obtained from Table 1 (milligrams per 100 c.c.); the values of "serum inorganic phosphorus," in milligrams per 100 c.c., are found by consulting Table 2 or 3, under the division corresponding to the volume of aliquot used: The values of "total inorganic phosphorus" are obtained in similar manner but the dilution in the standard procedure being 20, multiply the "table value" by 2. When dilutions of 30 or 40 are employed, multiply the "table value" by 3 or 4, respectively.

A unit of phosphatase activity is defined as equivalent to the liberation of 1 mg. of phosphorus (as the phosphate ion) during the first hour of incubation, under the conditions described.

"Total inorganic phosphorus" *minus* "serum inorganic phosphorus" equals milligrams of "inorganic phosphorus liberated" from glycerophosphate by 100 c.c. of serum (or units of phosphatase activity per 100 c.c. *if hydrolysis is continued for one hour*).

When serum phosphatase activity is high the relatively large amounts of liberated inorganic phosphate retard the hydrolysis. Bodansky measured this effect, and established a correction, to be applied to the value of "inorganic phosphorus liberated," per 100 c.c. of serum, during the given period of incubation. A simple rule is: Divide this value by 30, and add the square of the quotient to the uncorrected value. Thus, if 60 mg. have been liberated per 100 c.c. of serum, the correction amounts to 4 mg., and the corrected value to 64 mg., per 100 c.c.

If half-hour or quarter-hour periods of incubation have been employed for hydrolysis, multiply the value of "inorganic phosphorus liberated" by 1.82 or 3.3 respectively (more rarely periods of two or three hours may be desirable: in such cases the factors are 0.55 or 0.39, respectively).

In laboratories where determinations of phosphatase are made infrequently, the incubation period may be kept uniformly at one hour, if desired.

TABLE 1

INORGANIC PHOSPHORUS ALIQUOTS, AT STATED COLORIMETRIC READINGS CORRECTED FOR DEVIATION FROM BEER'S LAW

0.02 MG. STANDARD SET AT 20 MM.

In column D are given the values for decrements corresponding to an increase of the readings by 0.1 mm., for use in interpolation.

Mm.	0.0	0.2	0.4	0.6	0.8	D
mm.	mg.	mg.	mg.	mg.	mg.	
5	0.0974	0.0931	0.0892	0.0856	0.0823	18
6	792	764	737	711	687	12
7	665	645	626	607	588	9
8	570	554	539	525	512	7
9	499	487	475	464	453	6
10	443	433	424	415	406	5
11	398	390	382	374	367	4
12	360	353	346	340	334	3
13	328	322	316	311	306	3
14	301	296	291	286	282	2
15	278	274	270	266	262	2
16	258	254	251	248	244	2
17	241	238	235	232	229	1+
18	226	223	220	217	215	1+
19	212	209+	207	204+	202	1
20	200	198−	195+	193	191	1
21	1890	1869	1849	1829	1809	10
22	1790	1771	1753	1735	1717	9
23	1700	1683	1666	1650	1634	8
24	1618	1602	1587	1572	1557	8
25	1543	1529	1515	1501	1487	7
26	1474	1461	1448	1435	1422	7
27	1410	1398	1386	1374	1362	6
28	1351	1340	1329	1318	1307	6
29	1297	1287	1277	1267	1257	5
30	1247	1237	1228	1219	1210	5
31	1201	1192	1183	1174	1166	4
32	1158	1150	1142	1134	1126	4
33	1118	1110	1102	1094	1087	4
34	1080	1072	1065	1058	1051	4
35	1044	1037	1030	1023	1016	4
36	1010	1003	997	991	984	3

When the stated correction for retardation of hydrolysis is applied to sera of high phosphatase activity a good approximation to correct value is obtained. The maximal error would not be great enough to impair the validity of clinical interpretation of the results obtained.

Table 4 indicates, approximately, the range of values of serum phosphatase in the normal state and in the several pathologic states in which

the concentration of serum phosphatase is raised. As a technical aid, it may be of some use to include in this table suggestions as to shorter periods of hydrolysis, when these are desired, and the approximate aliquot volumes indicated for the *lower* limit of each range.

TABLE 2

INORGANIC

PHOSPHORUS CONTENT, IN MILLIGRAMS PER 100 C.C., CORRECTED FOR DEVIATION FROM BEER'S LAW AND FOR EFFECTS OF TRICHLORACETIC ACID AND GLYCEROPHOSPHATE

Column D contains the values for decrements corresponding to an increase of the readings by 0.1 mm., for the use in interpolation.

The values are calculated for a dilution of 10× (as in "inorganic phosphorus filtrate"). When the dilution is 20, 30, or 40× (as in "total inorganic phosphorus" after incubation), these figures are to be multiplied by 2, 3, or 4 respectively.

FOR 6 C.C. ALIQUOTS (OR FOR 3 C.C. WHEN HALF VOLUMES ARE USED).

Mm.	0.0	0.2	0.4	0.6	0.8	D
mm.	mg.	mg.	mg.	mg.	mg.	
5	17.7	16.9	16.2	15.5	14.9	0.3+
6	14.4	13.8+	13.4	12.9	12.5	0.2+
7	12.0+	11.7	11.3	11.0	10.7	0.2−
8	10.4	10.1	9.8	9.6−	9.3	0.1+
9	9.06	8.83	8.61	8.41	8.22	0.10
10	8.04	7.87	7.70	7.54	7.38	0.08
11	7.23	7.09	6.95	6.81	6.67	0.07
12	6.54	6.42	6.30	6.18	6.07	0.06
13	5.96	5.86	5.76	5.66	5.56	0.05
14	5.47	5.38	5.29	5.21	5.13	0.04
15	5.05	4.97	4.90	4.83	4.76	0.04
16	4.69	4.62	4.56	4.50	4.44	0.03
17	4.38	4.32	4.26	4.20	4.15	0.03
18	4.10	4.05	4.00	3.95	3.90	0.02+
19	3.85	3.80	3.76	3.71	3.67	0.02
20	3.63	3.59	3.55	3.51	3.47	0.02
21	3.43	3.40	3.36	3.32	3.29	0.02−
22	3.25	3.22	3.18	3.15	3.12	0.02−
23	3.09	3.06	3.03	3.00	2.97	0.02−
24	2.94	2.91	2.88	2.86	2.83	0.01+
25	2.80	2.78−	2.75	2.73−	2.70	0.01+
26	2.68−	2.65	2.63	2.61−	2.58	0.01+
27	2.56	2.54	2.52−	2.50−	2.47+	0.01
28	2.45	2.43	2.41	2.39	2.37+	0.01
29	2.36−	2.34	2.32	2.30	2.28	0.01
30	2.27−	2.25−	2.23	2.21	2.20−	0.01
31	2.18	2.17−	2.15	2.13	2.12−	0.01
32	2.10	2.09	2.07	2.06	2.04	
33	2.03	2.02	2.00	1.99	1.97	
34	1.96	1.95	1.94	1.92	1.91	
35	1.90	1.88	1.87	1.86	1.85	
36	1.84	1.82	1.81	1.80	1.79	

TABLE 3

INORGANIC

PHOSPHORUS CONTENT, IN MILLIGRAMS PER 100 C.C., CORRECTED FOR DEVIATION FROM BEER'S LAW AND FOR EFFECTS OF TRICHLORACETIC ACID AND GLYCEROPHOSPHATE

Column D contains the values for decrements corresponding to an increase of the readings by 0.1 mm., for the use in interpolation.

The values are calculated for a dilution of 10× (as in "inorganic phosphorus filtrate"). When the dilution is 20, 30, or 40× (as in "total inorganic phosphorus" after incubation), these figures are to be multiplied by 2, 3, or 4 respectively.

When 2 c.c. aliquots are used, deduct 3 per cent from the value for the 4 c.c. aliquot and multiply by 2.

FOR 4 C.C. ALIQUOTS (OR FOR 2 C.C. WHEN HALF VOLUMES ARE USED).

Mm.	0.0	0.2	0.4	0.6	0.8	D
mm.	mg.	mg.	mg.	mg.	mg.	
5	25.8	24.7	23.7	22.7	21.8	0.5
6	20.9	20.1	19.4	18.8	18.2	0.3
7	17.6	17.1	16.6	16.1	15.6	0.2+
8	15.1	14.7	14.3	13.9	13.5	0.2
9	13.2	12.9	12.6	12.3	12.0	0.1+
10	11.7	11.5	11.2	11.0	10.7+	0.1+
11	10.5+	10.3	10.1	9.9	9.7	0.1
12	9.52	9.34	9.17	9.00	8.83	0.08+
13	8.67	8.51	8.36	8.22	8.09	0.07
14	7.96	7.83	7.71	7.59	7.47	0.06
15	7.35	7.24	7.13	7.02	6.92	0.05
16	6.82	6.72	6.63	6.54	6.45	0.05
17	6.36	6.28	6.20	6.12	6.04	0.04
18	5.96	5.89	5.82	5.75	5.68	0.04
19	5.61	5.54	5.48	5.42	5.36	0.03
20	5.30	5.24	5.18	5.12	5.07	0.03
21	5.02	4.96	4.91	4.86	4.81	0.03–
22	4.76	4.71	4.66	4.61	4.56	0.03–
23	4.52	4.47	4.42	4.38	4.34	0.02
24	4.30	4.26	4.22	4.18	4.14	0.02
25	4.10	4.06	4.02	3.98	3.94	0.02
26	3.91	3.87	3.83	3.79	3.76	0.02
27	3.73	3.69	3.66	3.63	3.60	0.02–
28	3.57	3.54	3.51	3.48	3.45	0.01+
29	3.43	3.40	3.38	3.35	3.33	0.01
30	3.30	3.28	3.25	3.22	3.20	0.01
31	3.18	3.15	3.13	3.11	3.09	0.01
32	3.07	3.04	3.02	3.00	2.98	0.01
33	2.96	2.94	2.92	2.90	2.88	0.01
34	2.86	2.84	2.82	2.80	2.78	0.01
35	2.77	2.75	2.73	2.71	2.70	
36	2.68	2.66	2.64	2.63	2.61	

In Bodansky's experience, group 1 included osteomalacia, some cases of hyperparathyroidism, mono-ostotic osteitis deformans (and healed polyostotic disease), "marble bones," osteogenic sarcoma, carcinoma of bone and healing or healed rickets; group 2 included some cases of mono-ostotic and polyostotic osteitis deformans, hyperparathyroidism, carcinoma of bone, fragilitas ossium with osteomalacia and moderately active rickets; group 3 included polyostotic osteitis deformans, extensive carcinoma of bone and active rickets; group 4 included severe polyostotic osteitis deformans and severe rickets. The phosphatase values in clinical involvement of the liver, with or without jaundice, have frequently been similar to those in groups 1 and 2. Anemia and cretinism in children are associated with lower values; adult values are to be expected in sexually precocious children.

TABLE 4

A SUMMARY OF PHOSPHATASE VALUES IN CLINICAL CASES

The figures in parentheses indicate, approximately, the aliquot volumes, in cubic centimeters, suitable at the corresponding phosphatase activities.

	Phosphatase activity, units per 100 c.c.		Dilution, times.	Period of incubation, hours.
	Minimal.	Maximal.		
Adult, normal	2 (6)	4	20	1
Child, normal	5 (4)	15	20	1
	(6)		20	½
Group 1, slightly active process	5 (4)	20	20	1
Group 2, moderately active process	20 (2)	50	20	1
	(4)		20	½
Group 3, active process	50 (2)	100	40	1
	(4)		40	½
Group 4, very active process	100 (2)	250	40	½

Photelometric Determination of Inorganic Phosphate.[1]—In this method the Roe and Whitmore[2] modification of the Fiske and Subbarow[3] method for determining inorganic phosphate and Bodansky's technic for determining phosphatase are used but other colorimetric methods may be adapted to the photelometer. The ammonium molybdate reagent used in this method must be made up every month. The calibration curve is made by using a Cenco orange filter number 4.

[1] Hoffman, W. S.: Photelometric Clinical Chemistry. New York, Wm. Morris and Company, 1941, pp. 138–150.

[2] Roe, J. H., and Whitmore, E. R.: Clinico-pathologic Application of Serum Phosphatase Determinations with Special Reference to Lesions of the Bones. Amer. Jour. Clin. Path. *8*:233–254 (May), 1938.

[3] Fiske, C. H., and Subbarow, Yellapragada: The Colorimetric Determination of Phosphorus. Jour. Biol. Chem. *66*:375–400 (Dec.), 1925.

King and Armstrong Alkaline Phosphatase Method.—King and Armstrong[1] have studied phosphatase activity, making use of different units than those described in the previous method. With their test, one unit of phosphatase is that amount of enzyme which, when allowed to act upon an excess of disodium phenylphosphate at a *p*H of 9.0 for thirty minutes at 37.5° C., will liberate 1 mg. of phenol.

Reagents.—(*a*) Buffer substrate; a two hundredth molar solution of phenylphosphate in a twentieth molar solution of barbital. Dissolve 10.3 Gm. of barbital sodium and 1.09 Gm. of disodium phenylphosphate in 1 liter of distilled water. Add a few drops of chloroform and keep well stoppered in the refrigerator when not in use. (Should be prepared freshly each month.)

(*b*) Phenol reagent of Folin-Ciocalteu.[2] (This reagent may be purchased from a laboratory supply house.) In a 1500-c.c. Florence flask dissolve 100 Gm. of sodium tungstate and 25 Gm. of sodium molybdate in 700 c.c. of distilled water. Add 50 c.c. of 85 per cent phosphoric acid and 100 c.c. of concentrated hydrochloric acid. Heat gently with a reflux condenser for ten hours. Add 150 Gm. of lithium sulfate, 50 c.c. of distilled water and a few drops of bromine. Boil the mixture for fifteen minutes without a condenser to remove the excess of bromine. Cool, dilute to 1 liter and filter. There should be no greenish tint to the reagent. For use in the test, one part of this reagent is diluted with two parts of distilled water.

(*c*) Twenty per cent solution of sodium carbonate.

(*d*) Stock standard phenol solution (100 mg. per 100 c.c.). Dissolve 1 Gm. of crystalline phenol in tenth normal hydrochloric acid and make up to 1 liter with tenth normal hydrochloric acid. Transfer 25 c.c. of this solution to a 250 c.c. flask. Add 50 c.c. of a tenth normal solution of sodium hydroxide. Heat to 50° C. Add 25 c.c. of tenth normal solution of iodine. Stopper the flask and let it stand at room temperature for thirty or forty minutes. Add 5 c.c. of concentrated hydrochloric acid and titrate the excess of iodine with a tenth normal solution of sodium thiosulfate. Each cubic centimeter of tenth normal solution of iodine corresponds to 1.567 mg. of phenol. Dilute the phenol solution so that 1 c.c. contains 1 mg. This solution will keep indefinitely.

(*e*) Diluted stock standard. Dilute stock standard (*d*), one part to ten with distilled water (exactly 10 mg. of phenol per 100 c.c.). This solution keeps at least three months in the icebox.

(*f*) Standard phenol solution and reagent (1 mg. phenol per 100 c.c.). Take 5 c.c. of diluted stock standard solution (*e*) and 15 c.c. of diluted phenol reagent (*d*) and make up to 50 c.c. with distilled water. This solution must be made up daily.

Method.—1. In each of two test tubes place 10 c.c. of buffer substrate

[1] King, E. J., and Armstrong, A. R.: A Convenient Method for Determining Serum and Bile Phosphatase Activity. Canad. Med. Assn. Jour. *31*:376–381 (Oct.), 1934.

[2] Folin, Otto, and Ciocalteu, Vintila: On Tyrosine and Tryptophane Determinations in Proteins. Jour. Biol. Chem. *73*:627–650 (June), 1927.

(*a*). Heat in a water bath at 37.5° C. for five minutes or more. Without removing the tubes from the bath, add exactly 0.5 c.c. of clear, well centrifuged serum to each. Stopper, mix and allow to remain in the bath exactly thirty minutes.

2. In each of two test tubes place 10 c.c. of buffer substrate (*a*). Add 0.5 c.c. of serum and 4.5 c.c. of diluted phenol reagent (*b*). Pipet 10 c.c. of filtrate from test mixture (1) and 10 c.c. of this control solution into clean test tubes. Add 2.5 c.c. of 20 per cent solution of sodium carbonate (*c*) to each tube. Mix and place tubes in the water bath at 37.5° C. for five minutes.

3. Prepare a standard by taking 10 c.c. of standard phenol solution and reagent (*f*) and 2.5 c.c. of the 20 per cent solution of sodium carbonate. Place the unknown solution on the left side of the colorimeter and set the scale at 30 mm. Place the standard on the right side and match the colors. Also test the control in the colorimeter in the same manner.

Calculation:

$$\frac{\text{Reading of the Standard}}{\text{Reading of the Unknown}} \times \text{strength of the standard} \times \frac{15}{10} \times \frac{100}{0.5} = \text{mg.}$$

of phenol liberated per 100 c.c. of serum in the test and also in the control. When the reading for the unknown solution is 30 mm. and the strength of the standard is 0.1 mg., the equation becomes

$$\frac{\text{Reading of the Standard}}{30} \times 0.1 \times \frac{15}{10} \times \frac{100}{0.5}$$

As all of the figures in this equation cancel out, the milligrams per 100 c.c. = the reading of the standard; therefore, the units of phosphatase per 100 c.c. = the reading of the standard (against the test) − the reading of the standard (against the control).

As long as the enzyme content of the serum is less than 30 units, the colorimetric method and calculation are both carried out as described. If more than 30 units but less than 60 units are present, the unknown should be set at 15 mm. In such a case, the reading of the standard (against the test) must then be multiplied by 2 to obtain mg. of phenol per 100 c.c. of serum. For routine work, if the number of units is very high, it is satisfactory to dilute this serum with physiologic salt solution so that the number of units per 100 c.c. of diluted serum will not be in excess of 60. The values for the King and Armstrong units are about three times those for the Bodansky units. This makes the normal range up to about 15 units with this method.

Acid Phosphatase.—Occasionally it is of interest to determine the amount of phosphatase in serum that will act with acid buffers.[1]

Reagents.—(*a*) Buffer substrate. Two hundredth molar disodium mono-

[1] Gutman, A. B., and Gutman, Ethel B.: An "Acid" Phosphatase Occurring in the Serum of Patients with Metastasizing Carcinoma of the Prostate Gland. Jour. Clin. Invest. *17*:473–478 (July), 1938.

phenylphosphate in Sørensen's tenth molar citrate—hydrochloric acid buffer solution adjusted to *p*H 4.8.*

Method.—The method is the same as that described for the determination of alkaline phosphatase. The normal range is about two to three King and Armstrong units.

J. Iron

Iron in the Blood.—The determination of the amount of iron in the blood furnishes an accurate method for determining the hemoglobin content; the results are comparable to those which are obtained with the oxygen capacity. It can be used as a research method, or for calibration of simpler hemoglobin methods which are described in the chapter on Blood, page 209.

Osterberg's Modification.[1]—Osterberg has combined the method of Kennedy[2] with that of Wong[3] to make a reliable and accurate method.

Reagents Required.—(*a*) Concentrated sulfuric acid.

(*b*) A 60 per cent solution of perchloric acid.

(*c*) A 20 per cent solution of potassium thiocyanate (or 20 per cent solution of sodium thiocyanate may also be used).

(*d*) Wong iron standard solution. Weigh accurately 0.7 Gm. of crystallized ferrous ammonium sulfate (dried to constant weight) and dissolve in about 50 c.c. of distilled water. Add to the solution 20 c.c. of dilute (10 per cent) iron-free sulfuric acid, warm slightly, and then add decinormal solution of potassium permanganate to oxidize the ferrous salt completely. Dilute to exactly 1 liter with distilled water. One c.c. will contain 0.1 mg. of iron.

Method.—1. Place exactly 0.5 c.c. of whole oxalated blood in a 50-c.c. Nessler digestion tube.

2. Add 2 c.c. of concentrated sulfuric acid (*a*) and 1 c.c. of perchloric acid (*b*).

3. Digest over a microburner until the solution is practically colorless and fumes of sulfur trioxide appear.

4. After the solution has cooled slightly, dilute with distilled water exactly to the 50-c.c. mark, and mix by inverting several times.

5. Place exactly 20 c.c. of the solution in a large test tube and add 5 c.c. of thiocyanate solution (*c*).

6. In a test tube which is accurately graduated to 25 c.c., place exactly 1 c.c. of the standard iron solution (*d*). Add 0.8 c.c. of concentrated sulfuric acid (*a*), and dilute exactly to the 20-c.c. mark, and then add exactly 5 c.c. of the thiocyanate solution (*c*).

* Sørensen citrate—Hydrochloric acid buffer solution, *p*H 4.8. Dissolve 21.008 Gm. crystalline citric acid in 200 c.c. normal sodium hydroxide and make up to 1 liter with water. Take 900 c.c. and mix with 100 c.c. of tenth normal hydrochloric acid.

[1] Personal communication.

[2] Kennedy, R. P.: The Quantitative Determination of Iron in Tissues, Jour. Biol. Chem., *74*:385–391 (Aug.), 1927.

[3] Wong, S. Y.: Colorimetric Determinations of Iron and Hemoglobin in Blood, Jour. Biol. Chem., *77*:409–412 (May), 1928.

7. Compare the unknown with the standard in a colorimeter; the standard is set at 20 mm.

Calculation:

$$\frac{20}{\text{Reading of Unknown}} \times 50 = \frac{1000}{R} = \text{mg. of iron per 100 c.c.}$$

Divide by 3.35 to obtain grams per 100 c.c. of hemoglobin, as hemoglobin contains 0.335 per cent of iron.

A "short-cut method" of calculating is to set the unknown at 25 mm. and match the standard against it. Then multiply the reading of the standard by 2 to obtain milligrams of iron per 100 c.c.

The unknown may also be set at 14.9, and the reading of the standard which is matched against it will give approximately grams of hemoglobin per 100 c.c.

K. Sulfates

The consideration of the importance of sulfur in the blood has been of interest to biochemists for several years. Most attention has been paid to the oxidized portion of the nonprotein sulfur, that is, the inorganic and conjugated sulfates. The methods for determination are somewhat intricate, and extremely delicate, owing to the small quantity of these materials in normal blood serum. The normal range for inorganic sulfates in blood serum is 2.50 to 5 mg. per 100 c.c. of serum. In renal insufficiency occurring in glomerular nephritis, pyelonephritis, prostatic hypertrophy, polycystic kidneys, renal arteriosclerosis, and nephritis of pregnancy, there is an increase in the value for the inorganic sulfates in blood serum. This increase may occur before other tests reveal any change in kidney function. As the renal insufficiency advances, the sulfates in the serum continue to increase. Values may be as high as 50 to 100 mg. of sulfates in 100 c.c. of serum.

The best methods for determination depend on the precipitation, by benzidine in acetone, of the small quantity of sulfates which are present in trichloracetic acid filtrates of serum. The resulting precipitate of benzidine sulfate may be quantitatively measured colorimetrically, by direct titration with standard alkali, or by complete oxidation with potassium dichromate and subsequent iodometric determination of the amount of oxidizing agent used. All of these procedures require the utmost care in technic. The latest volumetric benzidine method of Power and Wakefield[1] is given.

Inorganic Sulfate in Serum (Method of Power and Wakefield).—*Glassware.*—If the centrifuge tubes are soaked in sulfuric acid-dichromate solution, extreme care is required to remove the last trace of it. Rinse the tubes three or four times with tap water and two or three times with distilled water, then heat them an hour or more with hot distilled water; finally, again rinse the tubes four or five times with distilled water. Certain soft

[1] Power, M. H., and Wakefield, E. G.: A Volumetric Benzidine Method for the Determination of Inorganic and Ethereal Sulphate in Serum, Jour. Biol. Chem., *123*:665–678 (May), 1938.

glass centrifuge tubes that have rolled rims sometimes retain solution under the rims. This might constitute a source of contamination if such tubes were used for the precipitation of protein.

Reagents.—(*a*) Trichloracetic acid (redistilled[1]). A solution containing 20 Gm. in 100 c.c. is prepared as follows: The acid is melted in a water bath at about 65° C., 25 c.c. of the melted acid is transferred to distilled water by means of a previously warmed pipet, and the volume is made up to 200 c.c.

(*b*) Ethyl alcohol, 95 per cent. Ordinary alcohol is redistilled; this gives the same results as aldehyde-free preparations.

(*c*) Benzidine in alcohol. One gram of benzidine is dissolved in alcohol (*b*) and the solution made to a volume of 100 c.c. Solutions of purified[2] benzidine will keep from one to two weeks in a brown, glass-stoppered bottle.

(*d*) Sodium hydroxide. A 0.01 to 0.001 N solution is used, depending on the size and graduation of the buret to be used. For routine determinations the use of 0.002 N solution in a 5-c.c. buret graduated to 0.01 c.c. and provided with a Shohl[3] needle tip (Fig. 195) is convenient; one filling of the buret serves for several titrations. The solution of the alkali is prepared essentially free of silicate and carbonate according to the method of Fiske and Logan.[4] The solution is preserved in a bottle coated with a purified grade of paraffin and provided with a soda lime guard tube. The factor of a 0.001 or 0.002 N solution does not change materially for several months. The buret should be filled without exposure of the solution to air. Fiske and Logan described a suitable procedure, and various other ways will suggest themselves. Since filling through the tip introduces traces of grease from

[1] Trichloracetic acid was purified by distillation *in vacuo*, in a 2000 c.c. pyrex distilling apparatus (No. 1370), connected by means of a rubber stopper to a suction flask as received. Hot water was used in the condenser. Considerable bumping occurred, in spite of the use of various antibumping devices; nevertheless, in the distillation of about 1 Kg. of the acid, to which 1.0 Gm. of benzidine was added to combine with the sulfate, a colorless and practically sulfate-free product was obtained.

[2] For the purification of benzidine, the method of Bing (Jour. Biol. Chem., *95*:387 [March], 1932) was modified as follows: 20 Gm. of benzidine was dissolved in 150 c.c. of 70 per cent alcohol (7 volumes of 95 per cent alcohol plus 3 volumes of water) at about 70° C., allowed to crystallize in the refrigerator, filtered with suction and washed with a little cold 50 per cent alcohol. The crystals were redissolved in about 135 c.c. of 70 per cent alcohol, 1 Gm. of decolorizing carbon (carboraffin) was added, and the mixture heated an additional one or two minutes, then filtered immediately on a hot-water funnel through a paper which previously had been washed with some of the warm alcohol; finally, the flask and filter were washed with about 30 c.c. of warm 50 per cent alcohol. The filtrate was cooled in the refrigerator, and the benzidine filtered with suction, and dried in a dark place. The air-dried substance contains water and traces of carbon which should be removed by recrystallization from dry benzene. The crystals were preserved in a bottle of brown glass.

[3] Shohl, A. T.: A Pipet for Micro-analyses. Jour. Am. Chem. Soc., *50*:417 (Feb.), 1928.

[4] Fiske, C. H., and Logan, M. A.: The Determination of Calcium by Alkalimetric Titration. II. The Precipitation of Calcium in the Presence of Magnesium, Phosphate, and Sulphate, with Applications to the Analysis of Urine, Jour. Biol. Chem., *93*:211–226 (Sept.), 1931.

the stopcock, which necessitates more frequent cleaning, it is preferable to fill through the top. A soda lime guard tube is then attached.

(*e*) Phenol red. A 0.01 per cent aqueous solution of phenol red is used. This solution should be clear and free of suspended particles.

Method.—1. *Deproteinization.*—Add 2 c.c. of 20 per cent trichloracetic acid (*a*) to 2 c.c. of serum in a centrifuge tube, mix with a glass rod, carefully breaking up the curdy precipitate, and allow the mixture to stand fifteen to thirty minutes, stirring thoroughly once or twice again. Close the tube with a rubber cap, centrifuge, and filter the supernatant fluid through a small sulfate-free filter paper. Filtrates made in this manner contain substantially the same amounts of sulfate as do those from mixtures which are made up to a definite mark in a volumetric flask before filtration. If the quantity of sulfate in the serum is greater than normal, remove proteins at a dilution of 1 : 5 or 1 : 10 by using for one volume of serum four volumes of 11.5 per cent or nine volumes of 10 per cent trichloracetic acid, respectively. The acidity of these various filtrates will correspond to about 8.5 per cent trichloracetic acid for serum which contains a normal amount of protein. Variation of acidity from 7 to 10 per cent is without material influence on the result.

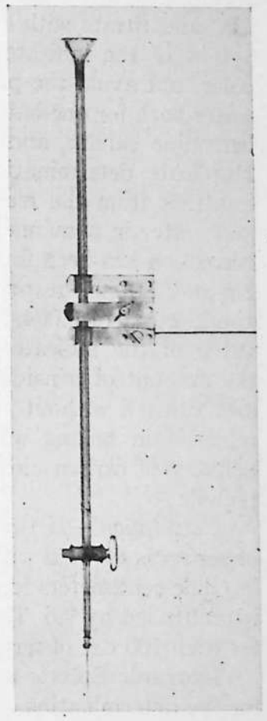


Fig. 195. — Microburet, with needle adapter for small gauge Luer hypodermic needle. (Gold.)

2. *Precipitation of Benzidine Sulfate.*—Select a pyrex centrifuge tube that is not scratched or etched on the inside and that is of such size and shape that the rounded end of a 3.0 to 3.5-mm. glass rod will make approximate contact with the inside surface at the tip. To 2 c.c. of filtrate in such a tube add 5 c.c. of benzidine reagent (*c*), stir the mixture with a glass rod, withdraw the rod, rinse it with a few drops of alcohol, close the tube with a rubber cap, and place it in the refrigerator. After an hour or more, centrifuge the tube for about ten minutes at 2500 to 3000 revolutions per minute (the radius of the centrifuge head used was 14 cm.), decant the supernatant fluid carefully, allow the last drop or two to fall onto a clean towel, then invert the tube over the spot so moistened. Allow to drain about five minutes, wipe the mouth of the tube with a clean towel, add 5 to 6 c.c. of alcohol (*b*), carefully rinse the sides from the top downward, pulverize the precipitate of benzidine sulfate with a moderately pointed pyrex rod, remove the rod, rinse with a little alcohol, mix the precipitate and wash fluid by rolling the tube between the palms of the hands, add 5 to 6 c.c.

more of alcohol, again rinse the sides, roll the tube between the hands again, cap the tube and centrifuge. Decant and drain over a moistened towel as before.

3. *Titration of Benzidine Sulfate.*—Add 2.0 to 2.5 c.c. of hot distilled water, rinse the walls of the tube, add 0.1 c.c. of phenol red (*e*) and a slender pyrex rod, and place the tube in a beaker of water boiling over an electric plate. After a few minutes the precipitate will usually dissolve completely, especially if broken up by means of the rod. Remove the tube from the water bath, introduce a fine pyrex jet for the delivery of carbon dioxide-free air,[1] and titrate with a 0.002 N solution of sodium hydroxide (*d*) until the yellow of the indicator undergoes the first easily recognizable change of color, but avoid the production of much pink. Place the tube back in the water bath for one-half to one minute to insure solution of any undissolved benzidine sulfate, and continue the titration if the yellow color returns. Duplicate determinations will usually agree to within 0.01 c.c. or less. Subtract from the reading a blank value obtained by titrating indicator and water in a manner similar to the titration of benzidine sulfate. This correction has been found to be around 0.008 and 0.012 c.c. at volumes of 2.5 and 5.0 c.c., respectively. Likewise standardize the alkali by titrating about 2 c.c. of 0.004 N acid as in an unknown titration, subtracting the value of the indicator-water blank before calculating the factor. When the amount of benzidine sulfate is large, the bulk of it may be dissolved and titrated without the introduction of the air stream; the tube then is replaced in boiling water to dissolve the remaining sulfate, the jet for delivery of carbon dioxide-free air is introduced, and the titration is completed.

Calculation.—If the benzidine sulfate from filtrate equivalent to 1 c.c. of serum is titrated with 0.002 N solution of alkali, the amount of solution in cubic centimeters required, minus the value of the indicator-water blank, is multiplied by 9.6. This gives the inorganic sulfate in terms of milligrams for each 100 c.c. of serum.

Inorganic Sulfate in Urine.—The *reagents* are the same as those used for the determination of serum sulfate except that (*a*) 10 per cent solutions of trichloracetic acid, and (*d*) 0.01 N solution of sodium hydroxide are used. Take 1 c.c. of urine, add 9 c.c. of 10 per cent trichloracetic acid, making a 1 : 10 dilution. Filter and transfer 2 c.c. of the filtrate into a 15-c.c. centrifuge tube. Add 5 c.c. of 1 per cent benzidine reagent, mix and proceed as in the serum method except that the titration is carried out with 0.01 N solution of sodium hydroxide. Multiply the titration value by 240 and by the correction factor for the sodium hydroxide reagent (if necessary) to obtain the amount of sulfate in milligrams for each 100 c.c. of urine.

The *sulfate clearance* may be determined in a manner similar to urea clearance (p. 174).

[1] Power and Wakefield have encountered samples of tubing which slowly liberate volatile bases in spite of careful cleaning with alkali or subsequent soaking in dilute acid. Such tubing must not be used for the delivery of carbon dioxide-free air.

L. Total Base: Sodium and Potassium

Total Base.—The determination of total base in blood and urine has been of interest in nephritis, acidosis, pyloric obstruction, diabetes, pneumonia, Addison's disease, and in studies of water balance generally. In the serum of normal human beings, total base is about 150 to 160 millequivalents, or amounts to about 360 mg. per 100 c.c. Osmotic pressure, and physiologic neutrality within physiologic limits are maintained primarily by the total base content in both extracellular and intracellular fluids. In Addison's disease, the values for the total base are low during the crisis. In pneumonia there is hypochloremia and a deficit in the serum base. For a method for the determination of the total base, the reader is referred to the technic which has been described by Stadie and Ross.[1]

Sodium.—Kramer and Gittleman[2] have described an iodometric method for the quantitative analysis of sodium in serum. The normal amount is about 325 to 350 mg. per 100 c.c. of serum. It is evident that changes in total base will be chiefly indicated by changes in sodium content. In acidosis, the value for sodium may be as low as 200 mg. per 100 c.c. of serum. The method for the determination of sodium is as follows: *Reagents.*—(*a*) Potassium pyroantimonate reagent. Dissolve 10 Gm. of potassium pyroantimonate (J. T. Baker) in 500 c.c. of boiling distilled water in a Pyrex flask. Continue boiling for three to five minutes. Then cool the flask under running water. When cool, add 15 c.c. of a 10 per cent solution of potassium hydroxide (alcohol-washed) which has been kept in a paraffined bottle. Filter through ash-free filter paper into a paraffined bottle. Ten cubic centimeters of this reagent will precipitate 11 mg. of sodium. Test 10 c.c. of this reagent with 2 c.c. of distilled water and 3 c.c. of 95 per cent alcohol. There should be no precipitate of sodium or of potassium pyroantimonate.

(*b*) 95 per cent alcohol redistilled over potassium hydroxide.

(*c*) 30 per cent alcohol.

(*d*) Ten normal hydrochloric acid (concentrated hydrochloric acid, 200, distilled water 60 c.c.).

(*e*) 20 per cent solution of potassium iodide.

(*f*) Tenth normal solution of sodium thiosulfate. Dissolve 24.822 Gm. of sodium thiosulfate in 1 liter of distilled water.

(*g*) 1 per cent solution of soluble starch freshly made each day.

Method.—1. Place 2 c.c. of serum in a large pyrex centrifuge tube.

2. Add 10 c.c. of the pyroantimonate reagent (*a*) and 3 c.c. of 95 per cent alcohol (*b*) drop by drop, stirring continuously with a rubber-tipped glass rod. Stopper and let stand for thirty minutes.

3. Centrifuge for thirty minutes, decant the supernatant fluid and drain for fifteen minutes.

[1] Stadie, W. C., and Ross, E. C.: A Micro Method for the Determination of Base in Blood and Serum and Other Biological Materials, Jour. Biol. Chem., *45*:735–754 (Oct.), 1925.

[2] Kramer, Benjamin, and Gittleman, I.: An Iodometric Method for the Determination of Sodium in Small Amounts of Serum. Jour. Biol. Chem., *62*:353–360 (Dec.), 1924.

4. Add 10 c.c. of 30 per cent alcohol (*c*) to the precipitate. Mix and centrifuge for twenty minutes. Decant the supernatant fluid carefully.

5. Add to the precipitate 5 c.c. of 10 normal hydrochloric acid (*d*) and stir thoroughly.

6. Transfer the mixture to a 125 c.c. flask with 10 c.c. of distilled water. Add 2 c.c. of 20 per cent solution of potassium iodide (*e*) and titrate with tenth normal solution of sodium thiosulfate (*f*). Add 1 c.c. of starch solution (*g*) when the color is almost gone and titrate drop by drop until clear.

Set up a blank of all of the reagents without serum and titrate in the same manner. (This titration should require about 0.05 c.c.)

Calculation.—Number of cubic centimeters of solution of sodium thiosulfate used in the titration of the unknown minus the number of cubic centimeters used in the titration of the blank × 1.15 × 50 = mg. of sodium per 100 c.c.

Potassium.—Kramer and Tisdall[1] have described a method for the quantitative determination of potassium in serum. The method may also be applied to determinations of potassium in urine. The normal value for potassium is about 20 mg. per 100 c.c. of serum. In acute fevers there is an increase in the concentration of potassium; values as high as 70 mg. or more may be found. The method for the determination of potassium is as follows:

Reagents.—(*a*) Sodium cobalti-nitrite reagent. Solution A. Dissolve 25 Gm. of cobalt nitrate crystals (J. T. Baker) in 50 c.c. of distilled water. Add 12.5 c.c. of glacial acetic acid. Solution B. Dissolve 120 Gm. of sodium nitrite (potassium-free) in 180 c.c. of distilled water. (The total volume of this solution will be about 220 c.c.) Add 210 c.c. of solution B to all of solution A. There will be an evolution of nitric oxide, all of which should be removed by drawing air through the solution. Place the reagent in the refrigerator and filter each time before using. It will keep at least a month.

(*b*) Fiftieth normal solution of potassium permanganate. Dilute tenth normal solution of potassium permanganate five times. (See page 403, calcium reagent [*d*] for the preparation of tenth normal potassium permanganate solution.)

(*c*) Hundredth normal solution of sodium oxalate. Dilute tenth normal solution of sodium oxalate ten times with distilled water to make hundredth normal solution. (To prepare tenth normal solution of sodium oxalate, dissolve 6.7 Gm. of Sørensen's sodium oxalate in a liter of distilled water with the aid of 5 c.c. of concentrated sulfuric acid.)

(*d*) Approximately fourth normal sulfuric acid. Dilute 20 c.c. of concentrated sulfuric acid to 100 c.c. with distilled water.

Method.—1. Measure exactly 1 c.c. of *clear serum* into a 15 c.c. graduated centrifuge tube. Add 2 c.c. of sodium cobalti-nitrite reagent (*a*) drop by drop and thoroughly mix. Place in the refrigerator for forty-five minutes.

[1] Kramer, B., and Tisdall, F. F.: A Clinical Method for the Quantitative Determination of Potassium in Small Amounts of Serum, Jour. Biol. Chem., *46*:339–349 (April), 1921.

2. Add 2 c.c. of water. Mix the contents of the tube and centrifuge at a speed of 1300 to 1400 revolutions per minute for thirty minutes.

3. Decant the supernatant fluid. Gently add 5 c.c. of distilled water to the residual reagent without disturbing the precipitate. Centrifuge for five minutes and again decant the supernatant fluid. Repeat this careful washing and centrifuge twice more, making three washings in all.

4. Add 1 c.c. of approximately fourth normal sulfuric acid (*d*) and 2 c.c. of fiftieth normal solution of potassium permanganate solution (*b*). Thoroughly mix the precipitate with the reagents by means of a glass rod. Heat in a boiling water bath for about a minute until there is no change in color.

5. Add sufficient hundredth normal solution of sodium oxalate (usually 2 c.c.) to decolorize the solution completely.

6. Titrate for the excess of oxalate with fiftieth normal solution of potassium permanganate (*b*) until a definite pink color is obtained.

A blank determination should be made on the reagents without serum.

Calculation.—Two cubic centimeters + the number of cubic centimeters of solution of potassium permanganate used in the final titration − the titration of the blank × 2 = the number of cubic centimeters of hundredth normal solution of potassium permanganate. The number of cubic centimeters of hundredth normal solution of potassium permanganate − 2 (c.c. of hundredth normal solution of sodium oxalate) = number of cubic centimeters of solution of potassium permanganate required to oxidize the potassium cobalti-nitrite. The total number of cubic centimeters of hundredth normal solution of potassium permanganate required to oxidize the potassium cobalti-nitrite × 7.1 = mg. of potassium per 100 c.c. of serum. The formula is: [(2 c.c. + titration − blank) × 2 − 2] × 7.1 = mg. of potassium per 100 c.c. of serum.

M. Bile Pigment

The faint yellowish tinge of normal blood plasma and blood serum is probably due to minute traces of bilirubin. In jaundice the yellow color becomes much deeper, and this may be evident in hepatic and hemolytic diseases at a time when no jaundice of the skin or sclera can be discerned.

Several years ago McNee[1] advanced a new theory for jaundice. He presented an interesting diagram which demonstrated the structure of the hepatic lobule, and the various ways by which jaundice might arise. These theories have been elaborated by others. The most recent working hypothesis, which has been presented by Elton,[2] is an ingenious diagrammatic explanation of the function of the hepatic lobules and circulation through the liver (Fig. 196). Accord-

[1] McNee, J. W.: Jaundice; a Review of Recent Work, Quart. Jour. Med., *16*:390–420 (July), 1923.

[2] Elton, N. W.: The Mechanism of Jaundice: A Working Hypothesis, Amer. Jour. Clin. Path., *5*:40–54 (Jan.), 1935.

ing to this theory, the hepatic lobule, from the standpoint of excretion of bilirubin, may be divided into two major functional zones and an intermediate zone. The "acceptance zone" is that portion of the lobule in which the Kupffer cells take up bilirubin and pass it on to the underlying hepatic parenchyma where in the "conversion, or intermediate zone" it is converted by neutralization to bilirubinate,

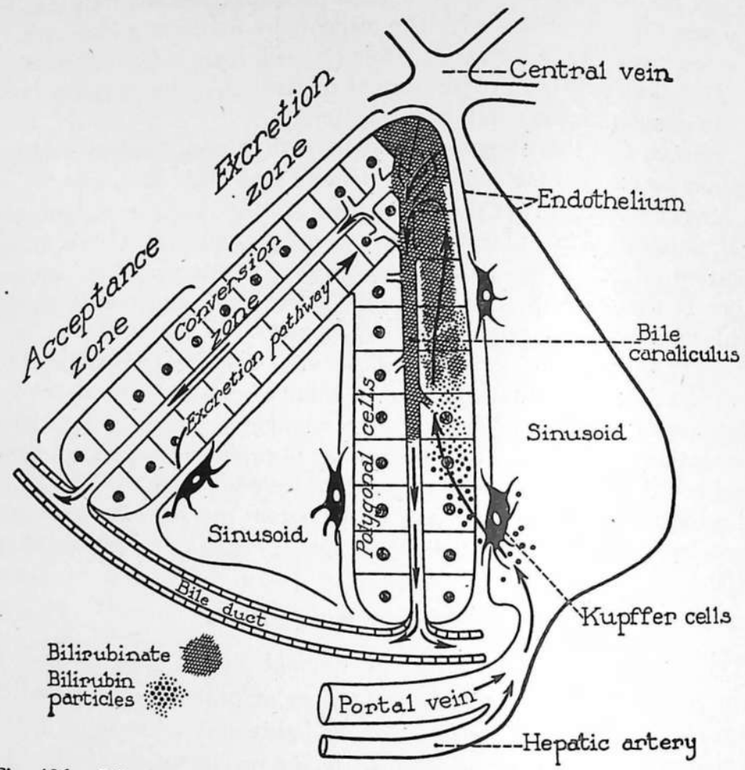


Fig. 196.—Bilirubin excretion mechanism. Radial structure of liver lobule causes marked narrowing of sinusoidal stream-bed as it converges toward the central vein and a marked reduction in number of polygonal cells carrying original pigment burden from peripheral parenchymal afferents (Norman W. Elton, in Am. Jour. Clin. Path., January, 1935, Williams and Wilkins Co., Publishers).

thereby becoming water soluble. The "excretion zone" is the central portion of the lobule where bilirubinate enters the bile canaliculi from the polygonal cells, and through which it passes as a bile constituent to the periphery and into the hepatic ducts. Jaundice, or leakage of bile, in which either bilirubin or bilirubinate may pass into the general circulation, may occur from the following causes according to Elton's hypothesis: (1) Mechanical obstruction of the bile ducts,

with a positive direct van den Bergh reaction; (2) dynamic elevation of the excretion threshold: existence of a reactive state in the liver, with overloading of the functioning lobules, and cessation of function in others, such as occurs occasionally in lobar pneumonia, in catarrhal jaundice, and in infectious jaundice; (3) adynamic elevation of the excretion threshold, as seen in familial icterus, where the fault may lie in the function of the Kupffer cells, or possibly in the size of the bilirubin particles; (4) pigment overload, which causes bilirubinate to leak into the blood stream; (5) damage to hepatic parenchyma from toxic agents of all sorts; and (6) patent ductus venosus, which Mann has pointed out is a natural "Eck fistula," may well explain icterus neonatorum. Two or more of the preceding factors may operate together to cause jaundice.

Mann,[1] and Mann and Bollman[2] have studied the function of the liver most extensively and have demonstrated that jaundice may be produced experimentally in dogs by methods which involve the liver. Complete removal of the liver produces jaundice of a hemolytic type in that bilirubin of the blood responds "indirectly" in the van den Bergh reaction. Occlusion of the bile ducts or hepatic injury increases the bilirubin content of the blood, producing a "direct" reaction.

The recognition of bile staining of the plasma is a very sensitive and very definite means of detecting slight grades of jaundice and of recognizing fluctuations in the severer grades.

A diet of carrots, especially popular for young children, may give a peculiar yellow tinge to the skin and blood serum, known as carotinemia. It does no harm and disappears after removing the vegetable from the diet.

Carotin in Blood Serum, or Plasma.—Place 10 c.c. of serum or plasma in an Erlenmeyer flask with about 40 Gm. of plaster of paris. Add 10 c.c. of alcohol, and mix thoroughly. Add 10 c.c. of low-boiling petroleum ether and shake vigorously, and filter. If carotin is present, the filtrate will be yellow. Evaporate to dryness on the steam bath. Add 10 c.c. of alcohol to the precipitate. Carotin is present if the precipitate is yellow and soluble in this alcohol.

In testing for bile pigment either plasma or serum may be used, but the blood must be secured with careful avoidance of hemolysis.

Test for Bile Pigment.—Gmelin's test may be applied by overlaying the yellow nitric acid with the serum or plasma. A broad white ring of

[1] Mann, F. C.: Hepatic Function in Relation to Hepatic Pathology; Experimental Observations, Ann. Int. Med., *8*:432–443 (Oct.), 1934.

[2] Mann, F. C., and Bollman, J. L.: Jaundice; A Review of Some Experimental Investigations, Jour. Am. Med. Assn., *104*:371–374 (Feb. 2), 1935.

coagulated protein appears at the zone of contact. The appearance of a narrow bluish-green ring in the midst of the white indicates an abnormal amount of bile pigment. The serum of normal persons will sometimes give a very faintly positive reaction, the colored ring becoming evident only after half an hour. The test is said to detect 1 part of bilirubin in 30,000 or 40,000 of serum.

"Icterus Index" Determination.—Bernhard and Maue, modifying Meulengracht's method, have made use of the Duboscq colorimeter in which the blood serum, diluted 1 : 2, 1 : 4, 1 : 10, and so forth, as may be necessary, is compared with a color standard consisting of 1 : 10,000 solution of potassium bichromate. The reading of the standard is divided by the reading of the unknown and this multiplied by the dilution to obtain the "icterus index." Stetten has found the normal by this method to lie between 2.5 and 5, average 3.6. Two drops of sulfuric acid added to 500 c.c. of the potassium dichromate solution is said to make a stable standard solution. It should also be kept in a dark bottle away from the light.

Van den Bergh Quantitative Method for Serum Bilirubin (*Modification of Thannhauser and Anderson*[1]).—Ehrlich found that when sulfanilic acid and sodium nitrite were added to a solution of bilirubin, a colored addition product, azobilirubin, was formed. This reaction is specific and will detect bilirubin in a dilution of 1 : 1,500,000. Van den Bergh precipitated the blood serum protein with alcohol, which also serves as a solvent for bilirubin, and showed that Ehrlich's reaction could be applied to this alcoholic extract. When a very small amount of bilirubin is present in the blood serum, with hemolysis as the source of the pigment, alcoholic extraction is necessary. This is the principle of the "indirect reaction." On the other hand, in obstructive jaundice with bile itself in the blood serum, alcoholic extraction is not necessary, as the characteristic color reaction appears at once on addition of the reagent. This is the "direct reaction." Either serum or plasma may be used for the test, but it must be clear and free from hemoglobin.

Reagents Required.—(*a*) Standard artificial bilirubin solution. (1) Dissolve 0.1508 Gm. of ammonium iron alum in 50 c.c. of concentrated hydrochloric acid, and add water to make 100 c.c. This solution will keep indefinitely.

(2) To 10 c.c. of (1) add 25 c.c. concentrated hydrochloric acid, and water up to 250 c.c. This solution keeps about six months.

(3) To 5 c.c. of (2) add 5 c.c. of 20 per cent potassium sulfocyanide in a glass-stoppered cylinder (50-c.c. capacity); add 20 c.c. of ether. Shake well and transfer ether extract to a colorimeter cup. This standard solution must be prepared each day a test is made, and represents 0.5 mg. of bilirubin in 100 c.c.

[1] Thannhauser, J. S., and Anderson, E.: Methodik der quantitativen Bilirubinbestimmung im menschlichen Serum, Deutsch. Arch. f. klin. Med., *137*:179–186 (Aug.), 1921; abst. Jour. Am. Med. Assn., *77*:1292 (Oct.), 1921. Greene, C. H., Snell, A. M., and Walters, Waltman: Diseases of the Liver. I. A Survey of Tests for Hepatic Function, Arch. Int. Med., *36*:248–272 (Sept.), 1925.

(*b*) Sulfanilic reagent. This is freshly prepared Ehrlich's diazo reagent. It is made of two solutions each of which keeps well, but the mixture must be made immediately prior to the test.

Solution A: Sulfanilic acid, 5 Gm.; concentrated hydrochloric acid, 50 c.c.; distilled water to make 1000 c.c.

Solution B: Sodium nitrite, 0.5 Gm.; distilled water, 100 c.c.

Take 0.8 c.c. of solution B and make up to 25 c.c. with solution A.

(*c*) Saturated solution of ammonium sulfate.

(*d*) Ethyl alcohol, 95 per cent.

Method.—1. To 2 c.c. of clear serum add 1 c.c. of freshly prepared sulfanilic reagent (*b*). If there is a marked color change this may be compared at once with the standard; the "direct reaction."

2. Add 2 c.c. saturated ammonium sulfate (*c*).

3. Add 10 c.c. 95 per cent alcohol (*d*).

4. Centrifugalize.

5. Compare with standard (*a*) in a colorimeter, setting standard cup at 20 mm.

Calculation:

$$\frac{\text{Reading of Standard (20)}}{\text{Reading of Unknown}} \times 3.75^{1} = \text{mg. of bilirubin in 100 c.c. of serum.}$$

Normal blood contains less than 1.5 mg. of bilirubin for each 100 c.c. of serum.

The technic which has been previously described is used in many laboratories, but a number of criticisms and modifications have been offered. They concern the standard, the dilution factor as it is modified by the precipitate in the centrifuge tube, and the method of observing the "direct reaction." For a discussion of these criticisms, the reader is referred to an editorial by Magath.[2] The standard that has been described matches more nearly in color than any other artificial bilirubin standard. Another standard that is used in many laboratories is the cobalt standard of McNee.[3]

McNee's Standard for Serum Bilirubin.—Dissolve 2.161 Gm. of anhydrous cobalt sulfate, or 3.92 Gm. of $CoSO_4.7H_2O$, in distilled water and dilute to 100 c.c. This is a permanent standard, and is preferable for that reason alone. The color matches rather well, but is not nearly so close a match as is the older ethereal standard.

There has been much discussion as to whether the original standard represents a dilution of 1 : 200,000 or 1 : 250,000; that is, whether 0.5 mg. or 0.4 mg. should be used in the calculation. It is obvious that this is a 20

[1] The serum, 2 c.c., is diluted 7.5 times by the addition of 13 c.c. of reagents (*b*) (*c*) (*d*). It is matched against a standard of 0.5 mg. to 100 c.c. Therefore for 1 mg. to 100 c.c. the factor is one half of 7.5 or 3.75.

[2] Editorial: The Serum Bilirubin Test, Jour. Lab. and Clin. Med., *18*:974 (June), 1933.

[3] McNee, J. W., and Keefer, C. S.: The Clinical Value of the van den Bergh Reaction for Bilirubin in Blood with Notes on Improvements in Its Technic, Brit. Med. Jour., *2*:52–54 (July 11), 1925.

per cent discrepancy. However, van den Bergh and Grotepass,[1] in reporting an improved method for the determination of bilirubin in blood serum, consider the concentration of azo bilirubin, as represented in the standard, to be 1 : 200,000, and not 1 : 250,000. The new method is an improvement only in the use of a Schott's green light filter (S53), transmitting light at 520 to 546 $\mu\mu$ and a fine black gauze screen made of wires of 0.35 mm. diameter, and mesh measuring 1 mm. No standard is necessary after the colorimeter readings have been calibrated. In the method that is described there is a considerable precipitate of protein which is brought down by the ammonium sulfate. The question arises whether the color is all in the alcohol above the precipitate, or whether some has been carried down with the precipitate. In the footnote explanation of the calculation, it is seen that the precipitate is disregarded. Some authors prefer to measure in cubic centimeters the alcohol layer above the precipitate and to use the following formula:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \text{volume of alcohol layer} \times 0.5 = \text{mg. bilirubin per 100 c.c. of serum.}$$

In their new method, which has been referred to previously, van den Bergh and Grotepass have attempted to prevent the adsorption of bilirubin to the albuminous precipitate by adding to the serum a mixture of reagent, diluted with 50 per cent alcohol which has been prepared with a buffer solution of *p*H 6.6. It must be remembered, however, that the method at best is only an "estimation" and has never been considered a very accurate quantitative method. In considering the type of reaction which goes by name of van den Bergh, several authors have advocated a method which first was described by Lepehne,[2] and in which the serum and reagent are brought together to form a simple "ring test." The direct reaction, as a result of the action of Ehrlich's diazo reagent on the water-soluble bilirubinate which is found in the blood in obstructive jaundice, is nicely demonstrated in a ring test which has been described by Elton,[3] as follows:

Place 1 c.c. of clear serum in a 15-c.c. graduated centrifuge tube. Slant the tube to a nearly horizontal position, and add 0.5 c.c. of diazo reagent in such a manner as to overlay the serum. Restore the tube to a vertical position and examine the contact zone of the two fluids for the development of a reddish ring. If no color appears within sixty seconds, gently shake the tube and thus lower the contact zone in the tube, and observe for another ten minutes. The quantitative test may then be carried out in the same tube by using half the quantities used in the Thannhauser and Anderson method as described.

[1] Van den Bergh, A. A. H., and Grotepass, W.: An Improved Method for the Determination of Bilirubin in Blood, Brit. Med. Jour., *1*:1157–1159 (June 30), 1934.

[2] Lepehne, G.: Weitere Untersuchungen über Gallenfarbstoff in Blutserum des Menschen, Deutsch. Arch. f. klin. Med., *135*:79–107 (Jan. 25), 1921.

[3] Elton, N. W.: The Mechanism of Jaundice: A Working Hypothesis, Am. Jour. Clin. Path., *5*:40–54 (Jan.), 1935.

Photelometric Determination of Serum Bilirubin[1,2,3,4].—Hoffman[2] stated that a thousandth normal solution of potassium permanganate gives the same reading with a green filter as does the azobilirubin solution, representing 10 mg. per 100 c.c. Therefore, for a standard, Hoffman prepared a five hundredths normal solution of potassium permanganate. Dilute 2 c.c. of this solution to 100 c.c. in a volumetric flask and mix. Read this solution in the photelometer, using Cenco number 2 green filter, with distilled water set at 100. Plot this reading as 10 mg. bilirubin per 100 c.c. Plot 0 mg. concentration at 100 on semilogarithmic paper. Draw a straight line through these two points to make a calibration curve which is just as accurate as one made from so-called pure bilirubin.

N. Tests of Liver Function

Following the great success of the phenolsulfonphthalein test of kidney function, Rowntree and his co-workers applied a similar test to the liver, using phenoltetrachlorphthalein, which they found to be excreted practically only by the liver and to be nontoxic. A definite dose was administered intravenously and the amount recoverable from the feces in forty-eight hours was taken as an index of the functional capacity of the liver. For various reasons the test did not meet general favor. It was later modified by H. L. McNeil, who determined the time required for the dye to appear in the bile and measured the amount which could be recovered from the duodenum within two hours by means of the duodenal tube, but obtained inconstant results. In 1922 Rosenthal introduced the method of measuring the amount which remains in the blood at definite periods after intravenous injection of 5 mg. of dye per kilogram of body weight. More recently he substituted bromsulfalein for phenoltetrachlorphthalein. He has found that a normal liver will remove most of the dye from the blood within fifteen minutes and practically all of it within an hour; while in animals whose livers have been damaged experimentally and in human beings with definite hepatic disease the dye is removed much more slowly, and considerable amounts may remain for three hours or longer. Twenty-four hours must elapse before a kidney function test using phenolsulfonphthalein is attempted; otherwise the kidney function test will not be reliable.

[1] Malloy, H. T., and Evelyn, K. A.: The Determination of Bilirubin with the Photoelectric Colorimeter. Jour. Biol. Chem., *119*:481–490 (July), 1937.

[2] Hoffman, W. S.: Photelometric Clinical Chemistry. New York, Wm. Morris and Company, 1941, pp. 231–243.

[3] Giordano, A. S., and Eager, Deane: The Quantitative Estimation of Bilirubin in the Blood Serum or Plasma. Amer. Jour. Clin. Path. *6*:286–292 (May), 1936.

[4] Osterberg, A. E.: New Van den Bergh Reaction for the Determination of Serum Bilirubin Utilizing the Photelometer. Jour. Lab. & Clin. Med. *22*:729–735 (Apr.), 1937.

Rosenthal's Bromsulfalein Method.[1]—The dye is conveniently used in 5 per cent solution. One-tenth c.c. then contains 5 mg. of the dye. This solution, sterilized and put up in sealed ampules, may be obtained of Hynson, Westcott, and Dunning, Baltimore, as may also the set of color standards. The *standards* may also be prepared by placing 0.08 c.c. of 5 per cent solution of bromsulfalein (4 mg.) in 100 c.c. of distilled water. Alkalinize with 0.25 c.c. of 10 per cent solution of sodium hydroxide. This standard is designated as 100 per cent. From this solution make dilutions with alkalinized water containing 80, 70, 60, 50, 40, 30, 20, 15, 10, and 5 per cent.

Method.—1. Weigh the patient, calculate the amount of dye required for the test, allowing 5 mg. for each kilogram of body weight. For each 10 Kg. or for each 22 pounds of body weight, take 1 c.c. of the sterile dye solution as furnished by the manufacturer.

(Originally 2 mg. for each kilogram of body weight was recommended as the test dose, and in some laboratories this dose is still preferred. If this dose is used the values are less for normal liver function.)

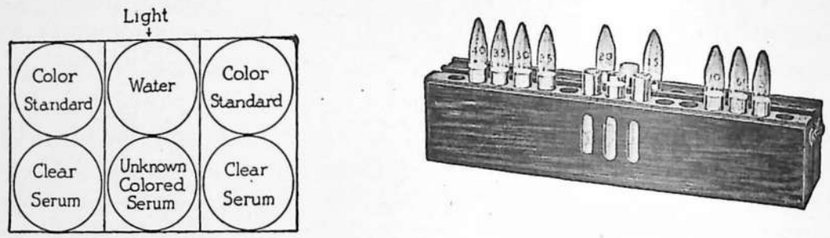


Fig. 197.—Rosenthal colorimeter for bromsulfalein liver function test. Supplied by Hynson, Westcott, and Dunning, Baltimore, Md.

2. Draw the required amount of dye into an accurately calibrated glass syringe, and inject the dye slowly, without diluting, into a vein in the patient's arm.

3. At the end of *thirty minutes* secure 5 c.c. of blood from the other arm, using a dry needle to avoid hemolysis. Collect the blood in a dry centrifuge tube and centrifugalize to separate the serum.

4. Determine the amount of dye in the thirty-minute and one-hour samples of blood. This determination is best made in the special colorimeter furnished by the manufacturer (Fig. 197). Equal quantities of the serum from each specimen are pipetted off into two small test tubes of the same diameter as the color tubes of the colorimeter. To one of the tubes add a drop of 10 per cent sodium hydroxide. Place the tubes in the colorimeter side by side and match by placing the standard tubes behind the tube without the alkali. (If there is a slight tinge of hemoglobin in the serum, a

[1] Rosenthal, S. M., and White, E. C.: Clinical Application of the Bromsulphalein Test for Hepatic Function, Jour. Am. Med. Assn., *84:*1112–1114 (Apr. 11) 1925.

drop of 5 per cent hydrochloric acid may be added to the control tube.) The percentage strength of the dye is marked on the standard tube.

In liver disease the dye is retained in the blood in concentrations that vary from 0 to 100 per cent of the amount injected. In many laboratories it has been found to be more useful to inject 5 mg. of dye for each kilogram of body weight. This amount is two and a half times the dose originally recommended. One cubic centimeter is injected for each 10 Kg., or 22 pounds, of body weight. The determination of dye retention is made at the end of exactly *one hour* after the intravenous administration. The standards for the 2 mg. dose may be used, but it must be remembered that in comparison, if a 5 mg. dose has been injected, the percentage of dye retained is only two-fifths of the percentage marked on the standard tube. It makes the calculation easier to relabel the standard tubes to conform with these percentages. If the dose of dye is 5 mg. per kilogram of body weight, the normal amount of retention in one hour is less than 6 per cent. Impairment of liver function will show a retention of dye between 6 per cent and 40 per cent or more.

Rose Bengal Test.—Instead of bromsulfalein, rose bengal, "special for intravenous use" (di-sodium-tetra-iodo-tetra-chlor-fluorescin) may be used to advantage according to Delprat[1] and his co-workers. Their test briefly is as follows: Inject intravenously 5 to 10 c.c. of 1 to 2 per cent rose bengal in physiologic saline solution. Two minutes later withdraw 8 c.c. of blood and place in a tube containing oxalate (see p. 360). The plasma of this sample is used as a "standard." *Exactly* six minutes later, withdraw another 8 c.c. of blood and use this as a test sample. It has been found, by experiment, that in six minutes the amount of color in the plasma is only 50 per cent of that in the standard plasma. Centrifugalize both samples. Take 3 c.c. of plasma from each sample, add 6 c.c. of acetone to precipitate the proteins, and recentrifugalize. If the plasma is yellow from bile pigment, add 3 drops of saturated solution of sodium hydrate, let stand in subdued light for fifteen minutes, centrifugalize again, and compare with the standard in a colorimeter. As the normal is only 50 per cent of the "standard," higher values mean lower liver function. The calculation is as follows:

$$200 - \frac{200\ RS}{Ru} = \text{per cent of normal liver function}$$

where RS is the colorimeter reading of the first (two-minute) sample and Ru of the second (eight-minute) sample.

Rose bengal is photosensitizing so patients should keep out of direct sunlight for a few hours, and they should be warned that their stools will be dyed red.

[1] Delprat, G. D.: Studies on Liver Function; Rose Bengal Elimination from Blood as Influenced by Liver Injury, Arch. Int. Med., *32:*401–410 (Sept.), 1923. Stowe, W. P., Delprat, G. D., and Weeks, Alanson: The Rose Bengal Test of Liver Function, Am. Jour. Clin. Path., *3:*55–60 (Jan.), 1933.

Galactose Tolerance Test.[1,2,3,4]—This test is not entirely reliable, but if carried out properly it may be of value in the differential diagnosis of jaundice. It depends on the use of a simple sugar, which is available in pure form, and which is promptly assimilated and converted into glycogen by a normal liver. The rest of the body seems unable to store galactose. Galactose is not a "renal threshold substance."

The patient should take the galactose in the morning, before breakfast. Save a morning sample of urine. Give exactly 40 Gm. of pure galactose in 500 c.c. of water which is flavored with lemon juice. Collect urine hourly for five hours, in separate containers. The patient may drink water, but should not take any food. Test each specimen for sugar by the Benedict qualitative method (p. 97). The samples of urine containing sugar are mixed and the total amount of sugar which has been excreted is determined by the Benedict quantitative method (p. 101). An excretion of more than 3 Gm. of reducing substance may indicate intrahepatic jaundice, although less than 6 Gm. may not be significant.

Hippuric Acid Test.—Hippuric acid, which results from the synthesis by the liver of benzoic acid and amino-acetic acid, is excreted in the urine normally at a nearly constant rate. Quick[5] has developed a simple test, which has proved useful in differential diagnosis. After the administration of sodium benzoate the normal excretion in urine of benzoic acid in the form of hippuric acid should be about 3 Gm. in four hours. A low output occurs in cases of catarrhal jaundice and various forms of hepatitis and usually in cases of malignant process with metastasis to the liver, and in cirrhosis. In cases in which cholecystitis, cholelithiasis, and biliary obstruction are due to stones in the common bile duct, the reaction to the test is normal.

The details of the test are as follows: The patient should eat a light breakfast of toast and coffee. One hour later administer *6 Gm. of sodium benzoate* dissolved in 30 c.c. of water, flavored with oil of peppermint, followed by one-half glass of water. Immediately after taking the drug the patient should void. Complete specimens of urine should be collected hourly for four hours. Measure each specimen and transfer it to a beaker. If any specimen exceeds 100 c.c. in amount, acidify with a few drops of acetic acid, and concentrate in a water bath to about 50 c.c. Acidify each of the four specimens of urine with 1 c.c. of concentrated hydrochloric acid.

[1] Bauer, Richard: Ueber die Assimilation von Galaktose und Milchzucker bei Gesunden und Kranken, Wien. med. Wchnschr., *56:*20–23, 1906.

[2] Shay, Harry, Schloss, E. M., and Bell, M. A.: The Metabolism of Galactose; Considerations Underlying Use of Galactose in Tests of Function of Liver, Arch. Int. Med., *47:*391–402 (Mar.), 1931.

[3] Shay, Harry, Schloss, E. M., and Rodis, Isadore: II. The Galactose Tolerance Test in the Differential Diagnosis of Jaundice, Arch. Int. Med., *47:*650–659 (Apr.), 1931.

[4] Banks, B. M., Sprague, P. H., and Snell, A. M.: Clinical Value of the Galactose Tolerance Test, Jour. Am. Med. Assn., *100:*1987–1993 (June 24), 1933.

[5] Quick, A. J.: Clinical Value of the Test for Hippuric Acid in Cases of Disease of the Liver, Arch. Int. Med., *57:*544–556 (Mar.), 1936.

Test with Congo red paper. If this does not turn blue, add more acid. Stir vigorously until the precipitation of hippuric acid is complete. Allow the precipitate to stand for one hour at room temperature and then filter off the crystalline hippuric acid on a small filter plate, or Buchner funnel. Wash with a small quantity of cold water and dry the precipitate in the air. Weigh to the second decimal place each dry sample of hippuric acid. If a balance is not available, dissolve the precipitate in hot water and titrate with 0.2 N solution of sodium hydroxide, using phenolphthalein as an indicator. One c.c. of 0.2 N solution of sodium hydroxide equals 0.0358 Gm. of hippuric acid. In each 100 c.c. of acidified urine 0.33 Gm. of hippuric acid will remain in solution at room temperature. Therefore, this figure must be added to the amount of hippuric acid determined by weight or titration in each specimen. Thus, for example, if the hourly specimen amounts to 70 c.c. and the amount of hippuric acid obtained by weight is 1.1 Gm., the total will be $1.1 + \left(0.33 \times \frac{70}{100}\right) = 1.33$ Gm. hippuric acid. Multiply by 0.68 to express the result in benzoic acid. Therefore, 1.33 Gm. $\times$ 0.68 = 0.91 Gm. of benzoic acid. Add the amount excreted in each of the four specimens together. The normal average amount excreted in four hours is 3 Gm. of benzoic acid with a variation owing to the size of the individual of from 85 to 110 per cent of this amount. An excretion of a less amount is indicative of impairment of hepatic function.

The Quick[1] prothrombin test (see p. 201) may be used as a liver function test. Some jaundiced patients have a prolonged prothrombin time in their plasma when tested by this method. If, after the administration of vitamin K in some form to such patients, the prothrombin time is not markedly shortened, it may be taken as evidence of serious hepatic damage.

Greene, Hotz and Leahy[2] have shown that patients with evident hepatic damage have *decreased combined cholesterol* in their blood (see p. 381). At times, there may be a decrease in the total cholesterol. In uncomplicated, obstructive jaundice the cholesterol esters rise along with the total cholesterol. In hepatic disease, the cholesterol esters tend to disappear from the blood regardless of the behavior of the total cholesterol. If there is a progressive decrease in the cholesterol esters, the prognosis is poor, while, if the esters are increasing in amount, the prognosis is more favorable.

The study of the size of the erythrocytes is of importance in liver disease. This has been pointed out by many authors. In damage to the parenchyma of the liver, there may be a *macrocytosis* of 8.8 microns or more for the average erythrocyte diameter.

[1] Quick, A. J.: The Clinical Application of the Hippuric Acid and the Prothrombin Tests. Amer. Jour. Clin. Path. *10:*222–233 (Mar.), 1940.

[2] Greene, C. H., Hotz, Richard, and Leahy, Evelyn: Clinical Value of Determination of Cholesterol Esters of Blood in Hepatic Disease. Arch. Int. Med. *65:*1130–1143 (June) 1940.

O. Enzymes in Duodenal Contents

Pancreatic Enzymes in Duodenal Contents.—The technic of the withdrawal of duodenal contents is described on page 464. Several methods for estimating the presence and amount of the various pancreatic enzymes have been devised. Simple and comparatively accurate methods are those of McClure, Wetmore, and Reynolds.[1] Their methods are for trypsin, amylase, and lipase. The first two methods will be described. It is rarely necessary to demonstrate lipase if the other enzymes are present.

Estimation of Trypsin.—*Reagents Required.*—(*a*) Phosphate mixture. Add 20 c.c. of a solution of potassium acid phosphate, which contains 27.234 Gm. to a liter, to 980 c.c. of a solution of disodium phosphate, which contains 35.628 Gm. to a liter. This buffer solution has a *p*H of 8.4.

(*b*) Casein solution. Place exactly 1 Gm. of soluble casein in a dry flask, add 100 c.c. of solution (*a*), and dissolve the casein by quickly rotating the flask. Heating the solution to 57° C. will facilitate solution, and care must be exercised to prevent the formation of a doughy, relatively insoluble mass. When the casein is dissolved, neutralize the casein by adding 4 c.c. of decinormal solution of sodium hydroxide.

(*c*) Metaphosphoric acid solution. This is used as a protein precipitant. Fuse metaphosphoric acid, as obtained on the market, in a graphite mortar until the white fumes of phosphorus pentoxide appear. Cool in a clean pie pan floating on cold water. Keep the fused metaphosphoric acid in a tightly stoppered bottle. Make a fresh 25 per cent solution by titrating the desired amounts of metaphosphoric acid and water in a mortar.

(*d*) The digestion mixtures. Folin and Wu reagent (*a*, p. 363) for determination of nonprotein nitrogen.

(*e*) Nessler's solution (*b*, p. 364).

(*f*) Standard ammonium sulfate solution (*c*, p. 364).

Method.—This consists of digesting the casein solution with diluted duodenal contents, precipitating the protein, and determining the nonprotein nitrogen content according to the method of Folin and Wu, which is described on page 364.

1. Centrifugalize the duodenal contents until clear. Decant into a clean tube. Place 1 c.c. of the clear fluid in a 50-c.c. volumetric flask and make up to the mark with phosphate solution (*a*).

2. Place 9 c.c. of casein solution (*b*) in a 100 x 10 mm. test tube. Heat in the water bath at 40° C. for five minutes.

3. Add 1 c.c. of diluted duodenal contents (1), mix, and incubate in a water bath for thirty minutes at 40° C.

4. Add 2 c.c. of freshly prepared solution of metaphosphoric acid (*c*). Mix thoroughly and filter. A colorless, clear filtrate should be obtained.

5. Place 1 c.c. of filtrate and 1 c.c. of digestion mixture (*d*) in a pyrex glass tube and carry out the digestion, as described on page 364.

[1] McClure, C. W., Wetmore, A. S., and Reynolds, Lawrence: New Methods for Estimating Enzymatic Activities of Duodenal Contents of Normal Man, Arch. Int. Med., *27*:706–715 (June), 1921.

6. After cooling, make up to 35-c.c. mark, add 15 c.c. of Nessler's solution, and centrifugalize to get rid of the sediment.

7. Place 2 c.c. of the digestion mixture in a 100-c.c. volumetric flask, add 3 c.c. of standard ammonium sulfate solution (*f*), about 60 c.c. of water, and 30 c.c. of Nessler's solution. Make up to the 100-c.c. mark.

8. Place standard in a colorimeter cup, set at 20, and read the unknown against the standard.

Calculation:

$$\frac{180}{\text{Reading of Unknown}} = \text{Gm. of nitrogen liberated per 100 c.c. of original duodenal contents.}$$

The normal range of nitrogen will amount to 7.5 to 15 Gm. per 100 c.c. of duodenal contents.

The Ågren and Lagerlöf[1] Method.—The determination of trypsin is an alternate method.

Reagents.—(*a*) Buffer solution. Equal parts of normal solution of ammonium chloride and normal solution of ammonium hydroxide.

(*b*) Casein. Dissolve 20 Gm. of casein in 300 c.c. of tenth normal solution of sodium hydroxide and dilute to 500 c.c. with distilled water.

(*c*) Indicator solution. A 2 per cent solution of thymolphthalein in 90 per cent alcohol.

(*d*) Potassium hydroxide solution. Dilute one part of normal solution of potassium hydroxide up to ten parts with absolute alcohol.

Method.—1. Place 2 c.c. of duodenal contents in an ordinary test tube. Add 2 c.c. of distilled water and 2 c.c. of buffer solution (*a*).

2. Add 0.6 c.c. of solution of casein (*b*) and simultaneously start a stop watch. Thoroughly mix the casein solution with the duodenal juice and immediately thereafter transfer 6 c.c. of the mixture into an Erlenmeyer flask containing 38 c.c. of 95 per cent alcohol and 3 c.c. of distilled water. This is the control. Heat the test mixture and the control for twenty minutes in a water bath at 30° C.

3. In exactly twenty minutes, discontinue the digestion by transferring the remainder of the mixture to another flask with alcohol of the same concentration and quantity as mentioned previously. Add 1 c.c. of indicator solution (*c*) to each flask.

4. Titrate with tenth normal alcoholic solution of potassium hydroxide to the first shade of blue in both flasks, at which point add 70 c.c. of 95 per cent boiling alcohol. The color then disappears. Continue the titration to the first blue color in both flasks.

Calculation.—The difference between the titration values gives the trypsin activity per cubic centimeter of duodenal juice expressed in cubic centimeters of the tenth normal solution of potassium hydroxide. The trypsin concentration is arbitrarily expressed as one trypsin unit, which is equal to 2 c.c. of tenth normal solution of potassium hydroxide. The normal

[1] Ågren, Gunnar and Lagerlöf, Henrik: The Pancreatic Secretion in Man after Intravenous Administration of Secretin. Acta Med. Scandinav. *90*:1–29, 1936.

range is from 0.6 to 3.2 units, with an average of about 1.6 units per c.c. Less than 0.6 units per c.c. may be considered as significance of the lack of trypsin secretory function of the pancreas.

Amylase Determination.—This method determines the amount of sugar that is formed from starch which has been digested for a definite period of time.

Reagents Required.—(*a*) Phosphate mixture which has been described for trypsin method.

(*b*) Starch-phosphate mixture. Completely dissolve 4 Gm. soluble starch in 100 c.c. of hot distilled water. Cool and dilute with an equal volume of phosphate mixture (*a*).

The other reagents which are required are those which are used for Folin and Wu's blood sugar method, and which are described on page 391.

Method.—1. Place 1 c.c. of centrifugalized duodenal contents in a 25-c.c. volumetric flask. Make up to the mark with phosphate mixture (*a*).

2. Place 9 c.c. of starch-phosphate mixture (*b*) in a test tube. Heat in a water bath at 40° C. for five minutes.

3. Add 1 c.c. of diluted duodenal contents, mix, and incubate for thirty minutes at 40° C.

4. Place 2 c.c. of the copper solution of Folin and Wu (*b*, p. 391) in a special blood-sugar tube.

5. Add 2 c.c. of the digested starch solution, rotate the contents, and place the tube in boiling water for six minutes.

6. Cool, and add 2 c.c. of molybdate solution (*c*, p. 391). Make up to the mark, mix, and compare the color with the Folin and Wu No. 1 glucose standard that has been prepared in the usual way.

Calculation:

$$\frac{50}{\text{Reading of Unknown}} = \text{Gm. of dextrose liberated per 100 c.c. of original duodenal contents.}$$

The normal amount of dextrose liberated by this method in thirty minutes is 2.5 to 5 Gm. per 100 c.c.

Ågren and Lagerlöf[1] Method for Amylase Determination.—*Reagents.*—(*a*) 1.5 per cent solution of sodium chloride.

(*b*) 1 per cent solution of starch. Stir 5 Gm. of Kahlbaum starch into a little cold distilled water. Add 200 c.c. of boiling distilled water to dissolve the starch. Add 250 c.c. of phosphate buffer of *p*H 6.8 (equal parts of Sørensen buffer solution, p. 836). Transfer to a 500 c.c. volumetric flask and make up to volume with distilled water.

(*c*) Tenth normal solution of sodium hydroxide.

(*d*) Alkaline ferricyanide solution. Dissolve 8.3 Gm. of potassium ferricyanide and 10.6 Gm. of sodium carbonate, water-free, in 500 c.c. of distilled water.

(*e*) 50 per cent acetic acid.

[1] Ågren, Gunnar, and Lagerlöf, Henrik: The Pancreatic Secretion in Man after Intravenous Administration of Secretin. Acta Med. Scandinav. *90*:1–29, 1936.

(*f*) Iodide solution. Dissolve 25 Gm. of potassium iodide, 250 Gm. of sodium chloride and 50 Gm. of zinc sulfate in one liter of distilled water.

(*g*) Twentieth normal solution of sodium thiosulfate.

Method.—1. Dilute 1 c.c. of duodenal contents with 100 c.c. of 1.5 per cent solution of sodium chloride (*a*). (Further dilutions of 1 : 400 or 1 : 800 or even more may be necessary.)

2. Place 2 c.c. of diluted contents in a test tube and heat for eight minutes in a water bath at 37° C.

3. Warm the starch solution (*b*) at 37° C. and, in exactly eight minutes, add 2 c.c. to the diluted duodenal contents, thus making the salt concentration 0.75 per cent, and continue the digestion for exactly fifteen minutes longer.

4. Transfer 2 c.c. of the enzyme starch solution to a test tube containing 1 c.c. of tenth normal solution of sodium hydroxide (*c*). This stops the digestion.

5. Set up a control test in a similar test tube containing 1 c.c. of tenth normal solution of sodium hydroxide (*c*), 1 c.c. of diluted duodenal juice and 1 c.c. of warm starch solution (*b*).

6. Add to both the control and the digested mixture, 2 c.c. of twentieth normal alkaline ferricyanide solution (*d*) and 12 c.c. of distilled water. Heat in a boiling water bath for twenty minutes.

7. Cool the tube in a water bath of room temperature and add 2 c.c. of 50 per cent acetic acid (*e*) and 10 c.c. of iodide reagent (*f*).

8. Three minutes later, titrate the liberated iodine with twentieth normal solution of sodium hydroxide (*g*) until the color disappears.

Calculation.—The difference in cubic centimeters between the titrations of the control and the unknown with the sodium thiosulfate solution = the number of cubic centimeters of ferricyanide solution reduced by the maltose that has been formed by the action of the enzyme on the starch. One gram of maltose is reduced by 0.465 c.c. of alkaline ferricyanide solution; therefore, difference in titration × 0.465 = milligram per cubic centimeter of duodenal contents. In ten minutes the duodenal contents will normally contain enough amylase to form from 0.34 to 1.72 Gm. of maltose per cubic centimeter according to the studies of Comfort and Osterberg.[1]

P. Serum Lipase and Serum Amylase

Cherry and Crandall[2] showed the specificity of pancreatic lipase, and demonstrated experimentally its appearance in the blood after pancreatic injury. The substrate used in the test is an emulsion of olive oil. The degree of enzyme action is indicated by the amount of fatty acid liberated, and is

[1] Comfort, M. W., and Osterberg, A. E.: Pancreatic Secretion in Man after Stimulation with Secretin and Acetylbeta Methylcholine Chloride; a Comparative Study. Arch. Int. Med. *66*:688–706 (Sept.), 1940.

[2] Cherry, I. S., and Crandall, L. A., Jr.: The Specificity of Pancreatic Lipase: Its Appearance in the Blood after Pancreatic Injury, Am. Jour. Physiol., *100*:266–273 (Apr.), 1932.

reported in cubic centimeters of twentieth-normal solution of sodium hydroxide. Comfort and Osterberg[1] reported on the use of this method slightly modified, as a clinical laboratory test. Comfort[2] found that the normal range is from 0.2 to 1.5 c.c. of twentieth-normal solution of sodium hydroxide for each cubic centimeter of serum. A sharp rise, even up to 9 or 10 c.c. of twentieth-normal solution of sodium hydroxide for each cubic centimeter may occur early in cases of acute pancreatitis. There may be also an elevation of serum lipase in cases of malignant lesions, especially if the lesions are associated with painless jaundice.

Determination of Serum Lipase.—*Reagents.*—(*a*) Emulsion of olive oil. This should be purchased from a commercial laboratory.* It is prepared in a homogenizer from equal parts of pure olive oil, free from fatty acid, and a 5 per cent solution of acacia, with 0.2 per cent of sodium benzoate added as a preservative.

(*b*) Phosphate buffer solution, *p*H 7. Prepare Sørensen phosphate buffer (p. 836) solution from a fifteenth-molar solution of primary potassium phosphate, and fifteenth-molar, secondary sodium phosphate solution, adjusted to *p*H 7.

(*c*) Twentieth-normal solution of sodium hydroxide.

(*d*) A 1 per cent solution of phenolphthalein in alcohol.

(*e*) Ethyl alcohol (95 per cent).

Method.—1. Place 1 c.c. of serum in a test tube. Add 2 c.c. of olive oil emulsion (*a*), 3 c.c. of distilled water and 0.5 c.c. of buffer solution (*b*). Shake the mixture and incubate at 38° C. for twenty-four hours.

2. Prepare a "blank." Place 1 c.c. of serum in a test tube. Add 3 c.c. of distilled water. Heat to 70° C. for about five minutes to destroy the enzyme. Add the buffer (*b*) and substrate (*a*). Shake the mixture and incubate at 38° C. for twenty-four hours.

3. After twenty-four hours, add 3 c.c. of alcohol (*e*) to each tube. Titrate to a permanent pink color of the indicator (*d*) with twentieth-normal solution of sodium hydroxide (*c*).

Calculation.—Subtract the number of cubic centimeters of sodium hydroxide used to neutralize the "blank" (tube 2), from the number of cubic centimeters used to neutralize the acidity in tube 1, and record the difference as cubic centimeters of twentieth-normal solution of sodium hydroxide for each cubic centimeter of serum.

Determination of Serum Amylase.—(Somogyi[3]) *Reagents.*—(*a*) Starch substrate (starch 75 mg. and sodium chloride 250 mg. per 100 c.c.).

(*b*) Iodine solution (0.002 normal). Dilute 10 c.c. of tenth-normal

[1] Comfort, M. W., and Osterberg, A. E.: Lipase and Esterase in Blood Serum: Their Diagnostic Value in Pancreatic Disease, Jour. Lab. and Clin. Med., *20:*271–278 (Dec.), 1934.

[2] Comfort, M. W.: Serum Lipase: Its Diagnostic Value, Am. Jour. Digest. Dis. and Nutrition, *3:*817–821 (Jan.), 1937.

* Abbott Laboratories, Chicago, Illinois, have prepared this emulsion.

[3] Somogyi, Michael: Micromethods for the Estimation of Diastase. Jour. Biol. Chem. *125:*399–414 (Sept.), 1938.

aqueous solution of iodine to 50 c.c. with 2 per cent solution of potassium iodide.

Method.—1. Place 4 c.c. of starch substrate (*a*) in a large test tube (14 to 16 mm. in diameter) and warm the solution in a water bath maintained at a constant temperature of 40° C. Add 1 c.c. of serum (or plasma) and simultaneously start a stop watch. Mix the plasma and starch solution and rinse out the pipet once with the mixture.

2. Measure 0.5 c.c. portions of iodine solution (*b*) into several test tubes about 2 mm. in diameter.

3. After incubation for five minutes, place 0.5 c.c. of the digestion mixture in one of the tubes containing iodine solution. If digestion has begun, the blue color produced by the reaction of iodine with starch will be changing to purple. If this has occurred, similar 0.5 c.c. samples must be tested at intervals of one or two minutes. *The exact time for determination of the end point is the instant that the purplish color disappears and the color has changed to the color of erythrodextrin.* Stop the watch at this time. If there is no color change in five minutes, it may be necessary to continue the incubation much longer, possibly thirty minutes. It may be necessary to dilute the starch substrate to contain only 1.5 mg. of starch instead of 3 mg., if the enzyme action is very slow. If the enzyme is very active, it may be necessary to dilute the serum.

Calculation.—The final estimation is in terms of copper reducing substance in milligrams for each 100 c.c. of serum (or plasma) produced by the enzyme action on the starch. The formula is D (diastase activity) = $K \times \frac{1}{(t \times V)}$. By experimentation it has been found that K = 1600; t = time of incubation; V = volume of plasma or serum used; and 1 = concentration of starch (3 mg.).

For example; if t = 12, then $D = \frac{1600}{12} = 133$ = milligrams per 100 c.c. of reducing substance produced by the enzyme.

Q. Determination of Cevitamic Acid in Blood[1]

The method used for the determination of cevitamic acid in the blood is essentially that of Taylor, Chase and Faulkner,[2] utilizing the suggestion of Pijoan and Klemperer[3] that potassium cyanide will preserve blood against the loss of cevitamic acid by oxidation. If cevitamic acid, however, is determined in the blood immediately after the drawing of samples of

[1] Magnusson, Arlene, and Osterberg, A. E.: Proc. Staff Meet. Mayo Clinic, *13:*700–702 (Nov. 2), 1938.

[2] Taylor, F. H. L., Chase, Dorrance, and Faulkner, J. M. The Estimation of Reduced Ascorbic Acid in Blood Serum and Plasma, Biochem. Jour., *30:*1119–1125 (July), 1936.

[3] Pijoan, M., and Klemperer, F.: Determination of Blood Ascorbic Acid, Jour. Clin. Investigation, *16:*443–445 (May), 1937.

blood, use of potassium cyanide can be eliminated. Magnusson and Osterberg did not find that potassium cyanide elevated the apparent concentration of cevitamic acid, as Farmer and Abt[1] have recently reported.

The reagents required are: (*a*) glacial acetic acid, (*b*) 10 per cent solution of metaphosphoric acid, (*c*) 5 per cent solution of potassium cyanide, (*d*) a starch indicator, (*e*) a solution of iodine; a hundredth normal solution of iodine made according to directions given by Treadwell and Hall[2] is used, (*f*) a standard solution of 1 mg. of cevitamic acid per milliliter; further directions will be given later, and (*g*) 2-6-dichlorphenol-indophenol (La Motte), a solution containing 0.2 mg. per milliliter.

The standard solution is prepared by dissolving 100 mg. of pure cevitamic acid in 100 ml. of boiled, distilled water. Since the reduced cevitamic acid is rapidly oxidized in solution, the strength of the solution should be checked daily by determining its reducing power with the hundredth normal solution of iodine as follows: to 1 ml. of the standard in a centrifuge tube, 1 drop of 0.5 per cent solution of starch is added and titrated to a permanent blue color in a microburet with a hundredth normal solution of iodine. The standard solution of cevitamic acid should be preserved in a brown glass-stoppered bottle and stored in the icebox.

Tillman's reagent is prepared as follows: 20 mg. of 2-6-dichlorophenol-indophenol indicator is dissolved in 75 ml. of boiling water, cooled, transferred to a 100 ml. volumetric flask, and diluted with distilled water to make 100 ml. This reagent should be replaced within two weeks in order to maintain a sharp end-point, and should be stored in a dark bottle in the icebox. This reagent is checked against the standard solution of cevitamic acid every other day in the following manner: to 0.2 ml. of the standard solution of cevitamic acid in a centrifuge tube is added a drop of glacial acetic acid and this is titrated with the dye to a definite pink color. The standard is measured in a micropipet and the dye is added from a 5 ml. microburet (Fig. 195).

The solution of metaphosphoric acid is made up fresh each week and is kept in the icebox.

Procedure.—1. Two drops of 5 per cent solution of potassium cyanide and 10 mg. of potassium oxalate are placed in a test tube. Blood is collected from the patient's vein when the patient is in a fasting state; about 6 to 7 ml. is placed in the tube and centrifuged.

2. Two ml. of plasma is transferred to a centrifuge tube. To this is added 2 ml. of distilled water and 6 ml. of the 10 per cent solution of metaphosphoric acid. The mixture is stirred rapidly with a glass rod for thirty seconds and allowed to stand for three minutes. It is then filtered through Whatman No. 40 filter paper.

3. Five ml. of the filtrate is placed in a 15 ml. centrifuge tube and

[1] Farmer, C. J., and Abt, A. F.: Invalidation of Plasma Ascorbic Acid Values by Use of Potassium Cyanide, Proc. Soc. Exper. Biol. and Med., *38*:399–404 (Apr.), 1938.

[2] Treadwell, F. P., and Hall, W. T.: Analytical Chemistry: Quantitative Analysis, Ed. 5, New York, John Wiley and Sons, Inc., 1919, Vol. 2, 940 pp.

titrated with Tillman's reagent. The titration is carried out rapidly to a standard end-point. For this end-point, employ the color produced by a definite amount of Tillman's reagent added to 5 ml. of boiled distilled water containing 1 drop of glacial acetic acid in a test tube similar to that used for the blood filtrate. The filtrate is titrated to a color matching the standard which will persist thirty seconds. The titration for the blank is deducted from the titration of the filtrate. A 5 ml. microburet is used for this titration.

Calculation:

$$\frac{0.88 \times (\text{ml. } I_2{}^* \doteqdot 1 \text{ ml. standard})}{5 \times (\text{ml. dye} \doteqdot 0.2 \text{ ml. standard})} \times 100 \times \text{ml. dye used for titration} = \text{milligrams of cevitamic acid per 100 ml. of plasma.}$$

One ml. of a hundredth normal solution of iodine represents 0.88 mg. of cevitamic acid. The values (ml. I_2 equivalent to 1 ml. standard and ml. dye equivalent to 0.2 ml. standard) are determined as described above.

Example:

$$\frac{0.88 \times 1.58}{5 \times 2.06} \times 100 \times 0.06 = 0.81 \text{ mg. of cevitamic acid per 100 ml. of plasma.}$$

Two tenths ml. of standard is equivalent to 2.06 ml. of dye. Dye solution contains 20 mg. per 100 ml. One ml. of the standard is equivalent to 1.58 ml. I_2; 1 ml. of plasma was used for the test and 0.06 ml. of dye was used for the titration.

We consider from 0.9 to 1.5 mg. per 100 ml. of plasma the normal value for cevitamic acid in blood plasma.

R. Determination of Cevitamic Acid in Urine

The method used for the determination of cevitamic acid in urine is the technic recommended by Harris and Ray.[1] This is a titration method, the principle of which is to take a measured amount of recently standardized dye (2-6-dichlorophenol-indophenol) and to determine the amount of urine which has to be added to discharge its color, the titration being carried out rapidly and in a solution made acid by the addition of 5 per cent by volume of glacial acetic acid.

The reagents required are: (*a*) glacial acetic acid, and (*b*) Tillman's reagent. Fifty mg. of 2-6-dichlorophenol-indophenol is dissolved in boiling water, cooled, transferred to a 50 ml. volumetric flask and diluted with distilled water to fill the flask. This solution is standardized against the standard solution of cevitamic acid described in the method for cevitamic acid in blood plasma as follows: to 1 ml. of standard in a centrifuge tube

* Solution of iodine. The sign $\doteqdot$ in chemistry means "equivalent to": quantities of one substance which will react with a given quantity of another substance so as to leave no excess of either.

[1] Harris, L. J., and Ray, S. N.: Diagnosis of Vitamin-C Subnutrition by Urine Analysis with a Note on the Antiscorbutic Value of Human Milk, Lancet, *1*:71–77 (Jan. 12), 1935.

add 1 drop of glacial acetic acid and titrate with the dye from a 5 ml. microburet to a definite pink color.

Procedure.—1. A twenty-four-hour specimen of urine is collected in a dark bottle containing enough glacial acetic acid to make a 5 per cent solution by volume.

2. The urine is placed in a microburet, if clear; if not, it is first filtered. The titration is carried out in a centrifuge tube. The dye is measured accurately with a 0.2 ml. micropipet. The amount of dye varies according to the concentration of cevitamic acid in the specimen under examination, that is, it should be such an amount as to need 1 to 2 ml. of urine for titration. For normal adults, 0.05 ml. of dye is found to be a suitable amount. If the concentration is low, less dye is used and if the concentration is unusually high so that less than 0.5 ml. of urine is required to discharge the color of the dye, the urine is diluted with a known amount of water. In carrying out the titration the end-point is best found by matching with a control tube containing urine but no dye.

Calculation:

$$\frac{0.88 \times (\text{ml. } I_2 \text{ eq. to 1 ml. standard}) \times \text{volume}}{(\text{ml. dye eq. to 1 ml. standard}) \times 20 \times (\text{ml. urine used to titrate 0.05 ml. dye})}$$

= milligrams of cevitamic acid in the specimen.

The titration value must be corrected for the amount of glacial acetic acid which it contains.

The values (ml. I_2 equivalent to 1 ml. standard and ml. dye equivalent to 1 ml. standard) are determined by titration as described above.

Example:

$$\frac{0.88 \times 1.58 \times 1050}{2.06 \times 20 \times 1.5} = 23.6 \text{ mg. of cevitamic acid in specimen of urine.}$$

One ml. of a hundredth normal solution of iodine represents 0.88 mg. of cevitamic acid; 1 ml. of the standard is equivalent to 1.58 ml. of the solution of iodine; 1 ml. of the standard is equivalent to 2.06 ml. of dye. The solution of dye contains 50 mg. per 50 ml.

The volume of urine (containing acetic acid) of the twenty-four-hour specimen = 1050 ml.

One and five-tenths ml. of urine (5 per cent of which is acetic acid) is used to discharge the color in 0.05 ml. of dye.

The normal daily output of cevitamic acid in the urine of adults is from 10 to 30 mg.

S. Sulfonamides

As a result of the widespread use of sulfanilamide and other sulfonamides in the treatment of streptococcic, gonococcic, pneumococcic and other infections, it has become apparent that the determination of the concentration of this drug in blood and in urine is a very important clinical laboratory procedure. Concentration between 1 to 10,000 and 1 to 5,000 can be maintained in the blood by administering the drug several times a day by mouth. When an equilibrium is established between intake and output,

nearly 100 per cent of the drug may be recovered from the urine either in a free or combined state. In cases of impaired renal function these drugs may be excreted slowly, and caution should be observed in such cases.

The methods for the determination of these drugs in blood and urine are those developed by Bratton and Marshall[1] and are given as follows:

Reagents.—(*a*) Dissolve 15 Gm. trichloro-acetic acid in distilled water and dilute to 100 c.c.

(*b*) One-tenth per cent solution of sodium nitrite.

(*c*) Dissolve 100 mg. N- (1-naphthyl) ethylenediamine hydrochloride in distilled water and make up to 100 c.c. Keep this solution in a dark-colored bottle.

(*d*) Saponin solution. Dissolve 0.5 Gm. of saponin in 1 liter of water.

(*e*) Fourth normal hydrochloric acid.

(*f*) Dissolve 0.5 Gm. ammonium sulfamate in 100 c.c. distilled water.

(*g*) Standard solutions. (1) Stock standard solution. Dissolve 20 mg. of sulfanilamide or other sulfonamide drug in 100 c.c. of water. This solution keeps several months in the refrigerator.

(2) Dilute standard. Dilute 2 c.c. of stock standard (1) with 18 c.c. of 15 per cent trichloro-acetic acid (*a*) and make up to 100 c.c. with distilled water in a volumetric flask. This makes a standard containing 0.4 mg. per 100 c.c.

For the sodium salts of other sulfonamides, the standards are made up as follows: Sodium sulfadiazine: Dissolve 21.76 mg. of sodium sulfadiazine in 100 c.c. of water in a volumetric flask. Sodium sulfathiazole: Dissolve 21.72 mg. sodium sulfathiazole in 100 c.c. of water in a volumetric flask. If the sesquihydrate has been used as a drug, the standard may be made by dissolving 23.4 mg. in 100 c.c. of water. Sodium sulfapyridine: Dissolve 23.21 mg. of sodium sulfapyridine in 100 c.c. of water.

Method for Blood.—(Preparation of filtrate.) Place 1 c.c. of whole oxalated blood in a small Erlenmeyer flask. Add 15 c.c. of saponin solution (*d*). Shake the mixture well while adding. Allow the mixture to stand for ten minutes and filter. This filtrate may be used for determining either the free sulfonamide or the combined sulfonamide.

Free Sulfonamide.—(Sulfanilamide, sulfapyridine, sulfathiazole, sulfadiazine.) 1. Pipet 10 c.c. of the filtrate into a small Erlenmeyer flask.

2. Pipet 10 c.c. of the standard solution (*g*, 2) into a similar flask, selecting the standard that is required for the drug that has been administered.

3. Add to each flask 1 c.c. of freshly prepared solution of sodium nitrite (*b*); mix well and allow to stand for four minutes.

4. Add 1 c.c. of ammonium sulfamate solution (*f*) to each flask; mix thoroughly and allow to stand for three minutes.

5. Add 1 c.c. of coupling reagent (*c*); mix well and allow to stand for two minutes.

6. Compare in the colorimeter with the standard set at 20 mm.

Total Sulfonamide.—It is advisable to start with 2 c.c. of oxalated blood,

[1] Bratton, A. C., and Marshall, E. K., Jr.: A New Coupling Component for Sulfanilamide Determination. Jour. Biol. Chem. *128*:537–550 (May), 1939.

30 c.c. of saponin and 8 c.c. of the trichloro-acetic acid and make the filtrate as described.

1. Place 10 c.c. of the filtrate in a hard glass test tube which is marked for a volume of 10 c.c.

2. Add 0.5 c.c. of fourth normal hydrochloric acid (*e*); mix well, and heat in a boiling water bath for one hour.

3. Cool and make the volume up to 10 c.c. Add the reagents to the test tube as indicated under the test for free sulfonamide, but do not transfer to an Erlenmeyer flask. The reading obtained with this solution gives the total sulfonamide. To obtain the conjugated sulfonamide, subtract the amount of free sulfonamide from the total.

Succinyl Sulfathiazole.—If this drug has been administered, the determination of the amount of the drug in the blood must be made as follows: Use 10 c.c. of the filtrate and add 0.5 c.c. of fourth normal hydrochloric acid (*e*). This mixture and the standard must be heated for one hour in a boiling hot water bath. After boiling, cool the mixture and make up to the 10 c.c. mark and proceed as with other sulfonamide determinations.

Sulfones.—(Promin.) The older Marshall[1] modified method for determining sulfanilamide is used for determining promin or other sulfones in blood and urine. In determinations for this drug, any possibility that sulfanilamide may be present must be excluded.

Reagents.—(*a*) Saponin solution. Dissolve 0.5 Gm. of saponin in 1 liter of water.

(*b*) Toluene-sulfonic acid. Dissolve 20 Gm. *p*-toluene sulfonic acid in distilled water and dilute to 100 c.c. If the solution is colored, mix with charcoal and filter.

(*c*) Sodium nitrite solution, 0.1 per cent. Dissolve 0.1 Gm. of sodium nitrite in 100 c.c. of distilled water. Make a fresh solution each day that the test is to be performed.

(*d*) 1 molar sodium dihydrogen phosphate solution.[2] Dissolve 0.5 Gm. of ammonium sulfamate and 13.8 Gm. of sodium dihydrogen phosphate in 100 c.c. of water.

(*e*) Dimethyl-*alpha* naphthylamine solution. Dissolve 1 c.c. of dimethyl-*alpha*-naphthylamine in 100 c.c. of 90 per cent ethyl alcohol. Keep in a dark-colored bottle.

(*f*) Standard solutions are prepared freshly each day. (1) First strength (100 mg. per 100 c.c.). Dilute 0.25 c.c. of 40 per cent promin solution (supplied in ampules) to 100 c.c. with distilled water (this standard is used for determining promin in urine).

(2) Second strength. Dilute 10 c.c. of first strength standard (1) to 100 c.c. with distilled water. To prepare standards for comparison with determinations of promin in blood samples, take 2 c.c. of normal blood, 13.6 c.c of saponin solution (*a*) and 0.4 c.c. of second strength standard (2). This standard is treated the same as is the unknown sample of blood, so that the 10 c.c. of filtrate that is used will contain 0.2 mg. of promin. (At times, it may be desirable to use other quantities of the second strength standard. If this is done, the amount of saponin solution is varied so that the total mixture will be 16 c.c. and the calculation will, of course, be changed according to the strength of promin in the standard.)

[1] Marshall, E. K., Jr. (with the technical assistance of Dorothea Babbitt): Determination of Sulfanilamide in Blood and Urine. Jour. Biol. Chem. *122*:263–273 (Dec.), 1937.
Todd, J. C., and Sanford, A. H.: Clinical Diagnosis by Laboratory Methods. Ed. 9, Philadelphia, W. B. Saunders Company, 1939, pp. 412–413.

[2] Marshall, E. K., Jr., and Litchfield, J. T., Jr.: The Determination of Sulfanilamide, Science, *88*:85–86 (July 22), 1938.

Method (for Blood).—1. Place 14 c.c. of saponin solution (*a*) in a small flask. Transfer exactly 2 c.c. of oxalated blood to this solution. Shake the flask and let the mixture stand for two or three minutes until the blood is completely laked. (If distilled water alone is used instead of saponin solution, laking will require at least one hour.)

2. Add 4 c.c. of 20 per cent *p*-toluene sulfonic acid (*b*). Shake well and filter.

3. Transfer exactly 10 c.c. of filtrate to a small flask. Add 1 c.c. of solution of sodium nitrite (*c*). Shake the flask and let the mixture stand for three minutes.

4. Add 1 c.c. of 1 molar solution of sodium dihydrogen phosphate (*d*). Let the mixture stand for two minutes.

5. Add 5 c.c. of 1 per cent alcoholic solution of dimethyl-*alpha*-naphthylamine (*e*).

6. After ten minutes compare in the colorimeter with the standard, which has been treated exactly as the unknown, following steps 3 to 6. Set the reading of the standard at 20 mm. and compare the unknown with the standard.

Calculation.—With a standard containing 0.2 mg., the formula becomes

$$\frac{40}{\text{Reading of the Unknown}} = \text{mg. of promin per 100 c.c. of blood.}$$

Method (for Urine).—Determine the promin in undiluted urine and also in urine diluted 1 : 5 and 1 : 10.

1. Treat 2 c.c. of each sample of diluted and undiluted urine in the same manner as in the method for blood.

2. Prepare a standard directly from the first strength standard for undiluted urine and from second strength standard for diluted urine. Express the final results in milligrams of promin per 100 c.c. of undiluted urine.

Calculations.—With undiluted urine and the first strength standard, the formula is $\frac{40}{\text{Reading of the Unknown}} = \text{mg. of promin per 100 c.c. of urine.}$

If the urine is diluted 1 : 10 and a dilute standard containing 0.4 mg. is used, the formula is $\frac{800}{\text{Reading of the Unknown}} = \text{mg. of promin per 100 c.c. of urine.}$

CHAPTER V

GASTRIC AND DUODENAL CONTENTS

I. EXAMINATION OF THE GASTRIC CONTENTS

STOMACH digestion consists mainly in the action of pepsin upon proteins in the presence of hydrochloric acid, and in the curdling of milk by rennin. The fat-splitting ferment, lipase, of the gastric juice has very little activity excepting upon previously emulsified fats such as those of milk and egg yolk.

Pepsin and rennin are secreted by the gastric glands as zymogens —pepsinogen and renninogen respectively—which are converted into pepsin and rennin by hydrochloric acid. Hydrochloric acid is secreted chiefly by the fundus end of the stomach. It at once combines loosely with the proteins of the food, forming acid metaprotein, the first step in protein digestion. Hydrochloric acid, which is thus loosely combined with proteins, is called "combined" hydrochloric acid. The acid, which is secreted after the proteins present have all been converted into acid metaprotein, remains as "free" hydrochloric acid and, together with pepsin, continues the process of digestion.

At the height of digestion the stomach contents consist essentially of: (1) Water; (2) free hydrochloric acid; (3) combined hydrochloric acid; (4) pepsin; (5) rennin; (6) mineral salts, chiefly acid phosphates of no clinical importance; (7) particles of undigested and partly digested food; (8) various products of digestion in solution. In pathologic conditions there may be present, in addition, various microscopic structures and certain organic acids, of which lactic acid is most important.

The results of gastric analysis are influenced by many intra- and extragastric factors, and can be interpreted only in the light of the clinical findings. Excepting in the very rare instances of recovery of good-sized bits of diseased tissue there are no pathognomonic signs.

A **routine examination** is conveniently carried out in the following order. The first two steps must, of course, be modified when the "fractional method" is adopted.

1. Give the patient a test meal upon an empty stomach, washing the stomach previously if necessary.
2. At the height of digestion, usually in one hour, remove the contents of the stomach with a stomach tube.

3. Measure and examine macroscopically.
4. Filter. A suction filter is desirable, and may be necessary when much mucus is present. On the other hand, some clinical pathologists prefer to titrate the gastric contents without filtering.
5. During filtration, examine microscopically and make qualitative tests for—(*a*) free hydrochloric acid; (*b*) lactic acid.
6. When sufficient filtrate is obtained, make quantitative estimations of—(*a*) total acidity; (*b*) free hydrochloric acid; (*c*) combined hydrochloric acid (if necessary).
7. Make whatever additional tests seem desirable, as for blood, pepsin, or rennin.

A. OBTAINING THE CONTENTS

Gastric juice is secreted continuously, but quantities sufficiently large for examination are often not obtainable from the fasting stomach. In clinical work, therefore, it is desirable to stimulate secretion with food—which is the natural and most efficient stimulus—before attempting to collect the gastric fluid. Different foods stimulate secretion to different degrees, hence for the sake of uniform results certain standard "test meals" have been adopted.

1. Test Meals.—It is customary to give the test meal in the morning, since the stomach is most apt to be empty at that time. If it be suspected that the stomach will not be empty, it should be washed out with water the evening before.

(1) **Ewald's test breakfast** consists of a roll (or two slices of bread), without butter, and two small cups (300 to 400 c.c.) of water, or weak tea without cream or sugar. It should be well masticated. The contents of the stomach are to be removed one hour afterward, counting from the beginning, not the end of the meal. This test meal has long been used for routine examinations. Its disadvantage is that it introduces, with the bread, a variable amount of lactic acid and numerous yeast cells. This source of error may be eliminated by substituting a shredded whole-wheat biscuit for the roll. The **shredded wheat test meal** is now widely used, and is probably the most satisfactory for general purposes. A simple, satisfactory meal of the Ewald type consists of feeding eight **arrowroot cookies** and a glass of water.

(2) **Riegel's test meal** consists of 400 c.c. of bouillon, a broiled beefsteak (about 150–200 Gm.), and 150 Gm. of mashed potato. Since it tends to clog the tube, it must be thoroughly masticated.

Although the above meals would seem to yield satisfactory results from a physiologic standpoint, there has been a great deal of discussion by gastro-enterologists as to the actual value of the findings

with ordinary test meals. The result has been to study gastric secretion without a meal using either a small amount of alcohol as a direct stimulant, or a small dose of histamine injected hypodermically. The effect is striking, but it must be remembered that the secretion that follows is, after all, a result of a type of stimulation which is not normal as compared with the normal stimulation following the ingestion of foodstuffs. The technic for the alcohol meal and for the histamine injection is given below.

(3) An **alcohol meal** consists of 50 c.c. of 7 per cent grain alcohol injected through a small tube. Aspirate the fasting stomach at five-minute intervals for fifteen minutes before feeding the alcohol, and afterward at ten-minute intervals for one hour, saving a 10-c.c. portion for testing, and returning the remainder to the stomach. It is also possible to siphon out the stomach contents by holding the mouth of the tube lower than the level of the stomach. Bloomfield and Keefer[1] and Cheney[2] have outlined procedures combining phenolphthalein with the alcohol. The concentration of the dye can be read with a colorimeter as in the test of renal function.[3] Plot a curve showing the volume and titratable acidity for each period.

(4) **Histamine hydrochloride** or preferably **histamine phosphate** is administered hypodermically with the patient having fasted for at least twelve hours. The dose originally advised, namely, 0.1 mg. for each 10 Kg. of body weight, may cause a disagreeable reaction. Gompertz and Cohen[4] have shown that *a total dose of 0.25 mg. may may be used satisfactorily*. The dose given is for histamine itself; the actual dose of histamine phosphate which is administered is 0.19 mg. per 10 Kg. (22 pounds); this is the equivalent of 0.1 mg. of histamine. The reaction comes on rapidly. Flushing of the face, quickening of

[1] Bloomfield, A. L., and Keefer, C. S.: Clinical Studies of Gastric Function, Jour. Am. Med. Assn., *88*:707–711 (Mar. 5), 1927.

[2] Cheney, G.: A Simplified Method of Gastric Analysis, Am. Jour. Med. Sci., *177*:110–115 (Jan.), 1929.

[3] Bloomfield uses the concentration of dye for determining the maximal and minimal possible amounts of juice secreted in a ten-minute period, and employs the following formulae: $\left(\frac{x}{y} \times A\right) - A$ = maximal possible amount of juice secreted in a ten-minute period, and $B - \left(\frac{y}{x} \times B\right)$ = minimal possible amount. A = number of cubic centimeters of fluid in the stomach at the beginning of the period; B = number of cubic centimeters of fluid at the end of the period; x = the concentration (percentage) of dye at the beginning of the period, and y = the concentration at the end.

[4] Gompertz, L. M., and Cohen, W.: The Effect of Smaller Doses of Histamine in Stimulating Human Gastric Secretion, Am. Jour. Med. Sci., *177*:59–64 (Jan.), 1929.

the pulse, and, at times, physical discomfort results. Caution must be exercised in the choice of patients, and the test is not to be used as a routine. The aspirated secretions are measured and titrated in the usual manner, and the results are best shown graphically by plotting curves.

The test meals that have been described are more or less standard, each type having some claim for recognition. The Ewald meal has been used for fifty years, is simple, and is physiologic. Alcohol is easily administered. Histamine is a quick, powerful stimulant, often proving gastric acidity to be normal when test meals have failed to do so. However, many other test meals have been used, and a few of these may be mentioned. **Water** has been used alone as a gastric stimulant, being administered through a small tube. **Caffeine,** 0.2 Gm. in 200 c.c. of water, may also be used. Lövi[1] compared the results which were obtained after stimulation with caffeine with those which were obtained after the administration of 50 Gm. of dextrose in 200 c.c. of water. The initial degree of acidity, which is produced, is not so great with sugar as it is with caffeine. If histamine is injected subsequently, there is a marked increase in acidity if a sugar meal has been used, while this reaction is not so evident if caffeine has been introduced into the stomach before giving an injection of histamine. Another meal, of the physiologic type, was proposed by Heckman.[2] He used 80 c.c. of freshly prepared **egg albumin** with 130 c.c. of distilled water, to which was added 4 Gm. of Witte's peptone. The mixture was stained with 2 drops of a 2 per cent solution of methylene blue, heated to body temperature, and filtered through gauze. This fluid meal may be administered through the gastric tube and 10 c.c. portions of the gastric contents may be removed at regular intervals for titration by the fractional method.

2. Withdrawal of the Contents.—The gastric fluid is obtained by aspiration through a tube. Stomach tubes are of two general types. The older type, which was the only one in general use until recently, and is still required in some cases, is a moderately stiff rubber tube about 12 mm. in diameter. It should have an opening at the tip, and one or two in the side near the tip. Near the other end is generally inserted a large bulb for use as an aspirator when necessary. The newer type of stomach tube, of which the Rehfuss model is most widely used, is a modification of Einhorn's duodenal tube (Fig. 202). It is a very flexible rubber tube about 3 to 4 mm. in diameter with a perforated, olive-shaped, metal tip. A large glass aspirating syringe accompanies the tube.

[1] Lövi, László: Zucker-Pröbefrühstücke, Wien. klin. Wchnschr., *45*:460–463 (Apr. 8), 1932.

[2] Heckman: Zur Frage der Belastungproben des Magens. I. Magensaftuntersuchungen mittels einer Eiweiss-Peptonlösung, Ztsch. f. d. ges. exp. Med., *87*:406–528, 1933.

The use of the former, the Boas tube with bulb, will be first described. It should be sterilized by boiling before and after using.

It is important confidently to assure the patient that introduction of the tube cannot possibly harm him; and that, if he can control the spasm of his throat, he will experience very little choking sensation. When patients are very nervous it is well to spray the throat with cocaine solution.

The tube should be dipped in warm water just before using, or chilled in ice water in order to reduce nausea; the use of glycerin or other lubricant is undesirable. With the patient seated upon a chair his clothing protected by towels or a large apron, and his head tilted forward, the tip of the tube, held as one would a pen, is introduced far back into the pharynx. He is then urged to swallow, and the tube is pushed boldly into the esophagus until the colored ring upon it reaches the incisor teeth, thus indicating that the tip is in the stomach. If, now, the patient cough, or strain as if at stool, the contents of the stomach will usually be forced out through the tube. Should it fail, the fluid can generally be pumped out by alternate compression of the tube and the bulb. If unsuccessful at first, the attempt should be repeated with the tube pushed a little further in, or withdrawn a few inches, since the distance to the stomach is not the same in all cases. The tube may become clogged with pieces of food, in which case it must be withdrawn, cleaned, and reintroduced. If, after all efforts, no fluid is obtained, another test meal should be given and withdrawn after a somewhat shorter period, since, owing to excessive motility, the stomach may empty itself in less than the usual time. Care must be exercised to prevent saliva or vomitus running down the outside of the tube, and mingling with the gastric juice in the basin. As the tube is removed it should be pinched between the fingers so as to save any fluid that may be in it.

A more satisfactory method of withdrawing the fluid with this tube is to remove the bulb section and attach a Politzer bag with a short section of glass tubing intervening. The fluid may then be aspirated into the bag without danger of admixture of saliva or vomitus.

The stomach tube must be used with great care, or not at all, in cases of gastric ulcer, aneurysm, uncompensated heart disease, and marked arteriosclerosis. Except in gastric ulcer, the danger lies in the retching produced, and the tube can safely be used if the patient takes it easily.

The above procedure is made much easier both for physician and patient by use of one of the newer types of stomach tube, of which the

Rehfuss tube is best known. The metal tip is placed well back in the patient's pharynx, and he is directed to swallow several times in rapid succession with his lips closed and with his tongue forming a groove for the tube, upon which he sucks between swallows. Deep breathing will aid in overcoming nausea. After it has reached the stomach, indicated by the colored ring reaching the incisor teeth, the heavy tip sinks to the most dependent portion, and as much or as little of the stomach contents as is desired may be drawn off by means of an aspirating syringe. A satisfactory tube, known as the "Sawyer tube,"[1] is on the market. The walls of the tube are of such thickness and the size of the tube is such that no wire stylet is necessary as in the Jutte tube, nor is it necessary to use a metal tip as in the Rehfuss tube.

With the practical appreciation that there is great variation in the time at which the height of digestion is reached the **"fractional method"** came into wide use. This is carried out as follows:

1. Insert a Rehfuss stomach tube before breakfast and empty the stomach as far as possible.
2. Remove the tube and give an Ewald test breakfast, which must be chewed thoroughly. If an alcohol meal is used it is administered through the tube.
3. Reinsert the tube and withdraw 5 c.c. of the stomach contents at fifteen-minute intervals, until the fluid is free from food particles or until the acidity has returned to the same level as was found in the fasting content. The tube is left in place during the whole procedure. Ordinarily it causes very little nausea.
4. Examine each of the 5-c.c. portions, and also the fluid from the fasting stomach for total acidity, free hydrochloric acid, and lactic acid.

By means of the Rehfuss tube a much larger quantity of gastric juice can often be obtained from the fasting stomach than was formerly believed possible. The quantity is very variable, ranging from 5 to 150 c.c. or even more, and averaging about 45 c.c. The acidity values are also variable. Averages for the fasting content and each of the fifteen-minute periods are shown in Fig. 198. In some normal individuals the height of the acidity curve may be reached earlier; in others ("slowly elaborating stomachs") much later.

Recent work has thrown much doubt upon the value of the fractional method as originally carried out. It has been shown that samples of fluid secured from different parts of the stomach at the same time may differ markedly in acidity, particularly in individuals with gastric disease. Samples obtained in rapid succession through the same tube often differ greatly. It

[1] This tube developed at The Mayo Clinic after many modifications of various types of tubes by Miss Sawyer, technician in the Gastric Laboratory, Section of Clinical Laboratories. may be purchased from V. Mueller & Co., Chicago.

has been suggested that just before each of the fifteen-minute samples is removed the contents be mixed by sucking a portion into the syringe, and forcing it back into the stomach. Probably the best method of fractional

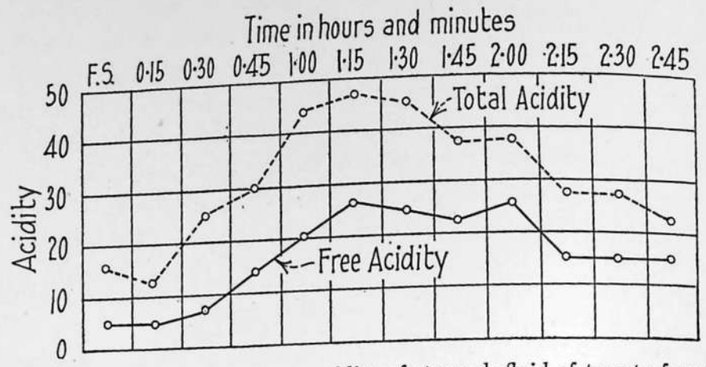


Fig. 198.—Diagram showing the average acidity of stomach fluid of twenty-four healthy persons studied by Talbot by the fractional method; F. S., Fasting stomach.

analysis is to give a series of test meals upon successive days, and to remove the entire contents each day at a different period of digestion. In any case only very marked changes in acidity are important.

B. Physical Examination

Under normal conditions 50 to 100 c.c. of fluid can be obtained one hour after administering Ewald's breakfast. Larger amounts point to motor insufficiency or hypersecretion; less than 20 c.c., to too rapid emptying of the stomach, or else to incomplete removal. Upon standing, it separates into two layers: the lower consisting of particles of food; the upper, of an almost clear, faintly yellow fluid. The extent to which digestion has taken place can be roughly judged from the appearance of the food particles.

The **reaction** is frankly acid in health and in nearly all pathologic conditions. It may be neutral or slightly alkaline in some cases of gastric cancer and marked chronic gastritis, or when contaminated by a considerable amount of saliva.

A small amount of **mucus** is present normally. Large amounts, when the gastric contents are obtained with the tube and not vomited, point to chronic gastritis. Mucus is recognized from its characteristic slimy appearance when the fluid is poured from one vessel into another. It is more frequently seen in stomach washings than in the fluid removed after a test meal.

A trace of **bile** is common as a result of excessive straining while the tube is in the stomach. Large amounts are very rarely found,

and generally point to obstruction in the duodenum. Bile produces a yellowish or more frequently greenish discoloration of the fluid.

Blood is often recognized by simple inspection, but more frequently requires a chemical test for confirmation. It is bright red when very fresh, and dark, resembling coffee grounds, when older. Vomiting of blood, or *hematemesis*, may be mistaken for pulmonary hemorrhage, or *hemoptysis*. In the former the fluid is acid in reaction, and usually dark red or brown in color, and clotted, while in hemoptysis it is brighter red, frothy, alkaline, and usually mixed with a variable amount of mucus. When the blood is small in amount and bright red, the possibility that it originates from injury done by the tube must not be overlooked.

Particles of food eaten hours or even days previously may be found, and indicate deficient motor power.

Search should always be made for **bits of tissue** from the gastric mucous membrane or new growths. These, when examined by a pathologist, will sometimes render the diagnosis clear.

C. Chemical Examination

A routine chemical examination of the gastric contents involves qualitative tests for free hydrochloric acid and organic acids, and quantitative estimations of total acidity, free hydrochloric acid, and sometimes combined hydrochloric acid. Other tests are applied when indicated. In the routine examination qualitative tests are done before quantitative. For this reason all of the qualitative tests are discussed before the quantitative procedures.

1. Qualitative Tests.—(1) **Free Acids.**—The presence or absence of free acids, without reference to the kind, is easily determined by means of Congo red, although the test is not much used in practice.

Congo-red Test.—Take a few drops of a strong alcoholic solution of Congo red in a test tube, dilute with water to a strong red color, and add a few cubic centimeters of filtered gastric juce. The appearance of a *blue color* shows the presence of a free acid (Plate X, B, B′). Since the test is more sensitive to mineral than to organic acids, a marked reaction points to the presence of free hydrochloric acid.

Thick filter paper soaked in Congo-red solution, dried, and cut into strips may be used, but the test is much less delicate when thus applied.

(2) **Free Hydrochloric Acid.**—In addition to its digestive function free hydrochloric acid is an efficient antiseptic. It prevents or retards fermentation and lactic-acid formation, and is an important means of protection against the entrance of pathogenic organisms into the

body. It is never absent in health. The significance of variations in disease is discussed on page 456.

Dimethyl-amino-azobenzol Test.—To a little of the filtered gastric juice in a test tube, or to several drops in a porcelain dish, add a drop of 0.5 per cent alcoholic solution of dimethyl-amino-azobenzol. In the presence of free hydrochloric acid there will at once appear a *cherry-red color*, varying in intensity with the amount of acid (Plate XI, C). This test is very delicate; but, unfortunately, organic acids, when present in large amounts (above 0.5 per cent), give a similar reaction. The color obtained with organic acids is, however, more of an orange red.

Boas' Test.—This test is less delicate than the preceding, but is more reliable, since it reacts only to free hydrochloric acid. It is probably the best routine test.

In a porcelain dish mix a few drops of the gastric juice and the reagent, and slowly evaporate to dryness over a flame, *taking care not to scorch.* The appearance of a *rose-red color*, which fades upon cooling, shows the presence of free hydrochloric acid (Plate X, 3).

Boas' reagent consists of 5 Gm. resublimed resorcinol, and 3 Gm. cane sugar, in 100 c.c. alcohol. The solution keeps well, which, from the practitioner's viewpoint, makes it preferable to Günzburg's phloroglucin-vanillin reagent (phloroglucin, 2 Gm.; vanillin, 1 Gm.; absolute alcohol, 30 c.c.). The latter is just as delicate, is applied in the same way, and gives a sharper reaction (Plate X, 4), but is unstable.

(3) **Organic Acids.**—Lactic acid is the most common, and is taken as the type of the organic acids which appear in the stomach contents. It is a product of bacterial activity. Acetic and butyric acids are sometimes present. Their formation is closely connected with that of lactic acid, and they are rarely tested for. When abundant, they may be recognized by their odor upon heating. Butyric acid gives the odor of rancid butter.

Lactic acid is never present at the height of digestion in health. Although often present early in digestion, it disappears when free hydrochloric acid begins to appear. Small amounts may be introduced with the food. Pathologically, small amounts may be present whenever there is stagnation of the gastric contents with deficient hydrochloric acid, as in many cases of dilatation of the stomach and chronic gastritis. The presence of notable amounts of lactic acid (more than 0.1 per cent by Strauss' test) is strongly suggestive of gastric cancer, and is probably the most valuable laboratory sign of the disease.

As already stated, the Ewald test breakfast introduces a small amount of lactic acid, but rarely enough to respond to the tests

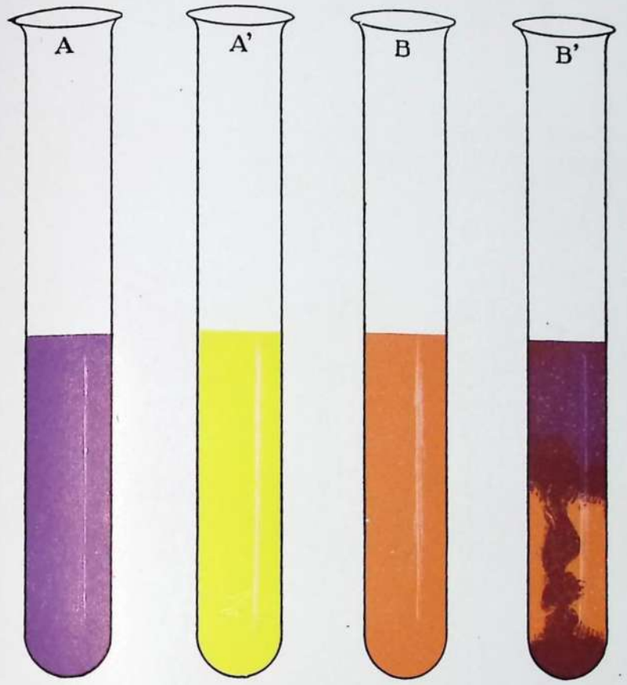


Fig. 1.—A, Uffelmann's reagent; A', A after the addition of gastric fluid containing lactic acid (Boston.)

Fig. 2.—B, water to which 3 drops of Congo red solution have been added; B', change induced in B when gastric fluid containing free hydrochloric acid is added (Boston).

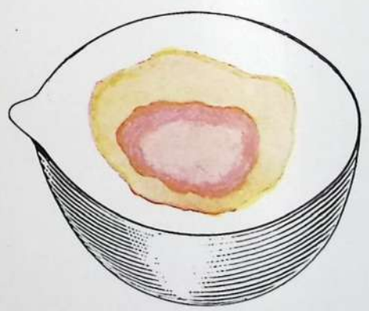


Fig. 3.—Resorcin-test for free hydrochloric acid (Boston).

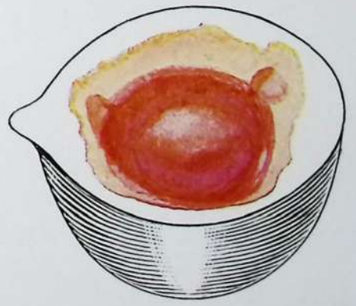


Fig. 4.—Günzburg's test for hydrochloric acid (Boston).

given here. In every case, however, in which its detection is important the shredded-wheat biscuit or Boas' test breakfast, consisting of a pint of oatmeal gruel, should be given, the stomach having been thoroughly washed the evening before.

Uffelmann's Test for Lactic Acid.—Thoroughly shake up 5 c.c. of filtered stomach fluid with 50 c.c. of ether for at least ten minutes. Collect the ether and evaporate. Dissolve the residue in 5 c.c. of water and test with Uffelmann's reagent as follows:

In a test tube mix 3 drops concentrated solution of phenol and 3 drops saturated aqueous solution of ferric chloride. Add water until the mixture assumes an amethyst-blue color. To this add the solution to be tested. The appearance of a *canary-yellow* color indicates the presence of lactic acid (Plate X, A, A^1).

Uffelmann's test may be applied directly to the stomach contents without extracting with ether, but is then neither sensitive nor reliable because of the phosphates, sugars, and other interfering substances which may be present.

Kelling's Test (*Simon's Modification*).—This is much more satisfactory than Uffelmann's. To a test tube of distilled water add sufficient ferric chloride solution to give a faint yellowish tinge. Pour half of this into a second test tube to serve as a control. To the other add a small amount of the gastric juice. Lactic acid gives a distinct yellow color which is readily recognized by comparison with the control. The color is best seen when the tubes are viewed from above over a sheet of paper.

Strauss' Test for Lactic Acid.—This is a good test for clinical work, since it gives a rough idea of the quantity present and is not sufficiently sensitive to respond to the traces of lactic acid which some test meals introduce. Strauss' instrument (Fig. 199) is essentially a separatory funnel with a mark at 5 c.c. and one at 25 c.c. Fill to the 5-c.c. mark with filtered stomach fluid and to the 25-c.c. mark with ether. Shake thoroughly for ten or fifteen minutes, let stand until the ether separates, and then, by opening the stopcock, allow the gastric juice to run out. Fill to the 25-c.c. mark with water and add 2 drops of a 10 per cent solution of ferric chloride. Shake gently. If 0.1 per cent or more lactic acid be present the water will assume a strong greenish-yellow color. A slight tinge will appear with 0.05 per cent.

(4) **Pepsin and Pepsinogen.**—Pepsinogen itself has no digestive power. It is secreted by the gastric glands, and is transformed into pepsin by the action of a free acid. Although pepsin digests proteins best in the presence of free hydrochloric acid, it has a slight digestive activity in the presence of organic or combined hydrochloric acids.

The amount is not influenced by neuroses or circulatory disturbances. Absence or marked diminution, therefore, indicates organic disease of the stomach. This is an important point in diagnosis

between functional and organic conditions. Pepsin is rarely or never absent in the presence of free hydrochloric acid.

Test for Pepsin and Pepsinogen.—With a cork borer cut small cylinders from the coagulated white of an egg, and cut these into disks of uniform size. The egg should be cooked very slowly, preferably over a water bath, so that the white may be readily digestible. The disks may be preserved in glycerin, but must be washed in water before using.

Place a disk in each of three test tubes.

Into Tube No. 1 put 10 c.c. distilled water, 5 grains pepsin, U. S. P., and 3 drops of the official dilute hydrochloric acid.

Into Tube No. 2 put 10 c.c. filtered gastric juice.

Into Tube No. 3 put 10 c.c. filtered gastric juice and 3 drops dilute hydrochloric acid.

Place the tubes in an incubator or in warm water for three hours or longer. At intervals observe the extent to which the egg albumin has been digested. This is recognized by the depth to which the disk has become translucent.

Tube No. 1 is used for comparison, and should show the effect of normal gastric juice.

Digestion of the egg in Tube No. 2 indicates the presence of both pepsin and free hydrochloric acid.

When digestion fails in Tube No. 2 and occurs in No. 3, pepsinogen is present, having been transformed into pepsin by the hydrochloric acid added. Should digestion fail in this tube, both pepsin and pepsinogen are absent.

Fig. 199.—Separatory funnel for Strauss' lactic-acid test (Sahli).

(5) **Rennin** is the milk-curdling ferment of the gastric juice. It is derived from renninogen through the action of hydrochloric acid. Deficiency of rennin has the same significance as deficiency of pepsin, and is more easily recognized. Indeed, the existence of rennin as a separate enzyme is in doubt, since it has never been isolated. The curdling of milk may, in reality, be due to pepsin.

Test for Rennin.—Neutralize 5 c.c. filtered gastric juice with very dilute sodium hydroxide solution, add 5 c.c. fresh milk, and place in an incubator or in a vessel of water at about 40° C. Coagulation of the milk in ten to fifteen minutes shows a normal amount of rennin. Delayed coagulation denotes a less amount.

(6) **Blood** is present in the vomitus in a great variety of conditions. When found in the fluid removed after a test meal it commonly points toward ulcer or carcinoma. Blood can be detected in nearly one half of the cases of gastric cancer. The presence of swallowed blood and blood from injury done by the stomach tube must be excluded.

Test for Blood in Stomach Contents.—Extract with ether to remove fat if this be present, which is usually not the case after a test meal. If the fluid be strongly acid, as frequently happens in artificial fluids carelessly prepared for class use, the blood pigment may go into solution in this ether and be unwittingly discarded.

To 10 c.c. of the fat-free fluid add 3 or 4 c.c. of glacial acetic acid and shake the mixture thoroughly with about 5 c.c. of ether. Let stand a short time, remove the ether, which forms a layer above the stomach fluid, and use half of it for the guaiac or benzidine test (p. 115). Separation of the ether may be facilitated by adding a small amount of alcohol. In the case of a positive reaction the remainder of the ether extract may be examined spectroscopically, after treating so as to develop the bands of hemochromogen (pp. 356, 358).

When brown particles are present in the fluid the hemin test may be applied directly to them.

2. Quantitative Tests.—(1) **Total Acidity.**—The acid-reacting substances which contribute to the total acidity are free hydrochloric acid, combined hydrochloric acid, acid salts, mostly phosphates, and, in some pathologic conditions, the organic acids. The total acidity is normally about *50* to *100 degrees* (see method below), or, when estimated as hydrochloric acid, about *0.2* to *0.3 per cent.* With Riegel's test meal the figures are a little higher.

Töpfer's Method for Total Acidity.—In an evaporating dish or small beaker take 10 c.c. filtered stomach contents and add 3 or 4 drops of the indicator, a 1 per cent alcoholic solution of phenolphthalein. When the quantity of stomach fluid is small, 5 c.c. may be used, but results are less accurate than with a larger amount. Add decinormal solution of sodium hydroxide drop by drop from a buret, until the fluid assumes a rose-red color which does not become deeper upon addition of another drop (Plate XI, A, A[1]). Most workers accept the first appearance of a permanent pink as the end-point, just as in other titrations with phenolphthalein as indicator; but, owing to interaction of phosphates, Wood advises that the titration of gastric juice be carried a little farther, as here indicated. When this point is reached all the acid has been neutralized. The end reaction will be sharper if the fluid be saturated with sodium chloride. A sheet of white paper beneath the beaker facilitates recognition of the color change.

In clinical work the amount of acidity is expressed by the number of cubic centimeters of the decinormal sodium hydroxide solution which would

be required to neutralize 100 c.c. of the gastric juice, each cubic centimeter representing one *degree* of acidity. Hence, multiply the number of cubic centimeters of decinormal solution required to neutralize the 10 c.c. of stomach fluid by 10. This gives the number of degrees of acidity. The amount may be expressed in terms of hydrochloric acid, if one remembers that each degree is equivalent to 0.00365 Gm. of hydrochloric acid. Some one suggests that this is the number of days in the year, the last figure, 5, indicating the number of decimal places.

Example.—Suppose that 7 c.c. of decinormal solution were required to bring about the end reaction in 10 c.c. gastric juice; then $7 \times 10 = 70$ *degrees* of acidity; and, expressed in terms of hydrochloric acid, $70 \times 0.00365 = 0.255$ *per cent.*

Preparation of decinormal solutions is described on page 833. The practitioner will find it best to have them made by a chemist, or to purchase from a chemical supply house.

(2) **Hydrochloric Acid.**—After the Ewald test breakfast the amount of **free hydrochloric acid** varies normally between 25 and 50 degrees, or about 0.1 to 0.2 per cent. In disease it may go considerably higher or may be absent altogether.

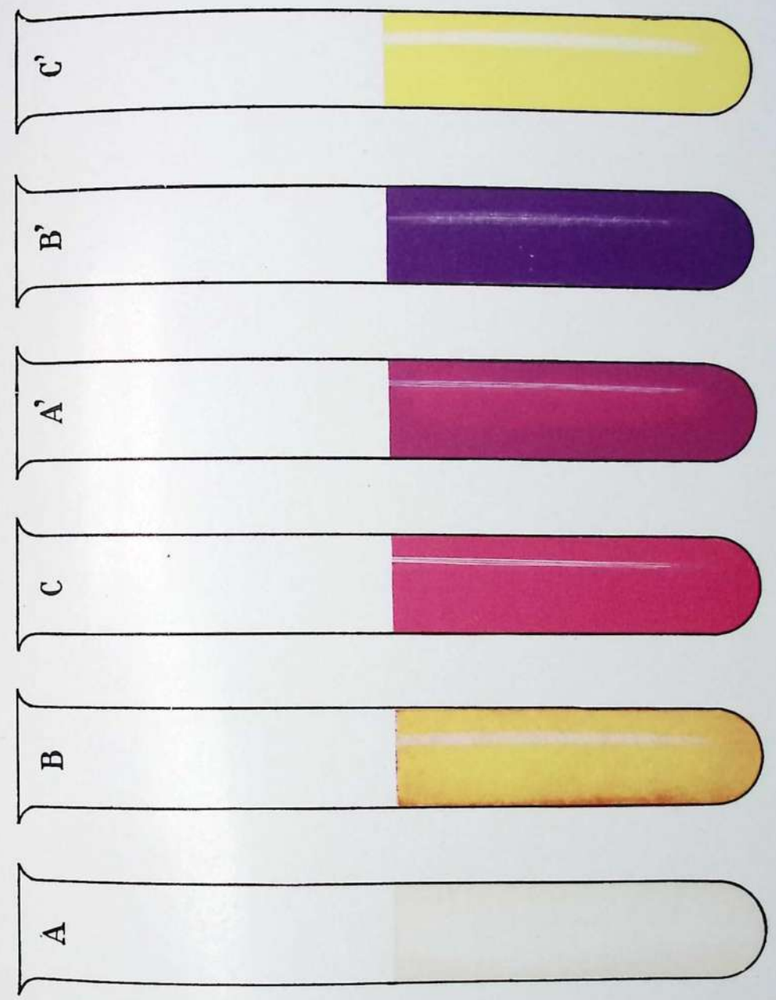
When the amount of free hydrochloric acid is normal, organic disease of the stomach probably does not exist.

Increase of free hydrochloric acid above 50 degrees (*hyperchlorhydria*) generally indicates a neurosis, but also occurs in most cases of gastric ulcer and beginning chronic gastritis. It has been found in normal persons.

Decrease of free hydrochloric acid below 25 degrees (*hypochlorhydria*) occurs in some neuroses, chronic gastritis, early carcinoma, pellagra, and most conditions associated with general systemic depression. Marked variation in the amount at successive examinations strongly suggests a neurosis. Too low values are often obtained at the first examination, the patient's dread of the introduction of the tube probably inhibiting secretion.

Absence of free hydrochloric acid (*achlorhydria*) occurs in most cases of gastric cancer and far-advanced chronic gastritis, in many cases of pellagra, and sometimes in hysteria and pulmonary tuberculosis. Achlorhydria is a constant and important symptom of pernicious anemia even during remissions. It sometimes appears long before any anemia is recognizable.

The presence of free hydrochloric acid presupposes a normal amount of **combined hydrochloric acid,** hence the combined need not be estimated when the free acid has been found. When, however, free hydrochloric acid is absent, it is important to know whether any



A, Gastric fluid to which a 1 per cent solution of phenolphthalein has been added; B, gastric fluid to which a 1 per cent solution of alizarin has been added; C, gastric fluid to which a 0.5 per cent solution of dimethylamino-azobenzol has been added; A', A after titration with a decinormal solution of sodium hydroxid; B', B after titration with a decinormal solution of sodium hydroxid; C', C after titration with a decinormal solution of sodium hydroxid (Boston).

acid is secreted, and an estimation of the combined acid then becomes of great value. The normal average after an Ewald breakfast is about 10 to 15 degrees, the quantity depending upon the amount of protein in the test meal. Somewhat higher figures are obtained after a Riegel test meal. Of greater significance than the amount of combined acid is the acid deficit, described later.

Töpfer's Method for Free Hydrochloric Acid.—In a beaker take 10 c.c. filtered stomach fluid and add 4 drops of the indicator, a 0.5 per cent alcoholic solution of dimethyl-amino-azobenzol. A red color instantly appears if free hydrochloric acid be present. Add decinormal sodium hydroxide solution, drop by drop from a buret, until the last trace of red just disappears, and a canary-yellow color takes its place (Plate XI, C, C′). For accuracy it is better (Benedict) not to carry the titration quite to the canary-yellow stage, although the end-point is then not so definite. Read off the number of cubic centimeters of decinormal solution added, and calculate the degrees or percentage of free hydrochloric acid, as in Töpfer's method for total acidity.

When it is impossible to obtain sufficient fluid for all the tests, it will be found convenient to estimate the free hydrochloric acid and total acidity in the same portion, and this is frequently adopted as a routine regardless of the amount of fluid available. After finding the free hydrochloric acid as just described, add 4 drops of phenolphthalein solution, and continue the titration. The total amount of decinormal solution used in both the titrations indicates the total acidity.

Töpfer's Method for Combined Hydrochloric Acid.—In a beaker take 10 c.c. filtered gastric juice and add 4 drops of the indicator, a 1 per cent aqueous solution of sodium alizarin sulfonate. Titrate with decinormal sodium hydroxide until the appearance of a violet color which has a slightly bluish tinge, and does not become deeper upon addition of another drop (Plate XI, B, B′). It is difficult, without practice, to determine when the right color has been reached. It is not always exactly the same, and it is better to watch the color change than to depend upon getting the precise shade. A reddish violet appears first. The shade which denotes the end reaction can be approximately imitated by adding 2 or 3 drops of the indicator to 5 c.c. of 1 per cent sodium carbonate solution.

Calculate the number of cubic centimeters of decinormal solution which would be required for 100 c.c. of stomach fluid. This gives, in degrees, *all the acidity except the combined hydrochloric acid*. The combined hydrochloric acid is then found by deducting this amount from the total acidity, which has been previously determined.

Example.—Suppose that 5 c.c. of decinormal solution were required to produce the purple color in 10 c.c. gastric juice; then $5 \times 10 = 50 =$ *all the acidity except combined hydrochloric acid*. Suppose, now, that the total acidity has already been found to be 70 degrees; then $70 - 50 = 20$ *degrees* of combined hydrochloric acid; and $20 \times 0.00365 = 0.073$ *per cent.*

When free hydrochloric acid is absent, it is probably more helpful to estimate the **acid deficit** than the combined hydrochloric acid. The acid deficit shows how far the acid secreted by the stomach falls short of saturating the protein (and bases) of the meal. It represents the amount of hydrochloric acid which must be added to the fluid before a test for free hydrochloric acid can be obtained. It is determined by titrating with decinormal hydrochloric acid, using dimethyl-amino-azobenzol as indicator, until the fluid assumes a red color. The amount of deficit is expressed by the number of cubic centimeters of the decinormal solution required for 100 c.c. of the stomach fluid.

(3) **Organic Acids.**—There is no simple direct quantitative method. After the total acidity has been determined, organic acids may be removed from another portion of the gastric filtrate by shaking thoroughly with an equal volume of neutral ether, allowing the fluids to separate, and repeating this process until the gastric fluid has been extracted with eight or ten times its volume of ether. The total acidity is then determined, and the difference between the two determinations indicates the amount of organic acids.

The **hydrogen ion concentration** may be determined by using either standard buffer solutions, or by comparison with the color chart that is now generally used. The range will be from 1.05 to 7; true "anacidity" with a *p*H of 7 is a rare condition. The indicators to be used and the *p*H of each are as follows: Thymol blue, 1.2 to 2.8; brom-phenol blue, 3 to 4.6; methyl red, 4.4 to 6; brom-cresol purple, 5.2 to 6.8; brom-thymol blue, 6 to 7.6; and, occasionally, phenol red, 6.8 to 8.4.

(4) **Pepsin.**—No direct method is simple enough for routine use. The following old methods are sufficient for clinical purposes:

1. **Hammerschlag's Method.**—To the white of an egg add twelve times its volume of 0.4 per cent hydrochloric acid (dilute hydrochloric acid, U. S. P., 4 c.c.; water, 96 c.c.), mix well, and filter. This gives a 1 per cent egg albumin solution. Take 10 c.c. of this solution in each of three tubes or beakers. To *A* add 5 c.c. gastric juice; to *B*, 5 c.c. water with 0.5 Gm. pepsin; to *C*, 5 c.c. water only. Place in an incubator for an hour, and then determine the amount of albumin in each mixture by Esbach's method. Tube *C* shows the amount of albumin in the test solution. The difference between *C* and *B* indicates the amount of albumin which would be digested by normal gastric juice. The difference between *C* and *A* gives the albumin which is digested by the fluid under examination. Schütz has shown that the amounts of pepsin in two fluids are proportionate to the squares of the

products of digestion. Thus, if the amounts of albumin digested in Tubes *A* and *B* are to each other as 2 is to 4, the amounts of pepsin are to each other as 4 is to 16.

Certain sources of error can be eliminated by diluting the gastric juice several times before testing. The most important of these are that the law of Schütz holds good only for comparatively dilute solutions, and that the products of peptic activity inhibit digestion.

2. **Mett's method** is generally preferred to the preceding. Put three or four Mett's tubes about 2 cm. long into a small beaker with diluted gastric juice (1 c.c. of the filtrate plus 15 c.c. twentieth-normal hydrochloric acid). Place in an incubator for twenty-four hours, and then measure as accurately as possible in millimeters the column which has been digested, using a millimeter scale and a hand lens or, better, a low power of the microscope and an eyepiece micrometer. Square the average length of this column (because of the law of Schütz mentioned above) and multiply by the degree of dilution, 16. The maximum figure obtained in this way is 256, representing a digested column of 4 mm.

Prepare Mett's tubes as follows: Beat up slightly the whites of one or two eggs and filter. Pour into a wide test tube, and stand in this a number of capillary glass tubes, 1 or 2 mm. in diameter. When the tubes are filled, plug their ends with bread crumbs, and coagulate the albumin by heating in water just short of boiling. Dip the ends of the tubes in melted paraffin, and preserve until needed. Bubbles, if present, will probably disappear in a few days. When wanted for use, cut the tubes into lengths of about 2 cm. Discard any in which the albumin has separated from the wall.

D. Microscopic Examination

A drop of unfiltered stomach contents is placed upon a slide, covered with a cover glass, and examined with the 16-mm. and 4-mm. objectives with the diaphragm opening much reduced. A drop of greatly diluted Lugol's solution allowed to run under the cover will aid in distinguishing the various structures. As a rule, the microscopic examination is of very limited value.

Under normal conditions little is to be seen except great numbers of starch granules, with an occasional epithelial cell, yeast cell, or bacterium. Starch granules are recognized by their concentric striations, and the fact that they stain blue with iodine solutions when undigested, and reddish, due to erythrodextrin, when partially digested. These colors, however, do not show clearly unless the iodine solution is very dilute.

Pathologically, remnants of food from previous meals, red blood corpuscles, pus cells, sarcinae, and excessive numbers of yeast cells and bacteria may be encountered (Fig. 200).

Remnants of food from previous meals indicate deficient gastric motility.

Red Blood Corpuscles.—Blood is best recognized by the chemical tests already given. The corpuscles sometimes retain a fairly normal appearance, but are generally so degenerated that only granular pigment is left. When only a few fresh-looking corpuscles are present, they usually come from irritation of the mucous membrane by the tube.

Pus Cells.—Pus is rarely encountered in the fluid removed after a test meal. Considerable numbers of pus corpuscles have been found in some cases of gastric cancer. The corpuscles are usually partially digested, so that only the nuclei are seen. The nuclei appear as small

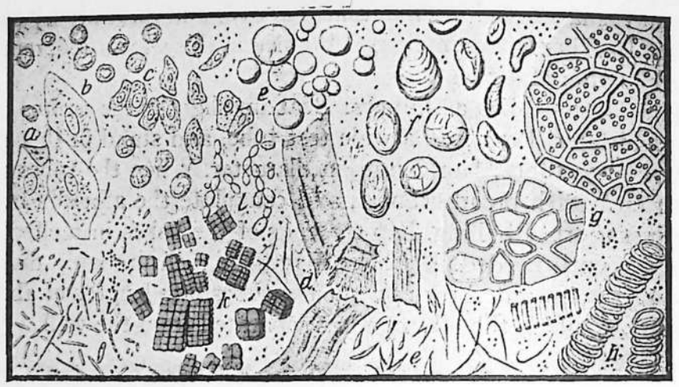


Fig. 200.—General view of the gastric contents: *a*, Squamous epithelial cells from esophagus and mouth; *b*, leukocytes; *c*, cylindric epithelial cells; *d*, muscle fibers; *e*, fat droplets and fat crystals; *f*, starch granules; *g*, chlorophyl-containing vegetable matters; *h*, vegetable spirals; *i*, bacteria; *k*, sarcinae; *l*, yeast cells (Jakob).

highly refractile bodies which lie in clusters of two, three, or four. Swallowed sputum must always be considered.

Sarcinae.—These are small spheres arranged in cuboid groups, often compared to bales of cotton. They frequently form large clumps and are easily recognized. They stain brown with iodine solution. They signify fermentation. Their presence in considerable numbers is some evidence against the existence of gastric cancer, in which disease they rarely occur.

Yeast Cells.—As already stated, a few yeast cells may be found under normal conditions. The presence of considerable numbers is evidence of retention and fermentation. Their appearance has been described (p. 159). They stain yellow to brown with iodine solution.

Bacteria.—Numerous bacteria may be encountered, especially in the absence of free hydrochloric acid. The *Boas-Oppler bacillus* is the only one of special significance. It occurs in the majority of cases of cancer, and is rarely found in other conditions. Carcinoma probably furnishes a favorable medium for its growth. It belongs to the *Bacillus bulgaricus* group.

Boas-Oppler bacilli (Fig. 201) are large (5 to 10 μ long), nonmotile, and usually arranged in clumps or end to end in zigzag chains. They stain yellow to brown with iodine solution, which distinguishes them from *Leptotrichia buccalis* (p. 56) which is not infrequently swallowed, and hence found in stomach fluid. They also stain by Gram's method. They are easily seen with the 4-mm. objective in unstained preparations but are best recognized with the oil lens, after drying some of the fluid upon a cover glass, fixing and staining with a simple bacterial stain or by Gram's method.

A few large nonmotile bacilli are frequently seen; they should not be reported as Boas-Oppler bacilli unless they are numerous and show something of the typical arrangement.

Fig. 201.—Boas-Oppler bacilli from case of gastric cancer (Boston).

E. The Gastric Contents in Disease

In the diagnosis of stomach disorders the practitioner must be cautioned against relying too much upon examinations of the stomach contents. A first examination is especially unreliable. Even when repeated examinations are made, the laboratory findings must never be considered apart from the clinical signs.

The more characteristic findings in certain disorders are suggested here:

1. Dilatation of the Stomach.—Evidences of retention and fermentation are the chief characteristics of this condition. Hydrochloric acid is commonly diminished. Pepsin may be normal or slightly diminished. Lactic acid may be detected in small amounts, but is usually absent when the stomach has been washed before giving the test meal. Both motility and absorptive power are deficient. The microscope commonly shows sarcinae, bacteria, and great numbers of yeast cells. Remnants of food from previous meals can be detected with the naked eye or microscopically.

2. Gastric Neuroses.—The findings are variable. Successive examinations may show normal, increased, or diminished hydrochloric acid, or even entire absence of the free acid. Pepsin is usually normal.

The presence of more than 100 c.c. of gastric juice in the fasting stomach has until lately been taken to indicate a neurosis characterized by continuous hypersecretion (gastrosuccorrhea), but recent studies of the fasting contents with the Rehfuss tube throw some doubt upon the condition. When the fluid contains food particles, it is the result of retention, not hypersecretion.

3. Chronic Gastritis.—Free hydrochloric acid may be increased in early cases. It is generally diminished in well-marked cases, and is often absent in advanced cases. Lactic acid is often present in traces, rarely in notable amount. Secretion of pepsin and rennin is always diminished in marked cases. Mucus is frequently present, and is very significant of the disease. Motility and absorption are generally deficient. Small fragments of mucous membrane may be found, and when examined by a pathologist may occasionally establish the diagnosis.

4. Achylia Gastrica (Atrophic Gastritis).—This condition may be a terminal stage of chronic gastritis. It is sometimes associated with the blood picture of pernicious anemia. It gives a great decrease, and sometimes entire absence of hydrochloric acid and ferments. The total acidity may be as low as 1 or 2 degrees. Small amounts of lactic acid may be present. Absorption and motility are not greatly affected. Achylia is not considered as significant as it was formerly thought to be, as it may occur in apparently healthy normal persons. For a complete review of recent thought on gastric anacidity the reader is referred to the work of Bloomfield and Polland.[1]

5. Gastric Carcinoma.—As far as the laboratory examination goes, the cardinal signs are absence of free hydrochloric acid and presence of lactic acid, and of the Boas-Oppler bacillus. These findings are, however, by no means constant, and in any case can be considered only as part of the evidence.

It is probable that some substance is produced by the cancer which neutralizes the free hydrochloric acid, and thus causes it to disappear earlier than in other organic diseases of the stomach. In early cases it may be diminished but slightly or not at all.

The presence of lactic acid may be a suggestive symptom of gastric cancer. In the great majority of cases its presence in notable amount (0.1 per cent by Strauss' method) after a test breakfast, the stomach having been washed the evening before, warrants a tentative diagnosis of malignancy.

[1] Bloomfield, A. L., and Polland, W. S.: Gastric Anacidity, Its Relations to Disease. New York, The Macmillan Co., 1933, 178 pp.

Carcinoma seems to furnish an especially favorable medium for the growth of the Boas-Oppler bacillus, hence this micro-organism is frequently present, and may also be found in the feces in large numbers (p. 485).

Blood can be detected in the stomach fluid by the chemical tests in nearly one half of the cases, and is more common when the new growth is situated at the pylorus. Blood is present in the stool in nearly every case.

Evidences of retention and fermentation are the rule in pyloric cancer. Tumor particles are sometimes found late in the disease. Sarcinae are rarely found.

6. Gastric Ulcer.—There is excess of free hydrochloric acid in about one half of the cases. In other cases the acid is normal or diminished. Blood is often present in the gastric contents, and is usually, although often intermittently, present in the feces as "occult blood." The diagnosis must be based largely upon the clinical symptoms, and where ulcer is strongly suspected, it is probably unwise to use the stomach tube.

II. ADDITIONAL EXAMINATIONS WHICH GIVE INFORMATION AS TO THE CONDITION OF THE STOMACH

1. Absorptive Power of the Stomach.—This is a very unimportant function, only a few substances being absorbed in the stomach. It is delayed in most organic diseases of the stomach, especially in dilatation and carcinoma, but not in neuroses. The test has little practical value.

Give the patient, upon an empty stomach, a 3-grain capsule of potassium iodide with a glass of water, taking care that none of the drug adheres to the outside of the capsule. At intervals test the saliva for iodides by moistening starch paper with it and touching with yellow nitric acid. A blue color shows the presence of an iodide, and appears normally in ten to thirty minutes after ingestion of the capsule. A longer time denotes delayed absorption.

Starch paper is prepared by soaking filter paper in boiled starch and drying.

2. Motor Power of the Stomach.—This refers to the rapidity with which the stomach passes its contents on into the intestines. It is very important: Intestinal digestion can compensate for insufficient or absent stomach digestion only so long as the motor power is good.

Motility is impaired to some extent in chronic gastritis. It is especially deficient in pyloric obstruction caused by malignant or benign new growths; in pyloric spasm, as in hyperchlorhydria; and in atony of the stomach wall which is usually associated with dilatation and gastroptosis.

The best evidence of deficient motor power is the detection of food in the stomach at a time when it should be empty, before breakfast in the morning. A special test meal containing easily recognized materials (rice pudding with currants, jam with seeds, raisins, or spinach) is sometimes given and removed at the end of six or seven hours. When more than 100 c.c. of fluid are obtained with the tube one hour after an Ewald breakfast, deficient motility may be inferred.

Ewald's salol test is scarcely so reliable as the above. It depends upon the fact that salol is not absorbed until it reaches the intestines and is decomposed by the alkaline intestinal juices.

The patient is given 15 grains of salol with a test breakfast, and the urine, passed at intervals thereafter, is tested for salicyluric acid. A few drops of 10 per cent ferric chloride solution are added to a small quantity of the urine. A violet color denotes the presence of salicyluric acid. It appears normally in sixty to seventy-five minutes after ingestion of the salol. A longer time indicates impaired motor power.

3. To Determine Size and Position of Stomach.—After removing the test meal, while the tube is still in place, force quick puffs of air into the stomach by compression of the bulb. The puffs can be clearly heard with a stethoscope over the region of the stomach, and nowhere else.

III. DUODENAL CONTENTS

1. Withdrawal of Contents.—The duodenal fluids are obtained by means of a slender, flexible, rubber tube, from 3 to 4 mm. in outside diameter, with a perforated metal tip (Fig. 202). It is marked with a series of black rings to indicate the distances from the incisor teeth to the cardia, to the pylorus, and to the duodenum. The tube was first demonstrated by Einhorn in 1909. There are many modifications, notably those of Jutte and of Rehfuss, which differ chiefly in the shape and weight of the metal tip, and in the arrangement of the perforations. The Jutte tube has a wire stylet which facilitates introduction as far as the stomach.

When chief interest centers in the pancreatic ferments it may be well to give a cup of bouillon a half-hour before the tube is introduced, but ordinarily no test meal is given. The patient abstains from food for about twelve hours, taking only occasional sips of water. The

tube is introduced into the stomach in the manner already described for the stomach tube (p. 448). The patient is then placed upon his right side with the hips elevated 6 or 8 inches. The movements of the stomach, aided by gravity, carry the metal tip through the pylorus into the duodenum, usually within thirty to forty-five minutes. Fluid should begin to drip from the free end of the tube soon after the metal tip has reached the stomach. If it does not do so, siphonage should be started by injecting a few cubic centimeters of warm water. The fluid is collected in portions of about 5 or 10 c.c. in a series of test tubes or small bottles. That which first appears is from the stomach, and may be recognized by its acid reaction. When the tube enters the duodenum, the fluid becomes slightly alkaline and is usually clear, light-yellow or colorless, and distinctly viscid. Only in case of gastric anacidity will there be much doubt as to the origin of the

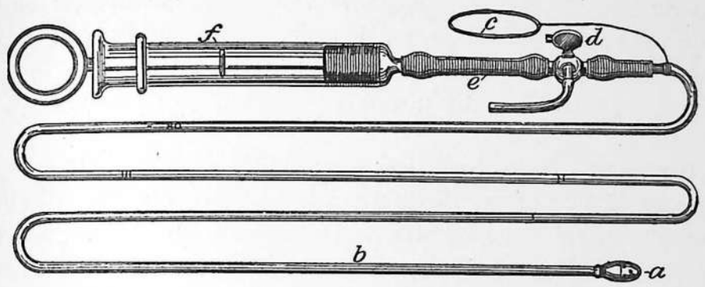


Fig. 202.—Einhorn's duodenal tube; *a*, Perforated metal capsule; *b*, rubber tube with marks at 40, 56, and 70 cm. from the metal capsule; *c*, rubber band which may be placed over ear of patient to hold tube in place; *d*, three-way stopcock; *e*, collapsible connecting tube; *f*, aspirating syringe. (Kemp.)

fluid. In such cases Einhorn advises giving the patient a few swallows of milk, and aspirating a portion of the fluid through the tube. If the milk appears, the tube is still in the stomach.

Should the flow be interrupted, injection of a few cubic centimeters of warm water will usually reestablish it. Occasionally, after the tube is in the duodenum, the fluid collected in some of the test tubes will be somewhat cloudy and opalescent, and less alkaline and less viscid than the usual duodenal fluid. This is due to admixture with stomach fluid which may have passed through the pylorus. Such fluid is to be discarded.

Although an Einhorn duodenal tube or some modification of this tube is most commonly used, a "double barrelled" tube designed by Ågren and Lagerlöf[1] is particularly useful in obtaining duodenal

[1] Ågren, Gunnar, and Lagerlöf, Henrik: The Pancreatic Secretion in Man after Intravenous Administration of Secretin. Acta Med. Scandinav. *90*:1–29, 1936.

contents to be examined for pancreatic enzymes (p. 432). One opening of the tube should lie in the stomach and the other in the duodenum. The terminal parts of both passages are perforated with small holes except for 10 cm. of the duodenal part which is meant for the pyloric region. The tube is so constructed that it will easily pass through the pylorus and not readily coil up in the stomach. Its passage should not be forced at any time if any obstruction is met, as resistance disappears in a few minutes, if the patient is placed on his right side. It may take an hour to five hours for the whole insertion of this tube to the proper position for duodenal drainage. When the tube is in the proper place, clear, alkaline, bile-colored juice should be obtained from the duodenal portion and gastric juice with no bile stain from the gastric portion. Roentgenologic examination may be used to confirm the position of the tube, or the patient may drink a glass of water colored with methylene blue. If the tube is placed correctly, methylene blue will be obtained only from the stomach tube. When the tube is in place, the gastric and duodenal juices are aspirated continuously by means of a suction pump, keeping negative pressure continuously at 20 to 30 mm. of mercury.

2. Physical Characteristics.—Normally duodenal fluid is clear, colorless or light yellow, distinctly viscid, and slightly alkaline to litmus. Admixture of acid gastric juice causes it to become somewhat cloudy and opalescent. Cloudiness due to bacteria and pus corpuscles may be present in inflammation of the duodenum or biliary passages.

3. Chemical Examination.—(1) **Ferments.**—Absence or great diminution of one or all of the pancreatic ferments, amylopsin, steapsin, and trypsin, would indicate deficient pancreatic secretion or occlusion of the pancreatic duct. Their estimation yields much the same information as their estimation in the feces, but is more reliable. Pancreatic fluid from a fistulous tract may show little or no proteolytic activity. The methods which are now considered most satisfactory are described in the chapter on Clinical Chemistry on page 432.

(2) **Bilirubin.**—There is generally a sufficient amount of unaltered bile pigment to give the duodenal contents at least a tinge of yellow, and the depth of color is a rough, though useful guide to the amount present, despite the presence of a variable amount of urobilin. The amount may be recorded as +, ++, or +++, indicating a small, moderate, or excessive amount, respectively. Sometimes the fluid is dark yellow, brown, or even chocolate brown. The presence of bile would rule out complete obstruction of the hepatic or common bile duct. Its absence does not mean that no bile reaches the duodenum at any time.

(3) **Urobilin** is a reduction product of bilirubin. Its nature and significance are discussed on page 476. A small amount of urobilin is present in the duodenal contents normally; but the chromogen, urobilinogen, is found only when urobilin is present in marked excess.

The presence of urobilinogen and an increase of urobilin have the same significance as an increase of these substances in the feces. In each case there are certain theoretic and practical objections, yet in clinical work estimations of urobilin in feces and duodenal contents appear to have about equal value, and constitute our most definite index of the activity of blood destruction. They are extremely valuable in the diagnosis of the hemolytic anemias, and the study of a case of anemia of doubtful origin is not complete without one or the other.

The most satisfactory clinical method for urobilin is that of Wilbur and Addis, which Schneider applies to the duodenal contents as follows:

1. To 10 c.c. of duodenal contents add 10 c.c. of saturated alcoholic solution of zinc acetate. Shake well and filter.
2. To 10 c.c. of the filtrate add 1 c.c. of Ehrlich's reagent (p. 477). Mix and let stand in a dark place for fifteen minutes.
3. Examine with a spectroscope and dilute with 60 per cent alcohol until the bands of both urobilin and urobilinogen have disappeared exactly as is described for urobilin in feces (p. 478). Calculate the dilution value for 1000 c.c. of duodenal contents, remembering that the filtrate used represented 5 c.c. of duodenal fluid. If, for example, the urobilin and urobilinogen bands disappeared when the 10 c.c. of filtrate was diluted to 80 c.c. and to 40 c.c. respectively, then the dilution value of 5 c.c. of duodenal fluid is 16 for urobilin and 8 for urobilinogen; and for 1000 c.c. it would be 200 times this, or 3200 and 1600, with a total dilution value of 4800.

Schneider found the maximum for healthy medical students to be about 1000 dilutions, with urobilinogen never present. In pernicious anemia and hemolytic jaundice urobilinogen is generally present, and the total dilution value usually reaches 3000 to 5000. Giffin, Sanford, and Szlapka found a striking decrease following splenectomy, particularly in pernicious anemia.

Schneider used these figures in the following formula, which aims to express the relation between blood regeneration and blood destruction in the form of an index number which he designated as the H-H (hematopoietic-hemolytic) Index: $\frac{Z + Y}{6}$ = H-H index, Z representing the total duodenal urobilin-dilution value in thousands, and Y the red corpuscle count in millions. Under normal conditions (urobilin dilutions 1000, red cell count 5,000,000) the index is 1. When hemolysis is very active it will be above

1 unless counterbalanced by very deficient blood formation. When blood regeneration fails the index will be below 1. Such an index is artificial and arbitrary, but is useful in impressing the importance of considering the ratio of blood formation to blood destruction in the course of an anemia.

4. Microscopic Examination.—The duodenal fluid must be examined within a few minutes after it is secured, otherwise the cellular elements may be damaged or destroyed by the ferments. The method is the same as for fresh urine. Normally only an occasional leukocyte or epithelial cell can be found. In pathologic conditions these may be present in increased numbers, but no definite diagnostic inferences can be drawn. A great excess of pus corpuscles would suggest inflammation of the duodenum or biliary tract. *Strongyloides stercoralis* and *Giardia lamblia* have been found, sometimes in great numbers. Cystic and vegetative forms of *Endamoeba histolytica* have also been found, and in such cases infection of the liver or bile passages is inferred.

5. Bacteriologic Examination.—At the present time very little of clinical value can be learned from a bacteriologic study. Normally the fluid is sterile or contains only a few Gram-positive cocci. Bacteria seen in the direct microscopic examination are mostly dead.

For bacteriologic examination the duodenal fluid is obtained in the usual way, with the following precautions to prevent contamination:[1]

Sterilize the tube by boiling. Slip over the metal tip a gelatin capsule which has been soaked in alcohol for several days. Dip the gelatin-covered tip in thin shellac several times, letting it dry after each coating. Introduce the tube in the usual way. When it has entered the duodenum, the gelatin bag may be removed by forcing in a little air or a few cubic centimeters of sterile water.

IV. EXAMINATION OF FRESH BILE

Following an observation of Meltzer upon the effect of magnesium sulfate applied locally to the mucosa of the duodenum, Lyon has advocated a procedure which he believes makes it possible to collect and segregate bile from the different parts of the biliary tract; and he holds that cytologic and bacteriologic study of this bile yields information of much value in the differential diagnosis of cholecystitis, cholelithiasis, and choledochitis.

The procedure may be outlined as follows: From 50 to 100 c.c. of sterile 25 per cent saturated magnesium sulfate are introduced

[1] For details see: MacNeal, W. J., and Chace, A. F.: A Contribution to the Bacteriology of the Duodenum, Arch. Int. Med., *12*:178–197 (Aug.), 1913.

into the duodenum through a duodenal tube, which is left in place. Magnesium sulfate introduced directly into the duodenum appears to relax the sphincter of the common duct and thereby to induce drainage of the entire biliary tract. The duodenal contents are aspirated into a series of sterile bottles. Golden-yellow bile from the common duct appears very soon, and is designated "A." After a few minutes this rather suddenly gives place to a darker, more viscid bile, which is supposed to come from the gallbladder and is designated "B." This portion usually amounts to from 30 to 75 c.c., and is succeeded by a clear light yellow bile of low specific gravity which is assumed to be freshly secreted bile from the liver, and is designated "C." The various portions are collected separately, and their color, viscosity, turbidity, and general appearance noted, as well as the presence or absence of mucus. They are also examined microscopically, chiefly for abnormal sediments, and culturally for bacteria.

Jones[1] has shown that in cholelithiasis the sediment, after high speed centrifugalization, will show characteristic bile-stained epithelium or leukocytes, cholesterin crystals, amorphous yellow bilirubin, or darker yellowish-brown crystals of calcium bilirubinate.

McClure and his colleagues[2] use 5 c.c. oleic acid in 45 c.c. warm water in place of magnesium sulfate which they found tended to depress the biliary function of the liver.

[1] Jones, C. M.: The Rational Use of Duodenal Drainage, Arch. Int. Med., *34*:60–78 (July), 1924.

[2] McClure, C. W., Mendenhall, W. L., and Huntsinger, M. E.: Studies in Liver Function. IV. A Procedure for the Uniform Stimulation of the Biliary Flow, Boston Med. and Surg. Jour., *193*:1052–1054 (Dec.), 1925.

CHAPTER VI

THE FECES

As commonly practiced, an examination of the feces is limited to a search for intestinal parasites or ova. Much of value can, however, be learned from other simple examinations, particularly a careful inspection. Anything approaching a complete analysis is, on the other hand, a waste of time for the clinician.

The normal stool is a mixture of—(*a*) water; (*b*) undigested and indigestible remnants of food, as starch granules, particles of meat, vegetable cells, and fibers; (*c*) digested foods, carried out before absorption can take place; (*d*) products of the digestive tract, as altered bile pigments, enzymes, mucus; (*e*) products of decomposition, as indol, skatol, fatty acids, and various gases; (*f*) epithelial cells shed from the wall of the intestinal canal; (*g*) harmless bacteria, which are always present in enormous numbers.

Pathologically, we may find abnormal amounts of normal constituents, blood, pathogenic bacteria, animal parasites and their ova, and biliary and intestinal concretions.

The stool to be examined should be passed into a clean vessel without admixture of urine. The examination should not be delayed more than a few hours, owing to the changes caused by decomposition. The offensive odor can be partially overcome with 5 per cent phenol or a little formalin. No disinfectant should be used when search for amebae is to be made; the vessel must be warm, and the stool kept warm until examined. For protozoa a saline cathartic may be given, and the first and the second stool examined. The first stool is usually too solid, and the later ones too greatly diluted. Some prefer to make the search for amebae in feces obtained in this way, using both the first and second stools. A jar or bottle which is sent to the laboratory nearly full of feces should be opened with great care, otherwise the gases which may have formed may force the fecal material out with a spurt and soil the hands.

I. MACROSCOPIC EXAMINATION

1. Quantity.—The amount varies greatly with diet and other factors. The average is about 100 to 200 Gm. in twenty-four hours. It is much larger upon a vegetable diet.

2. Frequency.—One or two stools in twenty-four hours may be considered normal, yet one in three or four days is not uncommon with healthy persons. The individual habit should be considered in every case.

3. Form and Consistence.—Soft, mushy, or liquid stools follow cathartics and accompany diarrhea. Copious, purely serous discharges without fecal matter are significant of Asiatic cholera, although sometimes observed in other conditions. Hard stools accompany constipation. Rounded scybalous masses are common in habitual constipation, and indicate atony of the muscular coat of the colon. Flattened, ribbon-like stools result from some obstruction in the rectum, generally a tumor or a stricture from a healed ulcer, most commonly syphilitic.

4. Color.—The normal light or dark brown color is due chiefly to urobilin, which is formed from bilirubin by reduction processes in the intestine, largely the result of bacterial activity. The stools of infants are yellow, owing partly to their milk diet and partly to the presence of unchanged bilirubin.

Diet and drugs cause marked changes: Milk, a light yellow color; cocoa and chocolate, dark gray; various fruits, reddish or black; spinach, dark green; large doses of calomel, green, due to biliverdin; iron and bismuth, dark brown or black; hematoxylin, red.

Pathologically the color is important. A golden yellow is generally due to unchanged bilirubin. Green stools are not uncommon, especially in diarrheas of childhood. They are sometimes met in apparently healthy infants, alternating with normal yellow stools, and have little significance unless accompanied by symptoms. The color is due to biliverdin or, sometimes, to chromogenic bacteria. Putty-colored or "acholic" stools occur when bile is deficient, either from obstruction to outflow or from deficient secretion. The color is due less to absence of bile pigments than to presence of fat. Similar stools, which have a greasy appearance and manifestly consist largely of fat or its derivatives, are common in conditions like tuberculous peritonitis, which interfere with absorption of fats, and in pancreatic disease.

Large amounts of blood produce tarry black, usually viscid stools when the source of the hemorrhage is the stomach or upper intestine, and a dark brown to bright red as the source is nearer the rectum. When diarrhea exists the color may be red, even if the source of the blood is high up. Red streaks of blood upon the outside of the stool are due to lesions of rectum or anus. Amounts of blood too small for recognition by simple inspection constitute "occult blood" and require chemical tests.

5. Odor.—Products of decomposition, chiefly indol and skatol, are responsible for the normal offensive odor. The strength of this odor depends largely upon the amount of meat in the diet, and the activity of putrefactive bacteria in the intestine. Upon a vegetable or milk diet the odor is much less. A sour odor due to fatty acids is normal for nursing infants, and is noted in mild diarrheas of older children. In the severe diarrheas of childhood a putrid odor is common. In adults, stools emitting a very foul stench are suggestive of malignant or syphilitic ulceration of the rectum or gangrenous dysentery.

6. Mucus.—Excessive quantities of mucus are easily detected with the naked eye, and signify irritation or inflammation. When the mucus is small in amount and intimately mixed with the stool, the trouble is probably in the small intestine. Larger amounts, not well mixed with fecal matter, indicate inflammation of the large intestine. Stools composed almost wholly of mucus and streaked with blood are the rule in dysentery, ileocolitis, and intussusception.

In the so-called "mucous colic or membranous enteritis," shreds and ribbons of altered mucus, sometimes representing complete casts of portions of the bowel, are passed, especially after an enema. In the ordinary formed stool they usually pass unrecognized unless the feces be well mixed with water. These may appear as firm, irregularly segmented strands, suggesting tapeworms. The mucus sometimes takes the form of brown or black jelly-like masses. In some cases it is passed at variable intervals, with colic; in others, with every stool, with only vague pains and discomfort. It is distinguished from inflammatory mucus by absence of pus corpuscles. The condition is not uncommon and should be more frequently recognized. It is probably a secretory neurosis, hence the name "membranous enteritis" is inappropriate.

7. Concretions.—Gallstones should be searched for on several successive days in every case of obscure colicky abdominal pain. Intestinal concretions (enteroliths) are rare. Intestinal sand, consisting of sandlike grains, has been found in neurotic conditions, such as mucous colitis. It attracts less attention than formerly, since in most cases the so-called "sand" proves to be vegetable matter, such as seeds of berries or bananas or the hard brown grains from about the seeds of pears. After ingestion of considerable amounts of olive oil, nodules of soap and fat often appear in the feces, and may be mistaken by the patient for gallstones, particularly when the oil has been given for cholelithiasis.

Concretions can be found by breaking up the fecal matter in a

sieve (which may be improvised from gauze) while pouring water over it. It must be remembered that gallstones, if soft, may go to pieces in the bowel. Gallstones are readily identified by their faceted surfaces. When facets are absent, the stones can be distinguished from other concretions by detecting cholesterol and bile pigment in them. The stone is broken up and as far as possible dissolved in ether. If now the ether be slowly evaporated in a watch glass, crystals of cholesterol will separate out. Addition of one half volume of alcohol to the fluid will cause it to evaporate more slowly with formation of more perfect crystals. The crystals may be identified microscopically by their characteristic form (Fig. 45); by a carmine color at the edges when treated with a drop of concentrated sulfuric acid on the slide;

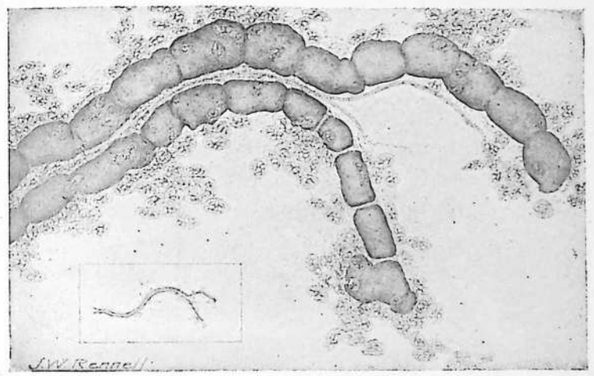


Fig. 203.—Undigested fiber from center of banana in feces (× 15). In the lower part of the figure the fiber is shown natural size. The segments are colored reddish brown when found in the stool. Such fibers in the stools of children with diarrhea are often reported as small tapeworms. In formed stools the segments are often broken apart, and appear as reddish-brown rectangular bodies.

or by a play of colors—blue, red, green, violet—when treated with a drop of Lugol's solution. To extract bile pigments treat the parts of the stone which have failed to dissolve in ether with chloroform and then with hot alcohol. A yellow color in the chloroform and a green in the alcohol show the presence of bilirubin and biliverdin respectively.

8. Animal Parasites.—Segments of tapeworms, and the adults of other parasites are often found in the stool. The method of recovering tapeworm heads is described on page 547. The smaller worms are sought as described for concretions, the material caught by the sieve being floated out in clear water and examined in a thin layer in a flat-bottomed dish over a dark background. The search should be preceded by a vermicide and a brisk purge or, when pinworms are

sought, by a copious enema. A hand lens or a very low power of the microscope may be needed to identify very small worms such as trichinella or the male pinworm. Patients often mistake vegetable tissue for intestinal parasites, and many times physicians have been known to make similar mistakes. The most frequent sources of confusion are long fibers from poorly masticated celery or "greens," which suggest round worms; cells from orange, which suggest pinworms; and fibers from banana, which, because of the segmented structure and the presence of oval cells, suggest tapeworms and ova (Fig. 203). Even slight familiarity with the microscopic structure of vegetable tissue will prevent the chagrin of such errors.

Larvae of insects are occasionally found, usually the result of accidental contamination, sometimes of swallowing eggs or larvae. They should be identified as larvae of insects without difficulty, but the determination of species is a problem for the trained entomologist.

9. Curds.—The stools of infants frequently contain whitish curd-like masses, due either to imperfect digestion of fat or casein, or to excess of these in the diet. When composed of fat, the masses are soluble in ether, and give the sudan III test. If composed of casein, they will become tough and fibrous-like when placed in formalin (10 per cent) for twenty-four hours.

II. CHEMICAL EXAMINATION

Complicated chemical examinations are of little value to the clinician. Certain tests are, however, important.

1. Reaction.—Normally this is either slightly acid or slightly alkaline. Much depends on the diet; excess of carbohydrates produces acidity, excess of protein, alkalinity. Pathologically, variations in reaction may result from intestinal indigestion of the respective foodstuffs. The reaction may be tested with litmus or with the alizarin indicator used in gastric analysis.

2. Fermentation.—Excessive carbohydrate fermentation, resulting from intestinal indigestion of carbohydrates, is manifested by gas formation and an acid reaction. The stool is usually soft and mushy, and bubbles of gas may be present. The bubbles become much more evident after it has stood in a warm place for twelve hours. Then the stool, when stirred with a stick, gives a crackling sound resembling râles. As the gas forms the reaction becomes increasingly acid. A similar bulky, frothy stool, which has these characteristics when freshly passed, is typical of **sprue.** Normal stool contains few or no gas bubbles even after standing twenty-four hours. Special diets and special procedures for the determination of gas formation, such as

Schmidt's well-known test, may be employed, but are not usually necessary.

Intestinal protein indigestion, on the other hand, is manifested by evidences of putrefaction, that is, very foul-smelling stools with strongly alkaline reaction and little gas formation, together with excess of indican in the urine.

3. Blood.—When present in large amount blood produces such changes in the appearance of the stool that it is not likely to be overlooked. Traces of blood (*occult hemorrhage*) can be detected only by special tests. Recognition of occult hemorrhage has its greatest value in diagnosis of gastric cancer and ulcer. It is constantly present in practically every case of gastric cancer, and is always present, although usually intermittently, in ulcer. Traces of blood also accompany malignant disease of the bowel, the presence of certain intestinal parasites, and other conditions.

Detection of Occult Hemorrhage.—Soften a portion of the stool with water, shake with an equal volume of ether to remove fat, and discard the ether. Treat 10 c.c. of the remaining material with about one third its volume of glacial acetic acid and extract with about 10 c.c. ether. Blood pigment is insoluble in neutral ether, but is readily soluble in acidified ether. Should the ether not separate well, add about one half its volume of alcohol and mix gently. Apply the guaiac or benzidine test to a portion of the ether as already described (p. 115). When much urobilin is present the color of a positive reaction may be purplish brown. In case the test is positive, it is a good plan to use the remainder of the ether for spectroscopic examination, treating it so as to produce the bands of hemochromogen (pp. 356, 358).

In all cases which give a positive reaction with the patient upon a full diet repeat the test after blood pigment has been excluded from the food by giving an appropriate diet—bread, milk, eggs, and fruit. At the beginning of the restricted diet give 1 Gm. of powdered charcoal or, better, 0.3 Gm. of carmine, in capsule, so as to mark the corresponding stool.

Alvarez and Wight[1] described a slightly modified Gregersen test for occult blood which is very satisfactory. Mix thoroughly barium peroxide, 40 Gm., with pure benzidine, 5 Gm., or in similar proportions, and store in bulk form in a dark, stoppered bottle. Dissolve immediately before using 0.225 Gm. of the powder (the amount may be estimated from its bulk on the end of a spatula) in 5 c.c. of 50 per cent acetic acid. Rub a small portion of feces into a few drops of water on a porcelain palette. Add a few drops of the benzidine solution, and make readings with a stop watch according to the Gregersen scale, which is as follows: A deep blue color inside of three

[1] Alvarez, R. S., and Wight, T. H. T.: The Gregersen Test, U. S. Vet. Bur. Med. Bull., *5*:888–890 (Nov.), 1929.

seconds, +++; a less vivid blue in fifteen seconds, ++; a blue-green color in an interval of from thirty to sixty seconds, +, doubtful in significance. The test is sensitive in a dilution of blood of 1 : 200,000, and gives no reaction with iron salts, or products from the ordinary diet.

4. Bile.—Normally, unaltered bile pigment is never present in the feces of adults. In catarrhal conditions of the small intestine bilirubin may be carried through unchanged. It may be demonstrated by the Schmidt test for urobilin, or, if a considerable amount is present, by filtering (after mixing with water if the stool be solid) and testing the filtrate by Gmelin's method, as described under The Urine.

5. Urobilin (Hydrobilirubin).—The urobilin of the urine and the hydrobilirubin which constitutes the principal normal pigment of the feces appear to be identical; and the present tendency is to use the name "urobilin" in both instances. In a general way the name covers both the pigment, urobilin, and the chromogen, urobilinogen, of which it is an oxidation product, since the two substances have exactly the same significance. For the mode of formation and the significance in the urine the reader is referred to the chapter on The Urine. Owing to constipation and other factors the amount of urobilin in the feces is subject to marked daily variations. The average of a number of successive daily estimations is, however, fairly constant. Ordinarily the twenty-four-hour stool gives a dilution value by the Wilbur and Addis method of 6000, and 9000 may be taken as the upper normal limit.

Since bilirubin, its mother substance, is a product of blood pigment, an abnormally large amount of urobilin in the feces or in the duodenal contents may be taken as definite evidence of excessive destruction of red blood cells within the circulation; and quantitative estimations are of great value whenever such increased blood destruction is in question, even though, as appears to be the case, urobilin excretion does not exactly parallel blood destruction. They have been found especially useful in distinguishing the anemias due to excessive hemolysis (for example, pernicious anemia) from other anemias in which hemolysis is not a prominent factor (carcinoma, hemorrhage); in following the progress of individual cases of pernicious anemia; and in studying the effect of splenectomy performed as a therapeutic measure in this disease. In progressing cases of pernicious anemia the Wilbur and Addis method usually gives urobilin dilution values of 20,000 to 30,000, and often much more; during remissions urobilin may return nearly to the normal. In connection with urobilin excretion the percentage of reticulated red corpuscles in the blood, which

serves as a measure of blood regeneration, should be studied, since it is chiefly the balance between blood regeneration and blood destruction which determines the clinical and hematologic pictures in anemia.

Urobilin is nearly or quite absent from the stool in cases of catarrhal jaundice and obstruction of the common or hepatic bile ducts. Its return will herald relief of the condition before any lessening of the jaundice is evident.

Detection.—The chemical tests mentioned on page 113 may be applied to a watery extract of the stool. Direct spectroscopic examination is impossible owing to the cloudiness of the suspension. The following test is also useful:

Schmidt's Test.—Rub a small quantity of the fecal matter with saturated mercuric chloride solution and let stand twenty-four hours. Urobilin will give a red color, which is likewise imparted to such microscopic structures as are stained with urobilin. A green color shows the presence of unchanged bilirubin and is not seen normally.

Quantitative Estimation.—The method of Wilbur and Addis is useful clinically. While it does not give the actual quantity of urobilin, it furnishes a rough comparative index which works very well in practice. Because of the instability of urobilin, methods which involve elaborate treatment of the feces are not applicable. Since urobilin and urobilinogen have the same significance and are so readily changed one into the other they are included together in the estimation. Estimations are of little value unless the average of six to ten, made on successive days, is taken.

Method of Wilbur and Addis.—1. Collect all the feces for twenty-four hours, keeping them in darkness.

2. Grind the whole quantity with water to a homogeneous paste.

3. Dilute to 1000 c.c. with tap water (or to 500 or 2000 c.c. if the amount of feces is unusually small or large).

4. Measure off 25 c.c. and add to this 75 c.c. acid alcohol (alcohol 64 c.c., concentrated hydrochloric acid 1 c.c., water 32 c.c.).

5. Place in a mechanical shaker for one-half hour. Constant shaking by hand for a similar period will answer.

6. Add 100 c.c. of saturated alcoholic solution of zinc acetate and filter.

7. To 20 c.c. of the filtrate add 2 c.c. of Erhlich's reagent (paradimethylaminobenzaldehyde, 20 Gm.; concentrated hydrochloric acid, 150 c.c.; water, 150 c.c.).

8. Keep in darkness until next day (or at least for six hours) and examine spectroscopically. In the presence of both urobilinogen and urobilin the two absorption bands indicated in Fig. 204, A and B, will be seen.

9. Dilute with 60 per cent alcohol, adding a few cubic centimeters at a time, until first one and then the other band has entirely disappeared, when the slit of the spectroscope is wide open, but still remains visible when the slit is partly closed. The end point is fairly definite after one has established his standard upon a series of normal stools. It is perhaps best to use an unvarying width of slit and to dilute until the bands have just disappeared with this opening. One may establish uniform conditions as to the thickness of the layer of fluid, the kind and strength of the light, and the distance from the light, and then adopt a width of slit which gives an average of about 6000 dilutions in a series of normals. When using the "pocket" type of spectroscope we now place the fluid in a standard serologic tube about 12 mm. in diameter and employ a 60-watt frosted Mazda lamp, placed about 6 inches from the spectroscope, which is mounted upon a temporary stand to insure steadiness. The eyes are protected from the light by a cardboard screen.

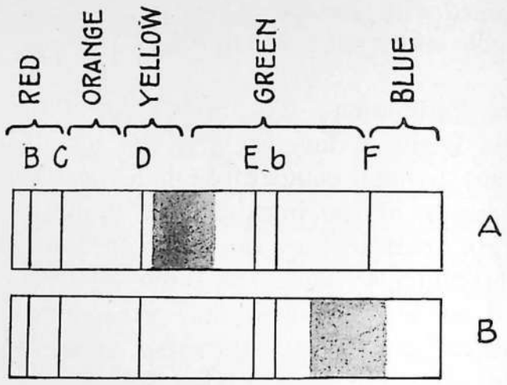


Fig. 204.—Absorption spectra: A, Urobilinogen in acid solution with Ehrlich's reagent; B, urobilin in acid solution with zinc acetate.

10. Calculate separately the number of dilutions necessary to cause disappearance of each of the absorption bands and add the two together. The calculation is based not upon the 20 c.c. of filtrate used, but *upon the 2.5 c.c. of fecal suspension represented by the filtrate*. The dilution value for the twenty-four-hour stool (1000 c.c. of fecal suspension) is then found by multiplying this figure by 400. When the fecal suspension was made up to 500 or 2000 c.c. the multiplier would, of course, be 200 or 800. This final result indicates the number of dilutions which would be necessary if all the urobilin and urobilinogen of the twenty-four-hour stool were concentrated in the 2.5 c.c. of fecal suspension examined.

Example.—Suppose that in Step 9 the urobilinogen band disappeared when the 20 c.c. of filtrate had been diluted to 25 c.c., and the urobilin band when the volume reached 30 c.c., then the dilution values for the 2.5 c.c. of feces would be 10 and 12 respectively and the combined value

$10 + 12 = 22$. The total dilution value of the twenty-four-hour stool would then be $22 \times 400 = 8800$.

If a more accurate quantitative method is desired the method developed by Watson[1] is the most accurate. Feces are collected for four days, well mixed, and a 10-Gm. portion emulsified in water is tested. The method depends upon the reduction of all the urobilin to urobilinogen with ferrous hydroxide, and combining the urobilinogen with modified Ehrlich's reagent (0.7 Gm. paradimethylaminobenzaldehyde, 150 c.c. of concentrated hydrochloric acid and 100 c.c. of distilled water) and a saturated solution of sodium acetate. The red color that develops can be compared with phenolsulfonphthalein standards. Watson has developed a formula by means of which the values in terms of milligrams of urobilinogen excreted per day can be calculated.

The normal range is between 40 and 280 mg. excreted in the feces each day; usually from 100 to 250 mg.

Obstruction to bile outflow owing to malignancy is usually characterized by diminution of the excretion of urobilinogen in the feces to less than 5 mg. each day. Partial obstruction caused by stone and diffuse hepatic disease have variable effects on the amounts of fecal excretion of urobilinogen. A marked increase in excretion of urobilinogen in feces indicates hemolysis. In hemolytic jaundice with marked increase in excretion of urobilinogen, there may be a rapid decrease in excretion after splenectomy.

6. Pancreatic Ferments.—Two of the ferments of the pancreatic juice—amylase and trypsin—are normally present in the feces. Lipase can usually not be detected. In pancreatic disease and in simple obstruction of the pancreatic duct these ferments are diminished or absent. Quantitative estimation of the pancreatic enzymes in the feces, which was formerly advocated, is reliable; but it is better to use duodenal contents, as described on pages 432 and 465.

III. MICROSCOPIC EXAMINATION

Care must be exercised in selection of portions for examination. A random search will often reveal nothing of interest. Samples from several different parts should be examined even when the stool is apparently homogeneous. A small bit of the stool, or any suspicious-looking particle, is placed upon a slide, thinned with water if necessary, and covered with a cover glass. The layer should be just thin enough to read newsprint through it when the slide is placed upon

[1] Watson, C. J.: Studies of Urobilinogen. I. An Improved Method for the Quantitative Estimation of Urobilinogen in Urine and Feces, Am. Jour. Clin. Path., *6*:458–475 (Sept.), 1936. II. Urobilinogen in the Urine and Feces of Subjects Without Evidence of Disease of the Liver or Biliary Tract, Arch. Int. Med., *59*:196–205 (Feb.), 1937. III. The Per Diem Excretion of Urobilinogen in the Common Forms of Jaundice and Disease of the Liver, Arch. Int. Med., *59*:206–231 (Feb.), 1937.

the paper. A large slide—about 2 by 3 inches—with a correspondingly large cover will be found convenient. Most of the structures which it is desired to see can be found with a 16-mm. objective. Details of structure must be studied with a higher power. Since size is always an important consideration in the identification of microscopic structures, and particularly so in the case of parasites and their ova, frequent use of the eyepiece micrometer is essential. When it is desired to study the food remnants as an index of the state of digestion a test meal should be given and a series of slides prepared as described on page 493.

The bulk of the stool consists of granular débris. Among the recognizable structures (Fig. 205) met in normal and pathologic con-

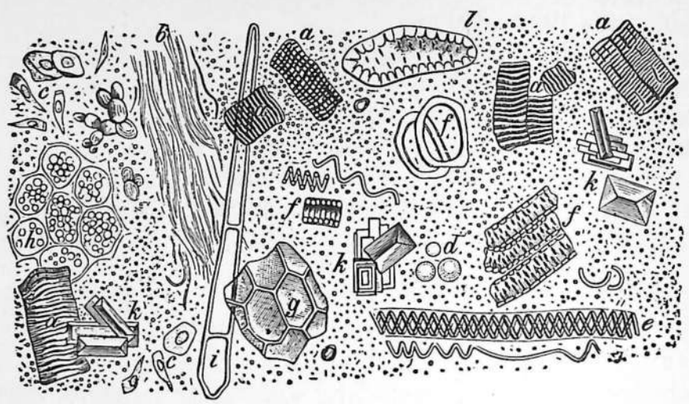


Fig. 205.—Microscopic elements of normal feces: *a*, Muscle fibers; *b*, connective tissue; *c*, epithelial cells; *d*, white blood corpuscles; *e*, spiral vessels of plants; *f–h*, vegetable cells; *i*, plant hairs; *k*, triple phosphate crystals; *l*, stone cells. Scattered among these elements are micro-organisms and débris (after v. Jaksch).

ditions are: Remnants of food, epithelial cells, pus corpuscles, red blood corpuscles, crystals, bacteria, protozoa, and ova of animal parasites.

1. Remnants of Food.—These include a great variety of structures which are very confusing to the student. Considerable study of normal feces is necessary for their recognition.

Vegetable fibers are generally recognized from their spiral structure or their pits, dots, or reticulate markings; **vegetable cells,** from their double contour and the chlorophyl bodies which many of them contain. These cells are apt to be mistaken for the ova of parasites. **Vegetable hairs** (Fig. 206) frequently look much like the larvae of some of the worms. Anything like a careful examination will, however, easily distinguish them because of the homogeneous and highly

refractile wall, the distinct central canal which extends the whole length, and, especially, the absence of motion. **Starch granules** sometimes retain their original form, but are ordinarily not to be recognized except by their staining reaction. Potato starch appears in colorless

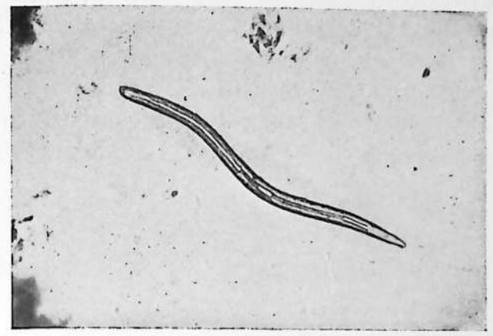


Fig. 206.—Vegetable hair (down from skin of peach) in feces (photograph × 150). Compare with Fig. 271.

translucent masses somewhat like sago grains or flakes of mucus. Starch strikes a blue color with Lugol's solution when undigested; a red color, when slightly digested. **Scales of sardines** have an appearance similar to starch granules, but can be differentiated with Lugol's

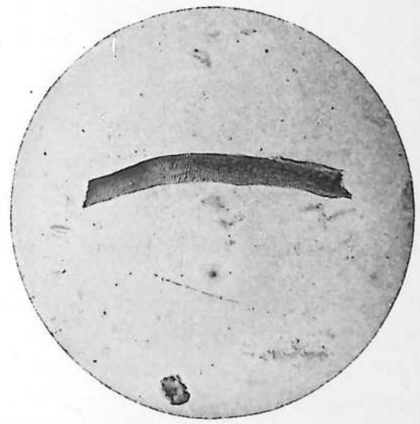


Fig. 207.—Poorly digested muscle fiber in feces showing striations and ragged ends (photograph × 200).

solution. **Muscle fibers** are yellow, and when poorly digested appear as short, transversely striated cylinders with rather squarely broken ends (Fig. 207). Generally the ends are rounded and the striations faint; or only irregularly round or oval yellow masses which bear

little resemblance to normal muscle tissue are found. If a little eosin solution be run under the cover, muscle fibers will take up the red color and stand out distinctly.

Fats occur in three modifications: Neutral fats, fatty acids, and soaps. *Neutral fats* are present in very small amounts or not at all on an ordinary diet. They appear as droplets or yellowish flakes, depending upon the melting point. They stain strongly with sudan III. *Fatty acids* take the form of flakes like those of neutral fat, or of needlelike crystals which are generally aggregated into thick balls or irregular masses in which the individual crystals are difficult to make

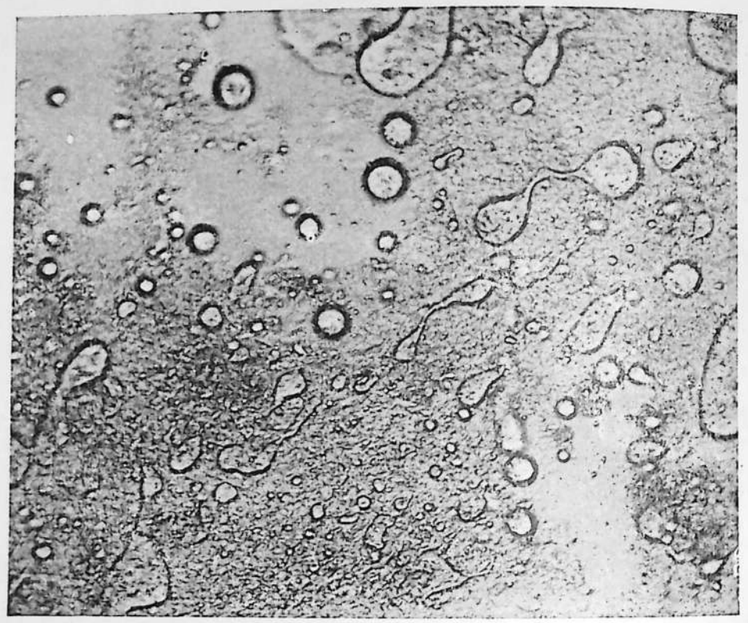


Fig. 208.—Microphotograph of castor oil in the stool (magnification × 150).

out. When treated with sudan III (p. 833 for formula) the amorphous flakes take a lighter orange than do the neutral fats, while the crystals do not stain. *Soaps*—chiefly calcium soap—appear partly as well-defined yellow amorphous flakes or rounded masses suggesting eggs of parasites, partly as coarse crystals. They do not stain with sudan III and do not melt into globules when warmed as do the fatty acids. **Mineral oil** or **castor oil** taken as a cathartic may appear in the stool in such an amount as to interfere with an examination for parasites (Fig. 208). **Connective tissue** consists of colorless or yellowish threads with poorly defined edges and indefinite longitudinal striations. When treated with 30 per cent acetic acid the fibers swell up and become

clear and homogeneous. **Elastic fibers,** which are often present along with the connective tissue, are more definite in outline and branch and anastomose. They are rendered more distinct by acetic acid.

Excess of any of these structures may result from excessive ingestion or deficient digestion, and interpretation should be based upon a standard test diet (p. 493).

2. Body Cells.—A few **epithelial cells,** derived from the wall of the alimentary canal, are always present. They show all stages of disintegration and are often unrecognizable. A marked excess has its origin in a catarrhal inflammation of some part of the bowel, usually the colon, when the cells are well preserved. Squamous cells come from the anal orifice, otherwise the form of the cells gives no clue to the location of the lesion.

Pus corpuscles are present in catarrhal and ulcerative conditions of the intestine. The number of the pus cells roughly corresponds to the extent and severity of the process except in amebic dysentery, where any considerable number of pus corpuscles indicates superimposed infection. When the pus is well mixed with the stool the source is high up, but in such cases it is likely to be more or less completely digested and hence unrecognizable.

Haughwout and others have called attention to microscopic findings which they regard as characteristic of bacillary dysentery. The mucus contains large numbers of polymorphonuclear pus corpuscles, together with a variable number of **macrophages.** These are large mononuclear phagocytic cells with large vesicular nuclei, and frequently contain remnants of ingested leukocytes or red corpuscles. These cells might be mistaken for endamebae, but the character of the nucleus should make the differentiation easy. They show various degrees of necrosis, and frequently there remains only the circular or oval rim of the cell with a few included granules ("ghost cell"). Haughwout states that "these two types of cells, macrophage and ghost cell, are the two constant and specific characters of the bacillary exudate." In the majority of cases the macrophages are about 2 per cent of the total number of cells present.

A marked excess of **eosinophils** has been noted in the masses of mucus found in the discharges of intestinal allergy.

Unaltered **red blood corpuscles** are rarely seen unless their source is the colon, rectum, or anus. A striking tendency of the red cells to form clumps of three or more is mentioned by Anderson as characteristic of amebic dysentery. When the bleeding is in the small intestine the red corpuscles can seldom be recognized as such, and chemical tests must be used (p. 475).

3. Crystals.—Various crystals may be found, but few have any significance. Slender, needlelike crystals of fatty acids and soaps (Fig. 45, p. 129) and triple phosphate crystals (Fig. 205) are common. Characteristic octahedral crystals of calcium oxalate (Fig. 47) appear after ingestion of certain vegetables. Charcot-Leyden crystals (Fig. 24, p. 40) are often seen in ulcerative conditions of the intestine. This may be a helpful point in distinguishing between amebic and bacillary dysentery. Yellowish or brown, needlelike or rhombic crystals of hematoidin (Fig. 45) may be seen after hemorrhage into the bowel. The dark color of the feces after administration of bismuth salts is due largely to great numbers of bismuth suboxide crystals. They resemble hemin crystals.

4. Bacteria.—In health, bacteria—mostly dead—constitute about one third of the weight of the dried stool. In general it appears that they are beneficial, although not necessary to existence. Ordinarily it is both difficult and unprofitable to identify them.

Altogether more than fifty different species have been isolated. Some of these are met with only occasionally; some are so constantly present as to be recognized as normal inhabitants of the human intestine. In nursing infants the majority are gram-positive bacilli of the *Bacillus acidophilus* group, which produce acid, but no gas. This accounts for the normal sour odor of the stools of infants. Most of these disappear soon after the child is weaned, and are replaced by a variety of bacteria, chiefly gram-negative bacilli of the colon bacillus group, which are the predominant organisms in adult life. A small number of the gram-positive, acidophilic bacteria generally persists.

For stained preparations the bacteria can be obtained comparatively free from food remnants by mixing a little of the feces with water, allowing to settle for a short time, and making smears from the supernatant fluid. In order to remove fat these films should be fixed by immersion in methyl alcohol for five minutes rather than by heat.

When particles of mucus are found in the fecal discharge they should be washed gently in sterile water, spread on a slide, dried, fixed, and stained. In Asiatic cholera the common bacillus can often be found in immense numbers in this way.

In some pathologic conditions the character of the intestinal flora changes so that, even in adults, gram-staining bacteria very greatly predominate. This change is sometimes striking in cases of cancer of the stomach owing to large numbers of Boas-Oppler bacilli, and is of some value in diagnosis. A "gram-positive stool," due to predominance of cocci, is suggestive of intestinal ulceration.

In cases of excessive intestinal putrefaction it is sometimes possible to check the putrefactive process by substituting for the putrefactive bacteria an acid-producing species such as is normal in the intestine of nursing infants. For this purpose the gram-positive *Bacillus acidophilus* is now generally employed. Milk cultures of this organism may be administered, or a food may be given which causes great multiplication of the bacilli of this type which are already present in the intestine. The change from gram-negative to gram-positive stool may be observed by counting the proportions of the two types of bacilli in film preparations stained by Gram's method. The technic is the same as when Gram's method is applied to other material (p. 780). Pyronine is a good counterstain. The deep purple gram-staining bacteria stand out more prominently than the pale red gram-negative organisms, and one may be misled into thinking them more numerous, even in cases in which they are much in the minority.

Owing to the difficulty of excluding swallowed sputum the presence of the **tubercle bacillus** is less significant in the feces than in other material. It may, however, be taken as evidence of intestinal tuberculosis when clinical signs indicate an intestinal lesion and reasonable care is exercised in regard to the sputum. Success in the search will depend largely upon careful selection of the portion examined. A random search will almost surely fail. Whitish or grayish flakes of mucus or blood-stained or purulent particles should be spread upon slides or covers and stained by the method given on page 156. In the case of rectal ulcers swabs can be made directly from the ulcerated surface. With young children, who swallow all their sputum, an examination of the stool for tubercle bacilli may be the means of diagnosing tuberculosis of the lung.

Bacteria of the **typhoid-dysentery group** are frequently sought as a means of diagnosis of intestinal disorders or as a means of detecting carriers. These bacteria can be found and identified only by cultural methods, which may be carried out as follows:

Mix a small quantity of the feces with about ten times its volume of sterile nutrient broth. Transfer a loopful of the suspension to a large Petri dish containing Endo medium, the surface of which must be dry, and make a series of streaks. Dilute the suspension 1 : 5, 1 : 20, 1 : 100, and 1 : 500, and streak a similar plate from each dilution. In acute dysentery select a particle of blood-stained mucus, wash in sterile water, and streak several freshly prepared plates. It is well to fit a piece of filter paper into the lid of the Petri dish to absorb moisture. Incubate twelve to twenty-four hours. Colon bacilli give red colonies on this medium and should be disregarded, although the red will not always appear in a short incubation period. If

gray colonies suggesting the typhoid-dysentery group are found, pick off a dozen or more; plant slants of nutrient agar, or better, Russell's double sugar agar, and incubate. Next day study the growth on these tubes, determine motility, make Gram stains, transfer to differential sugar media and, if possible, test agglutinability by immune serum (p. 662). This will usually suffice for identification. Should immune serum not be available, plant a series of sugar media, dextrose, lactose, saccharose, maltose, mannite, xylose, containing bromthymol blue as indicator. The cultural characteristics of the typhoid-dysentery group are given on page 798.

5. Yeasts and Molds.—Yeast cells, which show budding and form short chains, are often present in normal stools and may be very numerous in cases of intestinal fermentation. Sarcinae may be present under normal conditions. Molds are rare and are usually a contamination from unclean vessels or the air. The spores of molds as well as yeast may be mistaken for the cysts of intestinal protozoa.

In some cases of tropical sprue *Monilia psilosis*, a fungus related to the thrush fungus, has been isolated from the feces as well as from the lesions on the tongue. It is occasionally found in the stools of healthy persons. Ashford reported that sprue is an infection with *Monilia psilosis* upon a state of nutritional unbalance—chronic digestive incompetence—but the specificity of this organism as an etiologic factor has never been established. The organism is best found by making a series of streaks on Petri dishes of Sabouraud's glucose agar (p. 768). It forms raised, sharply outlined, shiny, creamy white colonies which are most characteristic after several days' growth.[1] Another culture medium found very useful in isolating this and similar organisms is the tartaric acid medium described on page 768.

A microscopic structure which is frequently found in feces, and the nature and significance of which have not been fully determined has been described under the name *Blastocystis hominis*. It appears to belong to the yeasts or molds. Lynch[2] has found it in the feces of more than 40 per cent of hospital patients in Charleston, S. C. It is a colorless, round, sometimes oval body, 5 to 15 μ in diameter, or, when actively growing, even 25 μ, and superficially resembles the cysts of some of the intestinal protozoa. It consists of a large central clear body surrounded by a narrow rim of cytoplasm containing a

[1] For details regarding *Monilia psilosis* and sprue see Ashford, B. K.: The Etiology of Sprue, Am. Jour. Med. Sci., *154*:157–176 (Aug.), 1917. Ashford, B. K. and Hernández, L. G.: Blood-serum Calcium in Sprue and Other Pathologic States in the Tropics, Am. Jour. Med. Sci., *171*:575–591 (Apr), 1926.

[2] Lynch, K. M.: Blastocystis hominis: Its Characteristics and Its Prevalence in Intestinal Content and Feces in South Carolina, Jour. Bacteriol., *2*:369–377 (July), 1917.

number of refractile spots or nuclei. The whole is surrounded by a delicate capsule (Fig. 209).

6. Animal Parasites and Ova.—Descriptions will be found in the following chapter. Only general considerations and methods of examination will be given at this place. Frequent use of the eyepiece micrometer is essential, since size is always an important consideration in the identification of parasites and ova.

Of all the **protozoa** which inhabit the bowel of the human being, only two, *Endamoeba histolytica* and *Balantidium coli*, are sufficiently harmful to warrant serious clinical consideration. Many authorities believe the better-known intestinal flagellates, *Giardia*, *Chilomastix*, and *Trichomonas*, to be entirely harmless. There is, however, clinical evidence to indicate that they may cause mild chronic diarrhea or at least aggravate an already existing inflammatory condition. They are rare in carnivorous animals, and Hegner therefore suggests a meat

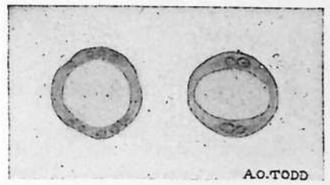


Fig. 209.—*Blastocystis hominis*, a peculiar structure related to the yeasts found in the feces; stained (× 1000) (after Lynch).

diet in the treatment of infection in man. He reports excellent results in a number of cases.

When protozoa are sought the feces should be obtained without admixture of urine or water. Urine might kill delicate organisms; water might introduce contaminations. In formed stools most protozoa appear in the encysted form, while the actively motile stages which are more easily recognized by the untrained worker, are ordinarily found only in liquid stools. For this reason it is advisable to give a saline cathartic, and to examine the first liquid stool. Castor oil is not satisfactory because the microscopic droplets of oil are confusing. Some experienced protozoologists prefer to make the diagnosis from the cysts, and advise against the use of a cathartic. Excepting when endamebae are in question it is not necessary to keep the stool warm, but it should not be cooled below room temperature. Examination should be made as soon as possible, preferably within a half hour, since parasitic protozoa in the vegetative or motile stage soon die and disintegrate. They do not encyst after leaving the body. Cysts

passed with the feces, on the other hand, remain unchanged for many days.

Not infrequently free-living protozoa are found multiplying in the feces (coprozoic protozoa). This may result from contamination after the feces are passed; or the protozoa may have been swallowed in the encysted stage with the food. Among these are free-living amebae, which can generally be distinguished from the parasitic species by the presence of a contractile vacuole, and by the continuance of motility for many hours after the stool has cooled. The most important coprozoic flagellates are *Cercomonas* and *Bodo* (p. 528), which were at one time regarded as parasites.

Special Methods for Protozoa.—While the finding of flagellates in feces is generally simple, their accurate identification is difficult. Much depends on the number and position of the flagella, but these are difficult to study in the living specimen. It may be impossible to see them at all until the parasite becomes less active as a result of exposure. Sometimes two or more flagella adhere together, with only the tips free, and thus appear as one. To bring them out more clearly Stitt recommended mixing a drop of Gram's iodine solution with the feces on the slide or, better, use of a dark-ground condenser.

At times, especially in formed stools, no active forms are present and only the cysts can be found. These may be very confusing to the inexperienced. Superficially they resemble yeasts or the spores of molds. To aid in their detection and differentiation Kofoid strongly recommends a modification of the iodine-eosin method originated by Donaldson:

Eosin, saturated solution in physiologic saline	2 c.c.
Iodine solution (physiologic saline, 100 c.c.; potassium iodide, 5 Gm.; iodine to saturation)	1 "
Physiologic saline	2 "

Break up a particle of the feces in a drop of physiologic saline on a slide, place near it a drop of the iodine-eosin solution, and apply a single large cover glass over both drops. Unstained portions can then be compared with portions showing various degrees of staining. In stained areas the fecal particles, bacteria, and most yeasts are pink, while protozoal cysts gradually take on a yellow to brown color and stand out distinctly, and their nuclei become more clearly defined. D'Antoni[1] recommended that a saturated solution of iodine be made up in a standardized 1 per cent solution of potassium iodide. This solution contains 0.8248 Gm. of iodine per 100 c.c. and does not stain as darkly as a stronger solution of iodine made with a 5 per cent solution of potassium iodide. After adding 1.5 Gm. of powdered iodine to 100 c.c. of a 1 per cent solution of iodide, the solution should be allowed

[1] D'Antoni, J. S.: Standardization of the Iodine Stain for Wet Preparations of Intestinal Protozoa. Amer. Jour. Trop. Med. *17*:79–84 (Jan.), 1937.

to stand four days and then filtered. It should be kept in a tightly stoppered bottle and should be discarded after four weeks of use. Vegetative forms of amebae may be readily recognized. They should be searched for first with the 16-mm. objective. When located, they are then studied with the 4-mm. objective. For a description of their appearance, see Chapter VII, pages 499–507. A mechanical stage should be used, and the entire area under the cover glass (usually 22 by 22 mm.) should be studied. Magath[1] has made a very interesting computation of the probabilities of finding a protozoon by this method. His calculations are presented in the following table.

PROBABILITIES OF FINDING A PROTOZOON IN A SPECIMEN OF STOOL

Concentration in stool, organisms per		Probability of finding a protozoon in the field examined,* fields examined (22 by 22 mm.).				
Gm.	20 mg.	¼ field (2½ mins.).	½ field (5 mins.).	1 field (10 mins.).	2 fields (20 mins.).	4 fields (40 mins.).
5	0.1	0.025	0.049	0.095	0.181	0.330
10	0.2	0.049	0.095	0.181	0.330	0.551
15	0.3	0.072	0.139	0.259	0.451	0.699
20	0.4	0.095	0.181	0.330	0.551	0.798
25	0.5	0.118	0.221	0.394	0.632	0.865
50	1.0	0.221	0.394	0.632	0.865	0.982
100	2.0	0.394	0.632	0.865	0.982	0.9997
150	3.0	0.528	0.777	0.950	0.998	
200	4.0	0.632	0.865	0.982	0.9997	
250	5.0	0.714	0.918	0.993	0.99995	
300	6.0	0.777	0.950	0.998		
350	7.0	0.826	0.970	0.9991		
400	8.0	0.865	0.982	0.9997		
450	9.0	0.895	0.989	0.99987		
500	10.0	0.918	0.993	0.99995		

* The calculations are based on the assumption that a single complete field contains 20 mg. of the specimen and that this is a random sample of the stool in which the organisms are mixed at random. If four fields are examined and no organism is found, there is "practical certainty" that there are fewer than fifty organisms per gram in the stool, or fewer than 2.5 organisms in 20 mg. of preparation. Similarly, if two fields are examined and no organisms found, it is "certain" that there are less than 100 organisms per gram, and so forth.

If two cover-glass preparations of the size specified are each examined for only five minutes, and if the slide is moved so that the entire area is examined, the probabilities are that, given properly selected material, an experienced person will find protozoa in a large majority of cases, if they are present.

For detection of *Giardia*, *Chilomastix*, and *Trichomonas*, when they cannot be found in the ordinary microscopic examination, Hegner recommends cultures in Hogue's ovomucoid medium. This is prepared by shaking

[1] Magath, T. B.: The Laboratory Diagnosis of Amebiasis, Clinical Lecture at Cleveland, Jour. Am. Med. Assn., *103*:1218–1224 (Oct. 20), 1934.

the whites of six eggs in a flask with glass beads, adding 600 c.c. of 0.7 per cent sodium chloride solution, heating over a water bath with stirring for from twenty to thirty minutes, and filtering through cotton with suction. The medium is then tubed and sterilized in an autoclave. A bit of feces about the size of a small pea is placed in a tube of this medium and incubated at 37° C. Flagellates in the motile stage should appear in from twenty-four to forty-eight hours.

For detailed study of flagellates and endamebae protozoologists now generally depend on stained preparations. Giemsa's stain may be used as described on page 642, or, better, one of the iron hematoxylin methods, of which the following is one of the simplest:

Heidenhain's Iron-Hematoxylin Stain.—This stain is by far the best devised to bring out nuclear structures. It has been modified so that it is possible to complete the procedure in one day.

Reagents Required.—(*a*) Heidenhain's hematoxylin. Dissolve 0.5 Gm. hematoxylin in 10 c.c. of alcohol; and add 90 c.c. of distilled water. Allow this mixture to "ripen" several weeks before using it.

(*b*) Schaudinn's solution. Make a saturated solution of bichloride of mercury. Take 2 parts of this solution and add 1 part of 95 per cent alcohol.

(*c*) Glacial acetic acid.

(*d*) Tincture of iodine.

(*e*) Alcohol solutions. Make up solutions containing 50 per cent alcohol, 70 per cent alcohol, and 95 per cent alcohol.

(*f*) Iron alum mordant. Make a 2 per cent solution of ferric ammonium sulfate.

(*g*) Solution of ammonium hydroxide.

(*h*) Acetone. A minute amount of eosin or malachite green may be added to a portion of acetone if a counterstain is desired.

(*i*) Xylol.

(*j*) Canada balsam.

Method.—1. Make a fixing solution by adding 2 c.c. of glacial acetic acid (*c*) to 50 c.c. of Schaudinn's solution (*b*).

2. Spread the feces evenly on microscopic slides with a wooden applicator, and place the slides in the fixing solution for thirty minutes. Use Coplin staining jars.

3. Wash in tap water for thirty minutes. Add a few drops of tincture of iodine (*d*) and rinse in tap water.

4. Dehydrate by placing the slides in staining jars, which contain 50 per cent alcohol (*e*), for five minutes. Transfer to 70 per cent alcohol for ten minutes, or longer. Transfer to 95 per cent alcohol for five minutes, then transfer back to 70 per cent alcohol for five minutes, and finally place in 50 per cent alcohol for five minutes.

5. Wash in 4 or 5 changes of tap water.

6. Place in 2 per cent iron alum mordant (*f*) for from thirty minutes to two hours.

7. Wash in several changes of water, the last washing being in distilled water.

8. Place in hematoxylin solution (*a*) for fifteen minutes, or longer.

9. Wash in tap water several times, and decolorize in iron alum solution (*f*) for about two or three minutes, and then examine the slide with a microscope. This part of the technic is most important and must be learned by experience. Keep the slides continually moist with the iron alum solution, and watch carefully for the appearance of the optimum amount of decolorization.

10. Wash in tap water, to which has been added 2 or 3 drops of ammonia (*g*).

11. Dehydrate in 50 per cent alcohol (*e*) for five minutes, then transfer to 70 per cent alcohol for five minutes, then place in 95 per cent alcohol for five minutes, and finally place in acetone (*h*) for five to ten minutes.

12. If a counterstain is desired, place the slides in another acetone solution, to which has been added a minute amount of eosin or malachite green and allow them to remain for thirty seconds.

13. Clear in xylol (*i*) for one or two minutes.

14. Mount immediately with Canada balsam (*j*) and long cover glasses before the slides dry.

Nearly all of the **intestinal worms** deposit ova which are characteristic of the species and which, if abundant, can be found without difficulty by direct microscopic examination. Ova of *Strongyloides* ordinarily hatch in the intestine and only the larvae appear in the feces. Ova of *Taenia saginata* for the most part pass out while still enclosed within the segments of the worm, but a variable number from disintegrated segments can usually be found in the feces. Ova, which are likely to be encountered in this country, are shown in Plate XII.

The error of mistaking vegetable cells for the eggs of parasites is needlessly frequent when one can so easily familiarize oneself with the appearance of vegetable cells by study of feces of persons on a mixed diet. When there seems room for doubt, the structure in question is probably not an ovum. Ova of a given species are fairly uniform in size and nearly always typical in appearance, hence measurement and comparison with the plates should make identification easy. Only very rarely need there be any real difficulty, as in the case of a very atypical unfertilized egg of the common round worm (Fig. 234).

Concentration of Ova.—To find ova when scarce they must be concentrated. Hookworm ova are easily demonstrated with Pepper's method which is described on page 571. Various methods have been used to demonstrate other ova and protozoan cysts. In some of these methods the speci-

men is washed repeatedly with water, is allowed to settle between washings, and the final sediment is examined with the microscope. Some workers use solutions, such as brine, which have a high specific gravity, to float the ova to the surface, from which they are skimmed and placed on a slide. The simplest and most effective method is the one which has been described be De Rivas.[1]

De Rivas' Concentration Method.—Place 1 Gm. of feces in a medium size test tube. Add 5 c.c. of 5 per cent acetic acid solution. If necessary break up the material with a wooden applicator or place a few glass beads in the tube. Close the tube with a rubber stopper and shake the mixture forcibly for half a minute. Let the mixture stand for half a minute. The coarse particles will rapidly sink to the bottom. Remove the supernatant homogeneous suspension with a pipet, or filter it off through a double layer of gauze into a centrifuge tube. Add an equal portion of ether. Stopper the tube and shake the mixture forcibly for a few seconds. Centrifugalize the mixture for a few minutes. There will be formed four distinct layers. The ethereal extract at the top may be removed and used for testing for occult blood (see p. 475). Below the ether will be a detritus plug, composed of layers of bile, soaps, and protein matter. Underneath this will lie the acetic acid solution, and in the tip of the tube will be found a very small amount of sediment. Collect this sediment in a capillary pipet, place it on a microscope slide, cover with a cover glass, and examine with a microscope for ova, cysts of protozoa, and all organized and formed bodies.

Faust and Tobie Concentration Method.—Faust, D'Antoni, Odom, Miller, Peres, Sawitz, Thomen, Tobie, and Walker[2] made a critical study of various technics for the diagnosis of protozoan cysts and helminth eggs in feces. They found that the largest number of cysts were recognized by using a direct fecal film stained with iodine or fixed and stained with iron hematoxylin combined with the zinc sulfate centrifugal flotation method performed as follows: Take a small lump of feces, about 5 Gm., and dilute it five times by mixing it thoroughly with about 20 c.c. of warm water. Strain 10 c.c. of the mixture through one layer of wet cheese cloth in a small funnel into a small test tube. Centrifuge the tube forty-five to sixty seconds at top speed. Pour off the wash water and add more water; break up the sediment and wash rapidly in this manner three or four times more until the supernatant fluid is clear after short, rapid centrifugation. Pour off the last wash water and add 3 to 4 c.c. of a 33 per cent solution of zinc sulfate (specific gravity 1.180). Break up the packed sediment and add the solution of zinc sulfate to within $\frac{1}{2}$ inch of the rim of the test tube. Centrifuge for forty-five

[1] De Rivas, Damaso: An Efficient and Rapid Method of Concentration for the Detection of Ova and Cysts of Intestinal Parasites, Am. Jour. Trop. Med., *8*:63–72 (Jan.), 1928.

[2] Faust, E. C, D'Antoni, J. S., Odom, Vada, Miller, M. J., Peres, Charles, Sawitz, Willi, Thomen, L. F., Tobie, John and Walker, J. H.: A Critical Study of Clinical Laboratory Technics for the diagnosis of Protozoan Cysts and Helminth Eggs in Feces. Amer. Jour. Trop. Med. *18*:169–183 (Mar.), 1938.

to sixty seconds at top speed. Remove several loopfuls of the material floating on the surface and place on a clean slide. Add one drop of iodine solution (D'Antoni's stain, p. 488). Cover the material with a cover glass.

IV. FUNCTIONAL TESTS

1. Schmidt's Test Diet.—Much can be learned of the various digestive functions from a microscopic study of the feces, especially when the patient is upon a known diet. For this purpose the standard diet of Schmidt is generally adopted. This consists of:

Morning.....0.5 liter milk and 50 Gm. toast.
Forenoon.....0.5 liter porridge, made as follows: 40 Gm. oatmeal, 10 Gm. butter, 200 c.c. milk, 300 c.c. water, one egg, and salt to taste.
Midday.....125 Gm. hamburger steak, with 20 Gm. butter, fried so that the interior is quite rare; 250 Gm. potato, made by cooking 190 Gm. potato with 100 c.c. milk and 10 Gm. butter, the whole boiled down to 250 c.c.
Afternoon.....Same as morning.
Evening.....Same as forenoon.

At the beginning of the diet the stool should be marked off with carmine or charcoal (p. 475). One should familiarize himself with the feces of normal persons upon this diet. A portion of the stool about the size of a walnut should be rubbed up with water to a consistency of thick soup, and examined macroscopically and microscopically. The microscopic examination may be facilitated by preparing four slides: One of the diluted feces untreated; one treated with a drop of dilute Lugol's solution; one with 30 per cent acetic acid; one with sudan III.

Deficiency of starch digestion is recognized by the number of starch granules which strike a blue color with iodine. With exception of those inclosed in plant cells none is present normally.

The degree of protein digestion is ascertained by the appearance of the muscle fibers. Striations are clearly visible on any considerable number of the fibers only when digestion is imperfect (Fig. 207). They are most clearly seen in the acetic-acid preparation. The striations usually disappear after the feces have stood for some time. According to Schmidt, the presence of nuclei in muscle fibers denotes complete absence of pancreatic function. The presence of connective-tissue shreds is generally believed to indicate deficient gastric digestion, since it has been accepted that raw connective tissue is digested only in the stomach. Recent work of J. Buckstein, however, indicates that it may, to some extent at least, be digested in the small intestine. These shreds can be recognized macroscopically by examining in a thin layer against a black background, and microscopically by their

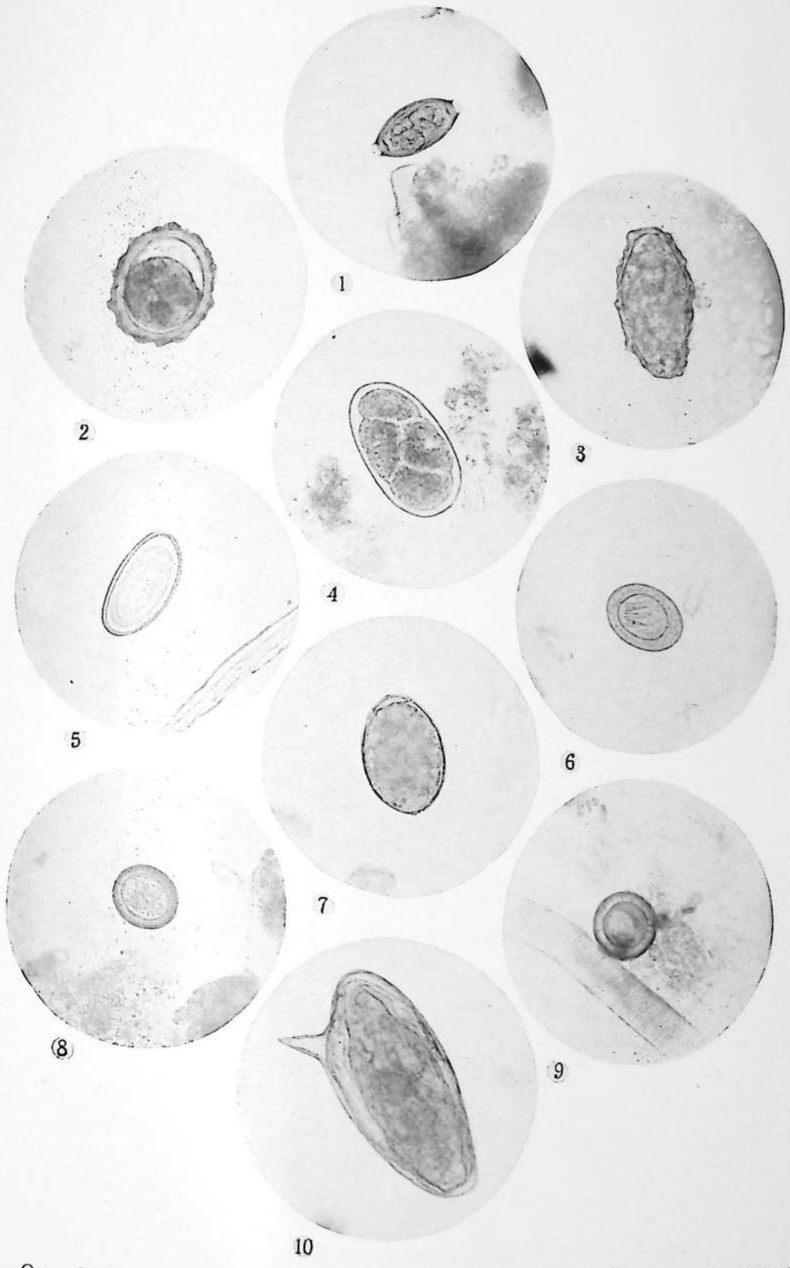
fibrous structure and the fact that they swell up and become clear and gelatinous when treated with acetic acid. The only structure likely to cause confusion is elastic tissue, and this is rendered more distinct by acetic acid.

Digestion of fats is checked up by the amount of neutral fat, which should not be present in appreciable quantity normally. It is best seen after staining with sudan III.

Schmidt's nuclei test for pancreatic insufficiency consists in the administration of a 0.5-Gm. cube of beef or, better, of thymus, tied in a little gauze bag with the test meal. The meat must previously have been hardened in alcohol and well washed in water. When the bag appears in the feces it is opened and its contents examined microscopically by pressing out small bits between a slide and cover. A drop of some nuclear stain may be applied if desired. If the nuclei are for the most part undigested, pancreatic insufficiency may be assumed, since it is probable that nuclei can be digested only by the pancreatic juice. Normally the nuclei are digested, provided the time of passage through the intestine is not less than eight or ten hours. Upon the other hand, if the time of passage exceeds thirty hours, nuclei may be partially digested in the complete absence of pancreatic juice.

2. Motility.—Ordinarily, with adults who are upon a mixed diet, twenty to thirty-six hours are required for the passage of ingested material through the gastro-intestinal tract. There is considerable variation among individuals, and usually not all of the residue from a single meal is evacuated at the same time. With infants the time is about one third as long. In diarrheal conditions it is usually much shortened. In intestinal stasis it may be much prolonged. The time of passage is ascertained by giving 0.5 Gm. of powdered charcoal or 0.3 Gm. of carmine in a capsule with a meal and watching for the resulting discolored feces. Alvarez and Friedländer gave a capsule containing fifty colored beads about 2 mm. in diameter. On the average about 15 per cent of the beads were recovered by the end of the first day, 40 per cent on the second, and 15 per cent on the third, while the others remained four or five days or even a week. This method has been criticized, on the ground that beads may be delayed by lodging in the stomach or in some pocket of the colon.

PLATE XII



Ova which may be found in the feces, showing comparative size (photographs, × 250); 1, *Trichuris trichiura* (whip-worm); 2, *Ascaris lumbricoides* (round-worm), fertilized; 3, *Ascaris lumbricoides*, unfertilized; 4, *Necator americanus* (hookworm), four-cell stage; 5, *Enterobius vermicularis* (pin-worm); 6, *Hymenolepis nana* (dwarf tapeworm); 7, *Diphyllobothrium latum* (fish tapeworm), the edge of the lid being out of focus; 8, *Tænia saginata* (beef tapeworm); 9, *Tænia solium* (pork tapeworm) lying beside a striated muscle-fiber; 10, *Schistosoma mansoni* (blood-fluke).

CHAPTER VII

ANIMAL PARASITES

ANIMAL parasites are common in all countries, but are especially abundant in tropical and subtropical countries, where, in some localities, almost every native is host for one or more species. Because of our growing intercourse with these regions, and because of our rapidly growing knowledge of parasitology, the subject is assuming increasing importance in this country. Many parasites, hitherto comparatively unknown here, will probably become fairly common.

Some parasites produce no symptoms even when present in large numbers. Others cause very serious symptoms. It is, however, impossible to make a sharp distinction between pathogenic and nonpathogenic species. Parasites which cause no apparent ill effects in one individual may, under certain conditions, produce marked disturbances in another. The disturbances are so varied, and frequently so indefinite, that diagnosis can rarely be made from the clinical symptoms. It must rest on detection, by the naked eye, the microscope, or other means of (*a*) the parasites themselves, (*b*) their ova or larvae, or (*c*) in a few cases, certain phenomena which depend on the reaction of the body tissues to the parasite, as complement fixation in echinococcus disease.

Unlike bacteria, the great majority of animal parasites multiply by means of alternating and differently formed generations, which require widely different conditions for their development. The exceptions are chiefly among the protozoa. Multiplication of parasites within the same host is thus prevented, as well as direct transmission from man to man. In the case of the hookworm, for example, there is no increase in the number of worms in the host's intestine except through reinfection from the outside. The ova are carried out of the intestine and the young must pass a certain period of development in warm, moist earth before they can again enter the human body and grow to maturity. This also explains the geographic distribution of parasites. The hookworm cannot flourish in cold countries; malaria can prevail only in localities in which the mosquito, *Anopheles*, exists, and then only after the mosquitoes have become infected from a human being.

In general, this alternation of periods of development takes place in one of three ways:

1. The young remain within the original host, but travel to other organs, where they do not reach maturity, but lie quiescent until taken in by a new host. A good example is *Trichinella spiralis*.

2. The young or the ova which subsequently hatch pass out of the host, and either (*a*) go through a simple process of growth and development before entering another host, as is the case with the hookworm, or (*b*) pass through one or more free-living generations, the progeny of which infect new hosts, as is the case with *Strongyloides stercoralis*.

3. The young or ova or certain specialized forms either directly (malarial parasites) or indirectly (tapeworms) reach a second host of different species, where a widely different process of development occurs. The host in which the adult or sexual existence is passed is called the *definitive* or final host; that in which the intermediate or larval stage occurs, the *intermediate* host. Man, for example, is the definitive host for *Taenia saginata*, and the intermediate host for the malarial parasites and *Taenia echinococcus*.

In the case of certain worms in which true alternation of generations is lacking, the ova pass out of the host and undergo a process of maturation before they become infective. Eggs of *Ascaris lumbricoides*, for example, are incapable of hatching for a month after they have left the host.

At this place a few words concerning the classification and nomenclature of living organisms in general will be helpful. Individuals which are alike *in all essential respects* are classed together as a *species*. Closely related species are grouped together to form a *genus;* genera that have certain characteristics in common make up a *family;* families are grouped into *orders;* orders, into *classes;* and classes, finally, into the *branches* or *phyla*, which make up the animal and vegetable *kingdoms*. In some cases these groups are subdivided into intermediate groups—subclasses, subfamilies, and occasionally slight differences warrant subdivision of the species into *varieties*. The name of the family ends in *-idae*, and that of a subfamily in *-inae*.

The scientific name of an animal or plant consists of two parts, both Latin or latinized words, and is printed in italics. The first part is the name of the genus and begins with a capital letter; the second is the name of the species and begins with a lower case letter, even when it was originally a proper name. When there are varieties of a species, a third part, the designation of the variety, is appended.

The author of the name is sometimes indicated in Roman type immediately after the name of the species. Examples: *Borrelia vincenti*, often abbreviated to *B. vincenti* when the genus name has been used just previously; *Staphylococcus pyogenes albus; Necator americanus*, Stiles.

The number of parasites which have been described as occurring in man and the animals is extremely large. Only those which are of medical interest are mentioned here. Some knowledge of their morphology, life histories, and means of dissemination, as well as the methods of diagnosis, is indispensable for the present-day physician. They belong to four phyla—Protozoa, Platyhelminthes, Nemathelminthes, and Arthropoda.

PHYLUM PROTOZOA

These are unicellular organisms, the simplest types of animal life. There is very little differentiation of structure. Each contains at least one, and some several, nuclei. Some contain contractile vacuoles; some have cilia or flagella as special organs of locomotion. They reproduce by division, by budding, or by sporulation. Sometimes there is an alternation of generations, in one of which sexual processes appear, as is the case with the malarial parasites. The protozoa are very numerous, the class Sarcodina alone including no less than 5000 species. Most of the protozoa are microscopic in size; a few are barely visible to the naked eye. The beginning student can gain a general idea of their appearance by examining water (together with a little of the sediment) from the bottom of any pond. Such water usually contains amebae and considerable variety of ciliated and flagellated forms.

The following is an outline of those protozoa which are of medical interest, together with the classes and subclasses to which they belong:

PHYLUM PROTOZOA

CLASS I. **SARCODINA.**—Locomotion by means of pseudopodia.

SUBCLASS **Rhizopoda.**—Pseudopodia form lobose or reticulose processes.

Genus	*Species*
Endamoeba.	*E. histolytica.*
	E. coli.
	E. gingivalis.
Dientamoeba.	*D. fragilis.*
Endolimax.	*E. nana.*
Iodamoeba.	*I. williamsi.*

CLASS II. **MASTIGOPHORA (FLAGELLATA).**—Locomotion by means of flagella.

SUBCLASS **Zoomastigophora.**—Forms in which animal characteristics predominate.

Genus	*Species*
Borrelia. (Classified by some parasitologists among the treponemata.)	*B. recurrentis.*
	B. vincenti.
	B. refringens.
	B. bronchiale.
Leptospira.	*L. icterohaemorrhagiae.*
	L. icteroides.
Treponema.	*T. pallidum.*
	T. mucosum.
	T. microdentium.
	T. pertenuis.
Trypanosoma.	*T. gambiense.*
	T. rhodesiense.
	T. cruzi.
	T. lewisi.
Leishmania.	*L. donovani.*
	L. tropica.
	L. infantum.
Trichomonas.	*T. hominis.*
	T. vaginalis.
	T. buccalis.
Chilomastix.	*C. mesnili.*
Giardia.	*G. lamblia.*

CLASS III. **SPOROZOA.**—All members parasitic. Propagation by means of spores. No special organs of locomotion.

SUBCLASS **Telosporidia.**—Sporulation ends the life of the individual.

Genus	*Species*
Isospora.	*I. hominis.*
Plasmodium.	*P. vivax.*
	P. malariae.
	P. falciparum.
Babesia.	*B. bigeminum.*

CLASS IV. **INFUSORIA.**—Locomotion by means of cilia.

SUBCLASS **Ciliata.**—Cilia present throughout life.

Genus	*Species*
Balantidium.	*B. coli.*
	B. minutum.

CLASS SARCODINA

SUBCLASS RHIZOPODA

These are protozoa the body substance of which forms changeable protoplasmic processes, or pseudopodia, for the taking in of food and for locomotion. They possess one or several nuclei. Their usual habitat in the body is the mouth and the intestine, although they are occasionally found in other situations. Only one species, *Endamoeba histolytica*, is definitely known to be pathogenic, but some degree of familiarity with a number of others is necessary in order to avoid confusion.

1. Genus Endamoeba.—(1) **Endamoeba histolytica.**—This organism is found, often in large numbers, in the stools of tropical dysentery, and in the pus and walls of hepatic abscesses associated with dysentery. It has also been found on occasions in duodenal contents, sputum, seminal fluid, urine, synovial fluid, and elsewhere. Infection is more common in this country than was at one time supposed, and is not at all rare in the Northern States, where it generally produces mild chronic dysentery, or, in slight infections, no definite symptoms at all. Records of The Mayo Clinic show cases from all of the Northern States, and from several provinces of Canada. Surveys made at the training camps during the World War indicate about 10 per cent of infection among the general population.

Like most of the intestinal protozoa, *E. histolytica* is found in the feces in two phases of its life history, its vegetative and encysted stages.

In the vegetative stage, which is found in acute dysentery, and also in quiescent cases when a liquid stool is obtained by catharsis, the parasite is a grayish or colorless, granular, motile cell, usually between 20 and 40 μ in diameter. There appear to be several races which differ only in size. Within a given race the size of individuals is fairly uniform, so that, in any particular sample of feces, the range in size is much less than is indicated by the above figures. The ectoplasm, seen most clearly as a broad zone at the ends of the pseudopodia, is homogeneous and refractile. The endoplasm is very granular and contains one small round very indistinct nucleus, and usually one or more digestive vacuoles with red blood corpuscles when blood is present in the stool. The presence of ingested red blood corpuscles is an extremely valuable diagnostic point, since these are seldom taken in by the nonpathogenic endamebae. Fragments of leukocytes and other cells may also be present. There is no contractile vacuole, a fact which serves to distinguish this and the other forms which inhabit the intestine of man from the free-living amebae which occasionally reach the feces as contaminations.

In cases of bacillary dysentery, certain large, phagocytic and vacuolated body cells (macrophages, p. 483) might be mistaken for endamebae. Some of these even contain red corpuscles. In general, however, they lack the glassy luster which *E. histolytica* shows. Moreover, the nuclei of body cells are much larger and more distinct.

not be kept warm. It should be thinned with water or 5 per cent formalin, and strained through cheesecloth or a sieve (Fig. 211) to remove coarse particles. It is then centrifugalized and the sediment washed several times with water or 5 per cent formalin to remove the innumerable bacteria which would otherwise cloud the field. The cysts are found without difficulty with the 16-mm. objective, and are then studied in detail with the 4-mm. objective. They are colorless, refractile, spheric bodies, 7 to 15 μ in diameter, with a clean-cut edge; and with the low power look not unlike colorless oil globules. When fully developed they contain four small nuclei. The nuclei lie at different levels and are usually not very distinct, but can generally be made out by careful focusing with the 4-mm. objective and reduced diaphragm opening. Young cysts, with one or two nuclei, contain several highly refractile, colorless granules of chromatin (chromidia), and a small amount of glycogen. A trace of Lugol's solution or of iodine-eosin (p. 488) added to the fecal material on the slide brings out the structure more clearly.

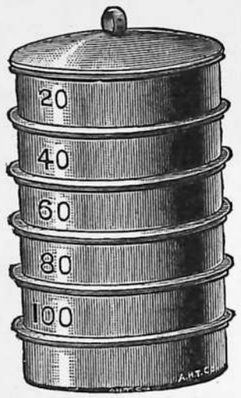


Fig. 211.—Nest of 5-inch sieves of graduated mesh, with cover and receiving pan. These are very useful for fecal examinations. The sieves may be obtained separately.

Encystment is a means of resisting unfavorable conditions, and of dissemination. At this stage alone is the organism infective. Boeck found the maximal length of life of cysts, in water, to be 153 days, and the thermal death point to be 68° C. The cysts reach a new host chiefly through the food, to which they are carried from the feces largely by house flies. They also have been transmitted undoubtedly by a contaminated water supply. The danger of "cross connections" in plumbing cannot be overemphasized from the standpoint of sanitary engineering. Patients with active dysentery are of little importance in transmitting the disease, since the vegetative forms die, without encystment, soon after leaving the host.

(2) **Endamoeba Coli.**—This organism is generally accepted as nonpathogenic, although some authorities believe that it may occasionally take on mildly pathogenic properties when local resistance is lowered. It is found on every continent, and surveys indicate that it is present in the stools of about 20 per cent of all persons. While it is thus less frequent than *Endolimax nana*, it has been known for a longer time, and has been more thoroughly studied. In general

appearance it resembles *E. histolytica*, but averages somewhat smaller, seldom exceeding 25 μ in diameter. It has, moreover, less distinct pseudopodia, less sharp differentiation between ectoplasm and endoplasm, much less active motion, and a much more distinct nucleus. Its food vacuoles contain bacteria and miscellaneous particles picked up from the feces, but hardly ever red blood corpuscles.

Under appropriate conditions the organism forms cysts similar to those of *E. histolytica*. Fully developed cysts (Fig. 212) are 15 to 22 μ

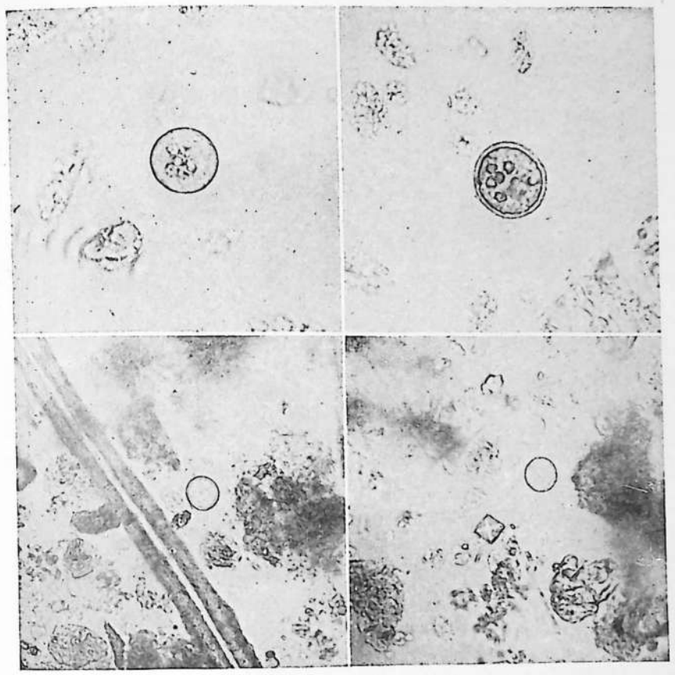


Fig. 212.—Photographs of cysts of *Endamoeba coli*. Above, two cysts as seen with oil-immersion objective and low-power eyepiece, one slightly tinged with iodine, one deeply stained with iodine and showing nuclei and double wall (× 525); below, two unstained cysts as seen with 4-mm. objective and low-power eyepiece, one lying beside a vegetable spine, one beside a small calcium oxalate crystal (× 250).

in diameter, averaging about 18 μ. They are colorless and highly refractile, with a glassy or, more accurately, a porcelain-like luster, and contain 8 to 16 small nuclei which lie at different levels, and are seen only by careful focusing. The cyst wall is well defined, and usually has a double contour. Their circular shape, sharp outline, and peculiar luster make the search for these cysts with the low power easy.

The principal points of distinction between *E. histolytica* and *E. coli* and *Endolimax nana* are included in the table on pages 506 and 507.

(3) **Endamoeba Gingivalis.**—That endamebae are common in the mouth and about the teeth has long been recognized, but they have generally been regarded as harmless or even as beneficial because they feed extensively upon bacteria. There is apparently only one species, which has been variously called *E. buccalis*, *E. dentalis*, and *E. gingivalis*, the last name being now accepted as correct. In some

Fig. 213.—*Endamoeba gingivalis*, pus corpuscles, red blood cells, spirochetes, and bacteria in a smear from a lesion of pyorrhea alveolaris. Giemsa's stain, without alkali twelve hours (× 850). The figure shows three endamebae, each with one round nucleus (red). The cytoplasm (deep sky blue) contains vacuoles and bacteria. The largest parasite contains ten nuclei (blackish purple) from ingested cells. A digestion vacuole is seen at each end of the long bacillus in the endameba near the bottom. The red corpuscles were salmon colored; nuclei of leukocytes, reddish purple; spirochetes, bluish purple.

parts of the slide from which Fig. 213 was made there were as many as 20 in a single field of the oil-immersion lens. It is always accompanied by myriads of bacteria, often by "spirochetes," and sometimes by other protozoa, and the rôle which each plays is difficult to determine.

Material is obtained for study by scraping between the teeth and the gum with a sterile wooden toothpick. When pus pockets exist the bottom and side of a pocket should be scraped with a dental scaler. This material may be examined in the fresh state by mixing

it with a little saliva and placing on a warmed slide. The organism is less active than *E. histolytica*, more so than *E. coli*. Unless motion is seen it will be difficult to recognize. Individuals range in size from 10 to 35 μ. *E. gingivalis* apparently forms cysts very rarely. Those which have been seen resembled cysts of *E. histolytica*.

In general, these endamebae are more easily identified in stained smears. The smears are made by streaking the toothpick three or four times across the slide. Often one of the streaks will contain many of the parasites and the others only a few. Giemsa's solution, applied as described for blood (p. 255), but allowed to act for three to twelve hours, is the most satisfactory stain. With this the cytoplasm of endamebae is blue, and shows the vacuoles clearly, the small round nucleus is red, ingested bacteria purple, and nuclei of ingested cells deep purple. In such preparations it is well-nigh impossible to mistake pus and epithelial cells for endamebae. Wright's stain gives a similar picture, but the differentiation is somewhat less sharp.

2. Genus Endolimax.—*Endolimax nana* is a small endameba which is now recognized as a frequent inhabitant of the normal colon. Kofoid found it in 28 per cent of American soldiers. It is 6 to 12 μ in diameter, is sluggishly motile, and never contains red blood corpuscles. In preparations stained with iron hematoxylin the nucleus is the most characteristic feature (Fig. 210, and the table on p. 506). The cysts are ovoid, about 7 to 9 μ in diameter, and usually contain one or two very small, faintly visible nuclei, although there are four when fully developed.

3. Genus Iodamoeba.—*Iodamoeba williamsi* resembles very small specimens of *Endamoeba coli*. It is about 9 to 13 μ in diameter, is sluggishly motile, and contains a small indistinct nucleus. As a rule, it dies very soon after leaving the intestine. The cysts were known as "iodine cysts" before their nature was recognized. They are spheric, ovoid, or irregular, 9 to 12 μ in diameter, and contain a single nucleus and a large, sharply defined mass of glycogen, which stains dark brown with iodine. Glycogen is usual in the young cysts of most endamebae, but the bodies are not so large or so sharply defined as in this case, and rarely persist in the mature cyst. Iodamoeba is harmless and rare.

4. Genus Dientamoeba.—*Dientamoeba fragilis*, also nonpathogenic, is the smallest of the amebae of the human intestine, usually about 8 μ in diameter. In its vegetative stage it possesses two very small nuclei, is actively motile, and has sharp demarcation between ectoplasm and endoplasm. It is very rare and degenerates quickly after leaving the body. The cysts have been described by Kofoid.

CHARACTERISTICS OF THE THREE IMPORTANT INTESTINAL AMEBAE

Vegetative Stage

Found in diarrheal stools and in liquid stools following a saline cathartic.

Endamoeba histolytica.	*Endamoeba coli.*	*Endolimax nana.*
Pathogenic.	Nonpathogenic.	Nonpathogenic.
Size varies with different races of the parasite, between 20 and 40 μ.	Size about the same, 20 to 30 μ.	Very small, 6 to 12 μ, average 8 μ. Important.
Ameboid motion very active in fresh feces. Parasite moves from place to place. Very characteristic.	Motion sluggish. Parasite rarely moves from place to place.	Motion moderately active in very fresh feces, but soon lost.
Ectoplasm hyaline, refractile sharply differentiated from endoplasm. Characteristic.	Ectoplasm slightly refractile and poorly differentiated from endoplasm.	Ectoplasm and endoplasm not sharply differentiated.
Red blood cells present in endoplasm when stool contains blood. Practically diagnostic. Few or no ingested bacteria.	Endoplasm contains many bacteria and fecal particles, but very rarely a red blood cell.	Endoplasm contains bacteria, but no red blood cells.
Nucleus in living specimen indistinct, often invisible.	Nucleus distinct.	Nucleus indistinct.
Nucleus in specimens stained with iron hematoxylin shows scanty chromatin arranged in a thin granular ring with a small granule (karyosome) in the center. Diameter 4 to 7 μ (Fig. 210).	Nucleus richer in chromatin and has a thicker peripheral ring of large granules. Karyosome larger and eccentrically placed. One or more granules of chromatin usually present between karyosome and peripheral ring. Diameter 4 to 7 μ (Fig. 210).	Nucleus very small, 1 to 3 μ. Chromatin arranged in several small masses connected by bands (Fig. 210).

5. Other Endamebae.—Under the name *Councilmania lafleuri*, Kofoid and Swezy recently described an exceptionally actively motile endameba which ingests red blood corpuscles and is probably pathogenic. Its cysts are very thick-walled, ovoid, 16 to 20 μ in diameter, and contain eight nuclei. It is not accepted by all protozoologists.

Brumpt described *Endamoeba dispar*, which he claimed was a new species found in the feces of man. He stated that it had four daughter-cell cysts, did not ingest red blood cells, and was nonpathogenic for man, but mildly pathogenic for kittens. This ameba is not generally accepted, being considered as a less virulent form of *E. histolytica* found in "carriers."

A number of similar organisms have been described as occurring in pus, and in ascitic and other body fluids, but it is probable that in many cases, at least, the structures seen were ameboid body cells.

Cystic Stage

The cysts are most frequently found in formed stools. If they can be properly studied they offer probably the best means of differentiating the species. They may be studied in the fresh condition with Gram's iodine, or iodine-eosin, or, better, in permanent preparations stained with iron hematoxylin (p. 490).

Endamoeba histolytica.	*Endamoeba coli.*	*Endolimax nana.*
Cysts spheric or slightly ovoid. Cyst wall single and delicate in young cysts; thicker and sometimes double outlined in older ones.	Similar, but double outline of wall more frequent and much more distinct. Entire cyst more refractile.	Cyst definitely ovoid, thin-walled.
Size differs with different races of the parasite, 7 to 15 μ.	Usually large, 15 to 22 μ.	Small, 7 to 9 μ.
Cytoplasm of 1- and 2-nucleated cysts granular, often with a poorly defined clear area which stains brown with iodine (glycogen). Presence of chromidia (brightly refractile spindle-shaped or irregular masses of chromatin) characteristic. Glycogen and chromidia disappear as cyst matures.	Similar, but glycogen more abundant and sharply defined when stained with iodine. Chromidia rare.	Glycogen sometimes present as a single mass, staining deeply with iodine. Chromidia absent.
Fully developed cysts contain four small nuclei seen by focusing at different levels.	Fully developed cysts contain eight to sixteen nuclei, eight being the normal number. The four-nucleate stage of developing cyst rarely seen.	Fully developed cysts contain four, very rarely eight, very small nuclei.
With iron hematoxylin the nuclei, although much smaller, show the same structure as in vegetative stage.	Structure of nuclei same as in vegetative stage.	Structure of nuclei same as in vegetative, but difficult to see owing to their small size.

Cultivation of Parasitic Endamebae.—Boeck and Drbohlav[1] developed a culture medium on which parasitic endamebae may be grown. Four eggs are broken carefully into a sterile flask containing beads, and shaken with 50 c.c. of sterile Locke's solution (p. 835). Slants are made in sterile test tubes, and inspissated at 70° C. and autoclaved at 15 pounds' pressure for twenty minutes. The slants are then completely covered with 8 parts sterile Locke's solution and 1 part of sterile inactivated human blood serum. In place of the blood serum, 10 per cent ascitic fluid in Locke's solution, or also dilute egg albumen in either Locke's or Ringer's solution, may be used. Dobell[2] suggested adding a 4-mm. loopful of rice starch to the fluid part of each tube of medium. The medium is inoculated with a loopful of feces containing endamebae. Multiplication of these organisms will be found to have taken place in twenty-four to forty-eight hours.

Cleveland and Collier[3] made a comprehensive study of slants of various culture media, which were covered with different liquids. These investigators found that the best slants were those which were made of Difco liver infusion agar. The culture medium is prepared by using 30 Gm. of Difco liver infusion agar and 3 Gm. of dibasic sodium phosphate (Na_2HPO_4) per liter of distilled water. The medium is sterilized in test tubes in the autoclave. The test tubes are removed from the autoclave and slanted. Other slants that are almost as satisfactory as these are those which are made by dissolving 80 Gm. of Difco Loeffler's dehydrated serum in a liter of distilled water at 42° C. The medium is first coagulated and then sterilized in the autoclave. After sterilization the slants should be covered with fresh serum-saline mixture, which is prepared by taking 1 part of sterile horse serum and adding 6 parts of sterile 0.8 per cent saline solution. A 5-mm. loopful of sterile rice flour is added to each tube. Cleveland and Collier also found that a 3 per cent solution of Difco hydrolyzed hemoglobin was a very satisfactory liquid to add to the slants, or to use as a liquid culture medium. This solution is sterilized in the autoclave and sterile rice flour is added subsequently.

Another excellent culture medium is one which has been proposed by Tsuchiya.[4] Ten Gm. of peptone, 3 Gm. of meat extract, and 3 Gm. of sodium chloride are mixed and sufficient distilled water is added to make 1 liter. The resulting solution has a *p*H of 6.8 to 7.4. This is placed in test tubes and sterilized in the autoclave. A starch-charcoal mixture is prepared by adding 2 parts of rice starch to 1 part of animal charcoal and triturating. This mixture is sterilized at 180° C. for forty-five minutes. A small amount

[1] Boeck, W. C., and Drbohlav, Jaroslav: Cultivation of *Endamoeba histolytica*, Am. Jour. Hyg., *5*:371–407 (July), 1925.

[2] Dobell, Clifford, and Laidlaw, P. P.: On the Cultivation of *Endamoeba histolytica* and Some Other Entozoic Amoebae, Parasitology, *18*:283–318 (Sept.), 1926.

[3] Cleveland, L. R., and Collier, Jane: Various Improvements in the Cultivation of *Endamoeba Histolytica*, Am. Jour. Hyg., *12*:606–613 (Nov.), 1930.

[4] Tsuchiya, H.: Further Studies on the Cultivation of *Endamoeba histolytica* and a Complement-fixation Test for Amebiasis, Jour. Lab. and Clin. Med., *19*:495–504 (Feb.), 1934.

of the starch-charcoal is then added to each test tube. This furnishes carbohydrate nutriment and is the most useful substance for the absorption of ammonia and hydrogen sulfide, which have a deleterious effect on the amebas. Tsuchiya advocated following the method of Hegner, in which the liquid stool is centrifugalized and the supernatant fluid is carefully decanted. The sediment is washed and centrifugalized at 1500 revolutions per minute for three minutes. The washing and centrifugalization should be repeated three or four times. Two loopfuls of sediment are planted in 8 c.c. of the rice-charcoal broth. Tsuchiya said that *E. histolytica* is the only organism which will grow in this culture medium in forty-eight hours.

Craig's Complement-fixation Method.—Craig[1] and others have reported a very significant percentage of positive results with a complement-fixation method for the demonstration of specific antibodies in the serum of patients who have amebiasis. The difficult part of the technic is the preparation of the *antigen*. One hundred and twenty tubes of cultures of actively growing organisms are required. All of the amebas at the junction of the liquid and the slant are withdrawn in a pipet, placed in a suitable centrifuge tube and centrifugalized. The sediment is extracted with seven and a half volumes of absolute alcohol at 37° C. for fifteen days. During this time the bottle is shaken several times daily. The mixture is filtered and extracted through fine filter paper. The antigenic properties of the extract are determined by titrating with the serum of a patient who has amebiasis. The freedom of the extract from hemolytic and anticomplementary properties should be proved. This extract, because of poor antigenic properties, must be used undiluted. Craig used the antihuman hemolytic system, and Tsuchiya employed a sheep cell system. While this test demonstrates the antigenic properties of *E. histolytica* in the host, and the antibody response, it is not suitable for routine clinical diagnosis, except possibly in the hands of an experienced serologist.

CLASS MASTIGOPHORA (FLAGELLATA)

Subclass Zoomastigophora

The protozoa of this class are provided with one or several whip-like appendages with lashing motion, termed flagella, which serve for locomotion and, in some cases, for feeding. They generally arise from the anterior part of the organism. Some members of the group also possess an undulating membrane—a delicate membranous fold which extends the length of the body and somewhat suggests a fin. When in active motion this gives the impression of a row of cilia. The flagellata do not exhibit ameboid motion, and, in general, maintain an unchanging oval or spindle shape, and contain a single nucleus. The cytoplasm contains numerous granules and, usually, several vacuoles, one

[1] Craig, C. F.: Amebiasis and Amebic Dysentery, Baltimore, Charles C. Thomas, 1934, 315 pp.

or more of which may be contractile. Encystment as a means of resisting unfavorable conditions is common.

Among the most important of the flagellates are the various spiral organisms commonly grouped under the name "spirochetes." Their classification and nomenclature are in an unsettled state. Bergey places them with the bacteria, in Order VI, as "protozoon-like" organisms (p. 809). Most of the organisms commonly called spirochetes are now classed with either the genus Borrelia, or Treponema, as the name Spirochaeta has been reserved for a different genus of spiral organisms.

Most of them break up into minute granules under certain conditions, each granule being capable of development into a new spirochete. In some cases at least the granules constitute the means of dissemination. Certain spirochetes are transmitted from man to man through the agency of an intermediate host; others by direct contact; still others by the air-borne infective granules.

For the study of the spiral micro-organisms no method is so satisfactory as direct observation of the living parasites by means of dark-ground illumination (p. 5), a method which has been utilized to the fullest extent by Noguchi. In stained preparations they lose many of their distinguishing characteristics, and even their form may be misleading, since they are distorted in the process of spreading the smear. Most of them stain poorly, and are likely to be overlooked in smears stained by the usual methods for bacteria. All, however, are stainable by the methods given for *Treponema pallidum* (p. 642).

1. Genus Borrelia.—The bacteriologists have placed the organisms here described in the genus *Borrelia*, while the parasitologists have been inclined to classify them in the genus *Treponema*. It is still an unsettled question whether they are animal or bacterial parasites. The mode of the transmission of the organism to man has provided an argument for their being protozoa.

(1) **Borrelia recurrentis.**—This "spirochete" was described by Obermeier as the cause of relapsing fever. It appears in the circulating blood during the febrile attack, and, unlike the malarial parasite, lives in the plasma without attacking the red corpuscles. The organism is an actively motile spiral, 15 to 20 μ long, with three to twelve wide, fairly regular turns. It can be seen in fresh unstained blood with a high dry lens, being located by the commotion which it creates among the red cells. For diagnosis thin films, stained with Wright's or some similar blood stain, are used (Fig. 214). In such preparations the spirals are not so regular.

The organisms of relapsing fever are disseminated by certain ticks, chiefly of the genus *Ornithodorus*, of which there are some 13 species.

At least seven of these have been cited as vectors of the spirochete causing relapsing fever in widespread areas in Africa, Asia, and the Americas.

It was believed that relapsing fever did not occur in the United States, but in 1915 Meader reported 5 cases which originated in Bear Creek Cañon, Colorado, and others have since been recognized in the same locality and in California. Spirochetes from one of these cases are shown in Plate XIII. Since then, this disease has attracted attention increasingly in North America. For a review of the reports of the spread of the disease as far north as British Columbia, the reader is referred to the interesting paper by Palmer and Crawford,[1] and Hearle.[2]

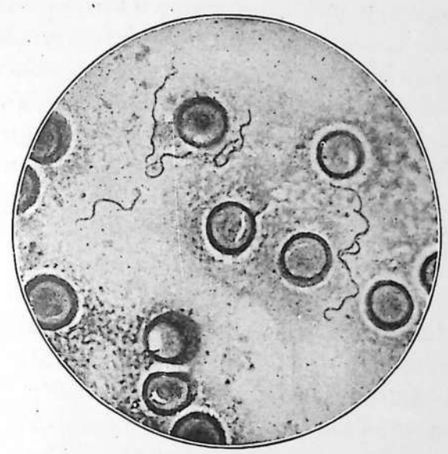


Fig. 214.—*Borrelia recurrentis* in the blood of a patient with relapsing fever (× 1000) (Karg and Schmorl).

Besides *Borrelia recurrentis*, a number of distinct strains have been described in connection with different types of relapsing fever in different localities: *B. novyi*, *B. kochi*, *B. duttoni*, and *B. carteri*.

Kemp, Moursund, and Wright[3] reported that Texas relapsing fever was caused by a spirochete proved serologically to be identical with

[1] Palmer, J. H., and Crawford, D. J. M.: Relapsing Fever in North America, with Report of Outbreak in British Columbia, Can. Med. Assn. Jour., *28*:643–647 (June), 1933.

[2] Hearle, Eric: Vectors of Relapsing Fever in Relation to an Outbreak of the Disease in British Columbia, Can. Med. Assn. Jour., *30*:494–497 (May), 1934.

[3] Kemp, H. A., Moursund, W. H., and Wright, H. E.: Relapsing Fever in Texas. I. The Identity of the Spirochete, Am. Jour. Trop. Med., *13*:425–435 (July), 1933. II. The Specificity of the Vector, *Ornithodorus turicata*, for the Spirochete, Am. Jour. Trop. Med., *14*:159–162 (Mar.), 1934.

B. novyi. In a later report they discuss the specificity of the vector, *Ornithodorus turicata*, a tick, for the spirochete of this form of relapsing fever, and the fact that it did not transmit laboratory strains of *B. novyi* in their experiments. This leaves the matter somewhat in doubt as to whether or not the Texas disease is caused by a separate species.

The history of tick-borne relapsing fever in the United States has progressed rapidly. Davis,[1] of the Rocky Mountain Laboratory, at Hamilton, Montana, has made a complete study of the relationship of ticks to relapsing fever. He reported six species of tick vectors, *Ornithodorus turicata*, *hermsi*, *parkeri*, *talaje*, *coriaceus*, and *coprophilus*. One or more of these have been reported from seventeen states from New York to California and from Washington to Florida. The disease itself has been reported in eleven states. *Ornithodorus parkeri* feeds readily on man and is the only known species in areas from which at least seventeen cases have been reported. One case of relapsing fever has been reported from California; in this case there was every evidence that disease had occurred following multiple bites by ticks of this species. (See page 579.) Davis stated that on the basis of a world wide survey of records, any species of Ornithodorus may be considered a potential vector of flagellates causing relapsing fever.

(2) **Borrelia vincenti.**—In stained smears from the ulcers of Vincent's angina (p. 614) are found what appear to be two organisms. One, the "fusiform bacillus," is a slender rod, 4 to 8 μ long, pointed at both ends, and sometimes curved and often lying in pairs end to end. The other is a slender spiral organism, 10 to 20 μ long, with three to ten comparatively shallow turns (Fig. 346, p. 641). The staining methods are given on pages 614, 642. The same organisms are quite constantly present in large numbers in ulcerative stomatitis, in noma, in erosive and gangrenous balanitis, and in certain leg ulcers in the tropics. Swabs from the surface of the lesions often show many other micro-organisms; deeper swabs generally show the spirochete, and the bacillus to dominate the picture. Unless both are present in considerable numbers they cannot be regarded as of etiologic significance. They are not infrequently found in small numbers in normal mouths.

[1] Davis, G. E.: Ticks in Relapsing Fever in the United States. Pub. Health Rep. *55*:2347–2351 (Dec. 20), 1940. *Ornithodorus turicata:* The Male; Feeding and Copulation Habits, Fertility, Span of Life, and the Transmission of Relapsing Fever Spirochetes. Ibid. *56*:1799–1802 (Sept. 5), 1941. *O. hermsi* and Relapsing Fever in Oregon. Ibid. *56*:2010–2012 (Oct. 10), 1941. *O. turicata* and Relapsing Fever Spirochetes in New Mexico. Ibid. *56*:2258–2261 (Nov. 21), 1941. Relapsing Fever: *O. parkeri*, a Vector in California. Ibid. *56*:2426–2428 (Dec. 19), 1941. *O. parkeri* and Relapsing Fever Spirochetes in Utah. Ibid. *56*:2464–2468 (Dec. 26), 1941.

2. Genus Leptospira.—This genus, created by Noguchi, includes the spirochete of infective jaundice.

Leptospira icterohaemorrhagiae is present in various organs, particularly the liver and kidneys, of persons suffering from infectious jaundice or Weil's disease. It averages 6 to 9 μ long, although there is considerable variation among individuals. There appear to be from two to five broad waves, but when examined closely, particularly by dark-ground illumination, its length is seen to be made up of a series of closely set coils. The disease is probably disseminated by rats, for the parasite has been found in the kidneys and urine of apparently healthy rats in many parts of the world, including the United States, although a form of infectious jaundice which is probably not of spirochetal origin is more frequent in the United States. The spirochetes are present in the blood early in the disease and may sometimes be found in blood films, although they are too scarce to make the method of value in diagnosis. They also appear in the urine where they may be very abundant during the third week, and can be found by centrifugalizing thoroughly at high speed and staining the sediment with any good spirochete stain. Since other spiral organisms are frequently present in the urine, one would have to be thoroughly familiar with the morphology of the spirochetes to make the diagnosis in this way. The most reliable means of diagnosis is inoculation of a light-skinned guinea-pig intraperitoneally with 5 c.c. of the patient's blood or urinary sediment. The animal usually dies in about ten days. Autopsy shows jaundice of the skin and widespread small hemorrhages into the tissues and organs. Larson[1] has reported that young white mice are extremely susceptible to infection with *L. icterohaemorrhagiae* when inoculated by intraperitoneal, subcutaneous or oral routes. They are, therefore, suitable animals for laboratory studies of leptospirosis. The same author stated that protective antibodies develop during the second week of the disease and persist for at least five years. He has devised a mouse protection test by using three-week-old white mice, inoculated with infectious material and with patient's serum, and he has found that this test is specific and the results are easily interpreted. Brown[2] has developed a rapid presumptive serologic test which may be carried out in about fifteen minutes with a properly prepared antigen.

[1] Larson, C. L.: Susceptibility of Young Mice (*Mus musculus*) to *Leptospira icterohaemorrhagiae*. Pub. Health Rep. *56*:1546–1556 (Aug. 1), 1941. A Protection Test in Mice for Identification of Leptospirosis icterohaemorrhagica (Weil's disease). *56*:1593–1609 (Aug. 8), 1941.

[2] Brown, H. C.: A Rapid Presumptive Serological Test for Weil's Disease. Brit. M. J. *2*:1183–1184 (Dec. 16), 1939.

3. Genus Treponema.—(1) **Treponema pallidum.**—This is the organism of syphilis. Its description and methods of diagnosis will be found on pages 641–644.

(2) **Treponema pertenue,** morphologically very similar to *Treponema pallidum*, was found by Castellani in yaws, a skin disease of the tropics.

4. Other "Spirochetes."—A number of harmless forms are of interest because of the possibility of confusing them with the more important pathogenic varieties. Of these, *Treponema mucosum* and *Treponema microdentium* are inhabitants of the normal mouth. When the teeth and gums are not in good condition they are often found in immense numbers (Fig. 213). The former is similar in morphology to *Borrelia vincenti*. *Treponema microdentium* (Fig. 215) is smaller (4 to 10 μ), more delicate, has deep curves, and may be easily mistaken for *Treponema pallidum*. It also stains reddish with Giemsa's stain. In

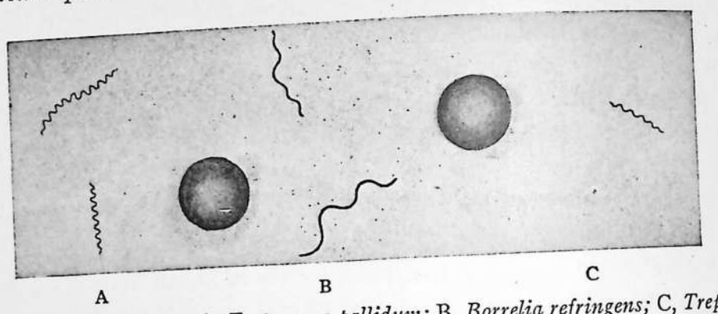


Fig. 215.—Spiral organisms: A, *Treponema pallidum;* B, *Borrelia refringens;* C, *Treponema microdentium*. Two red corpuscles are also shown (× 1200).

suspected syphilitic sores of the mouth it is, therefore, important to make smears from the tissue juices rather than from the surface (p. 642). *Borrelia refringens* is frequently present upon the surface of ulcers, especially about the genitals, and has doubtless many times been mistaken for *Treponema pallidum*. It can be avoided by properly securing the material for examination, but its morphology should be sufficient to prevent confusion. It is thicker than the organism of syphilis, stains more deeply, and has fewer and shallower curves (Figs. 215 and 346). Giemsa's stain gives it a bluish color. Spiral organisms of various kinds are present in the feces of many normal persons.

5. Genus Trypanosoma.—Trypanosomes have been found in the blood plasma of a great variety of vertebrates. Many of them appear to produce no symptoms, but a few are of great pathogenic importance. Many species have been described. The forms found in

PLATE XIII

PROTOZOAL PARASITES OF THE BLOOD.

(Photographs, × 1000; 1 mm. = 1 μ).

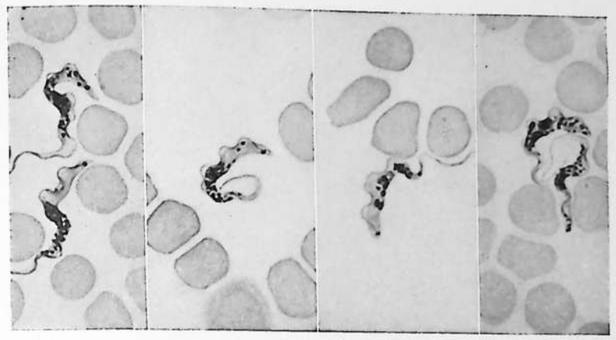


Fig. 1.—*Trypanosoma gambiense.*

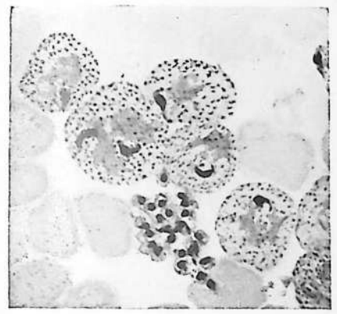


Fig. 2.—Half-grown tertian malarial parasites in stippled cells and a group of spores from a freshly ruptured segmenter. From a slide of double tertian malaria concentrated by F. M. Johns.

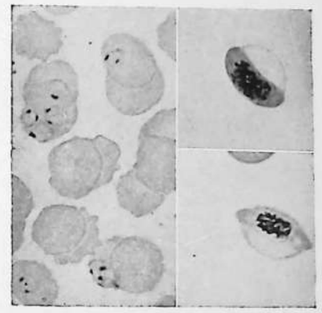


Fig. 3.—Estivo-autumnal malarial parasites, small ring forms and crescents.

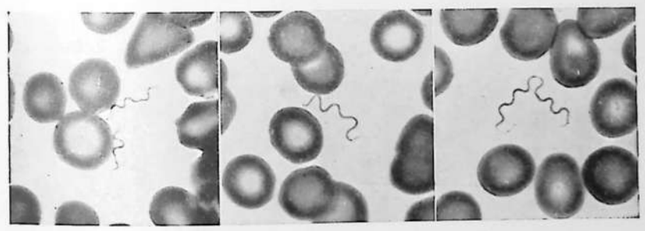


Fig. 4.—Spirochetes in the blood of a case of relapsing fever originating in Colorado. Reported by Dr. C. N. Meader.

the blood are easily recognized as trypanosomes, but accurate determination of species is difficult and may be impossible from morphology alone. They are elongated, spindle-shaped bodies, the average length of different species varying from 10 to 70 μ. Along one side there runs a delicate undulating membrane, the free edge of which appears to be somewhat longer than the attached edge, thus throwing it into folds. Somewhere in the body, usually near the middle, is a comparatively pale-staining nucleus; and near the posterior end is a smaller, more deeply staining chromatin mass, the micronucleus or blepharoplast. A number of coarse, deeply staining granules, chromatophores, may be scattered through the cytoplasm. A flagellum arises in the blepharoplast, passes along the free edge of the undulating membrane, and is continued posteriorly as a free flagellum. These details of structure are well shown in Plate XIII.

The life history of the trypanosomes is complicated and has not yet been worked out in all details. There is an alternation of hosts, various insects playing the part of definitive host. At least three species are pathogenic for man. These are pathogenic to a variable degree for some of the lower mammals, which in the wild state serve as "reservoirs" from which man may become infected through the agency of the insect host.

The best known trypanosome of human blood, *Trypanosoma gambiense* (Plate XIII), is an actively motile, spindle-shaped organism, from two to four times the diameter of a red corpuscle in length, with an undulating membrane which terminates at the anterior end in a long flagellum. It can be seen in stained films with medium power objectives, but is best studied with an oil-immersion lens. It will be necessary to search many slides. It is more abundant in the juice obtained by aspirating a lymph gland with a large hypodermic needle, and in the late stages when African "sleeping sickness" has developed; it is also found in the cerebrospinal fluid. Its length varies from 15 to 33 μ, there being short stumpy forms, long slender forms, and intermediates. It is transmitted by a biting fly, *Glossina palpalis*. A second species which causes sleeping sickness in Africa has been named *T. rhodesiense*. The chief point of distinction from *T. gambiense* is the situation of the nucleus close to, or even posterior to, the blepharoplast. It is transmitted by the fly, *Glossina morsitans*. The antelope and other large game animals are probably the reservoir for these African trypanosomes.

Trypanosoma (*Schizotrypanum*) *cruzi* is the cause of Brazilian trypanosomiasis, or Chagas' disease, and in the febrile stage is found in the peripheral blood without much difficulty. Its average length

is about 20 μ. The life cycle is very complicated. In the vertebrate host multiplication takes place in the muscles and certain internal organs where the parasites assume forms resembling Leishman-Donovan bodies. The early part of the flagellated stage is passed within red blood corpuscles, the latter part free in the blood plasma. The armadillo is probably the natural reservoir. The insect host, by which the trypanosome is transmitted to man, is a large bug belonging to the genus *Triatoma* which is abundant in the dwellings of the poorer classes in Brazil. There are several species, of which *Triatoma megista* is most important and best known. De Coursey[1] has reported a fatal case of Chagas' disease in Panama. Besides *T. megista*, other species of the family Reduviidae have been found as vectors. Reduviid

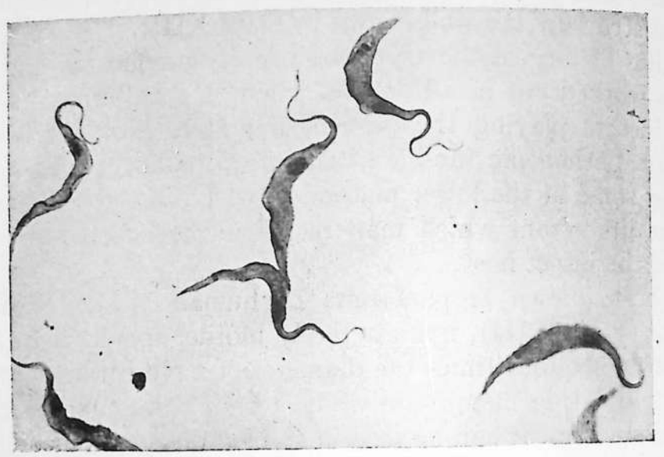


Fig. 216.—*Trypanosoma lewisi* in blood of rat. The red corpuscles were decolorized with acetic acid (photograph, × 1000).

bugs are found also in the United States. Opossums, armadillos, bats, dogs, and squirrels have all been found to be natural hosts.

Trypanosoma lewisi, a very common and apparently harmless parasite of gray rats, especially sewer rats, is interesting because it closely resembles the pathogenic forms, and is easily obtained for study. Its posterior end is more pointed than that of *T. gambiense* (Fig. 216).

Trypanosoma evansi, *T. brucei*, and *T. equiperdum* produce respectively surra, nagana, and dourine, which are common and important diseases of horses, mules, and cattle in the Philippines, East India, and Africa.

[1] De Coursey, Elbert: The First Fatal Case of Chagas' Disease Observed on the Isthmus of Panama, Am. Jour. Trop. Med., *15*:33–40 (Jan.), 1935.

6. Genus Leishmania.—The several species which compose this genus are apparently closely related to the trypanosomes, but their exact classification is undetermined. They have been grown outside the body and their transformation in cultures into flagellated trypanosome-like structures has been demonstrated.

They grow rather easily at room temperature either in citrated blood, or in N. N. N. (Novy, McNeal, Nicolle) medium (p. 766). Two to three weeks' incubation is necessary before calling cultures negative.

(1) **Leishmania donovani** is the cause of kala-azar, an important and common disease of India. With Wright's stain the "Leishman-

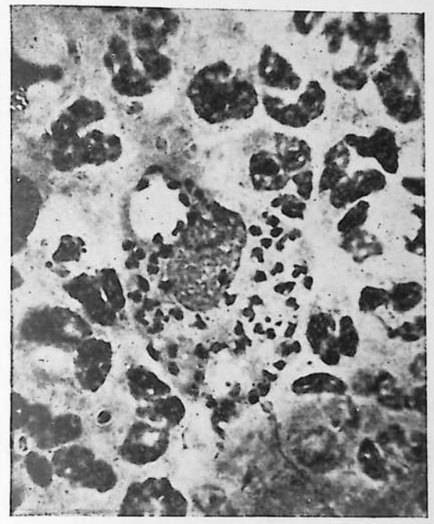


Fig. 217.—Smear from splenic pulp showing in the center a large mononuclear cell the cytoplasm of which is filled with Leishman-Donovan bodies. (Courtesy of C. I. Junkin.)

Donovan bodies" are round or oval, light blue structures, 2 to 3 μ in diameter, with two distinct reddish-purple chromatin masses, one large and pale (trophonucleus), the other small and deeply staining (blepharoplast) (Fig. 217). The parasites, which lie chiefly within endothelial cells, are especially abundant in the spleen; and splenic puncture has been resorted to for diagnosis, but is not without danger. They may also be found, although with less certainty, in material obtained by puncture of superficial lymph glands. While they have been seen within endothelial leukocytes in the peripheral blood, particularly late in the disease, they are extremely difficult to find in ordinary blood films. The search may be greatly facilitated by con-

centrating the leukocytes. The leukocytes will form a whitish layer on top of the solidly packed red corpuscles. They are skimmed off with a capillary pipet, spread on a slide, and stained with Wright's stain.

Napier's[1] serum test for kala-azar, which has proved useful in the field where laboratory facilities are not available, is performed as follows: Place 1 c.c. of clear serum in a small test tube. Add 1 drop of commercial formalin, containing about 40 per cent of formaldehyde. Shake the tube thoroughly and place in a rack. In three to twenty minutes the serum will coagulate and become white and opalescent if it is from an untreated case of kala-azar. Normal serum remains clear and fluid. Serum from patients who have leprosy, tuberculosis, or malaria gives a somewhat similar reaction to that which occurs in kala-azar, but to a very much less degree. Histoplasmosis (see p. 633) may be confused with kala-azar; however, the latter disease probably does not occur in the United States, while the disease described by Darling and commonly called "histoplasmosis" is being reported in the United States with increasing frequency.

(2) **Leishmania infantum** is found in a form of splenomegaly with severe anemia occurring in young children in the region around the Mediterranean, especially Italy, and known as infantile kala-azar. Morphologically the parasite is indistinguishable from *L. donovani*. Dogs are the natural reservoir, and the dog flea has been thought to transmit the disease, although recent work of Nicolle and Anderson would indicate that the flea is not implicated. The opinion of British workers is that the sand fly, *Phlebotomus*, may be the transmitting agent.

(3) **Leishmania tropica** resembles the preceding. It is found, lying intracellularly, in the granulation tissue of Delhi boil or Oriental sore. A variety, sometimes described as a separate species, *L. braziliensis* or *L. americana*, is similarly found in the ulcers of espundia, a very chronic form of mucocutaneous leishmaniasis, in South and Central America.

7. Genus Trichomonas.—(1) **Trichomonas hominis** is an oval or pear-shaped cell of somewhat changing shape (Fig. 218). The average length is about 10 to 15 μ, although there is considerable variation among individuals. In the anterior half of the cell is an oval nucleus which is not, however, well seen in unstained specimens; the cytoplasm contains food vacuoles. At the anterior or blunt end there is a cluster of four flagella of equal length, and along one side

[1] Napier, L. E.: A New Serum Test for Kala-azar, Indian Jour. Med. Res., *9*:830–846 (Apr.), 1922.

is an undulating membrane the thickened free edge of which is continued backward as a short flagellum. As to the number of flagella there is some confusion in the literature. Four is now accepted as the standard number, although there may be variation among individual parasites. Forms with three and five flagella have been called *Tritrichomonas* and *Pentatrichomonas*, respectively, but they are not generally regarded as separate species. Owing to the active motion of the flagella and undulating membrane these are not easily seen, and at first sight the parasite has much the appearance of a pus corpuscle moving busily about among the fecal particles. A drop of Gram's iodine solution added to the preparation on the slide kills the parasites and brings out the internal structure and the flagella more clearly. In infested feces a few hours old, a characteristic degenerating

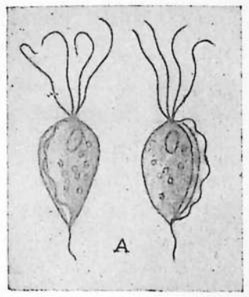


Fig. 218.—*Trichomonas hominis*, an intestinal flagellate (× 1000): Note the four anterior flagella and undulating membrane (composite drawing from various authors).

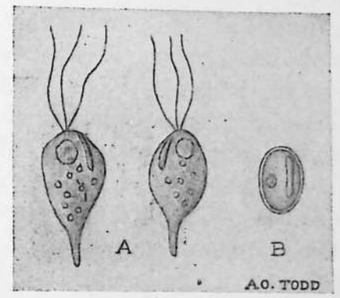


Fig. 219.—*Chilomastix mesnili*, an intestinal flagellate (× 1000): A, Flagellate form. Note the three anterior flagella, and the elongated cystostome or "mouth" (after Brumpt). B, Cyst. The cystostome can still be seen (Chandler, after Wenyon).

form of *Trichomonas* is common. The organism exhibits peculiar undulating, almost ameboid movements which may continue for many hours. The usual habitat is the colon, but it is not infrequently found in other situations: In the vagina, in the mouth, especially about the gums, and in the sputum of persons suffering from pulmonary gangrene and putrid bronchitis.

Unlike the other intestinal flagellates, it does not, as far as is known, become encysted, although cysts have been most carefully sought, and supposed cysts have from time to time been described. Infection, therefore, probably takes place through ingestion of the flagellate forms, which, according to Hegner and Becker, appear to be sufficiently resistant to pass through the stomach without injury when swallowed with contaminated food.

Trichomonas hominis is common in the tropics, and from clinical reports appears to be widespread throughout the United States. It is probable, however, that in many cases the organisms reported were really *Chilomastix* or *Giardia*, which are more frequent. Surveys among soldiers from all parts of the country indicate its presence in about 3 per cent of the population. Most authorities regard it as nonpathogenic; others believe that it may cause mild diarrhea of the dysenteric type, or that it at least may aggravate an already existing inflammatory condition. The parasites are often so abundant that four or more may be seen in a single field of the high dry objective.

(2) **Trichomonas vaginalis** is frequently found in vaginal discharges, and is thought by gynecologists to be a cause of colpitis and leukorrhea. It is the type species for the genus *Trichomonas* and is larger than the other trichomonads, measuring from 12 to 26 μ in length, and from 6 to 18 μ in width. Bland, Goldstein, and Wenrich[1] reviewed the literature very completely up to 1931. One of the chief differential points in the morphology of *T. vaginalis* is that the posterior flagellum forming the margin of the undulating membrane rarely reaches beyond the middle of the body, and is without a free portion. There are also four anterior flagella. The organism may be readily grown in a medium composed of Loeffler's dehydrated blood serum, 0.3 Gm.; sodium chloride, 0.7 Gm.; sodium citrate, 1 Gm.; egg albumin, 2 c.c.; distilled water, 100 c.c. The comparative size of *Trichomonas vaginalis*, *Trichomonas buccalis* and *Trichomonas hominis* is shown in Figure 220, from the drawings of these organisms made by Powell.[2]

8. Genus Chilomastix.—Chilomastix mesnili is widely distributed on all continents. Surveys among soldiers and others indicate that it is present in the feces of about 5 per cent of all persons in this country, and is thus the second most prevalent intestinal flagellate, standing next below *Giardia*. Its pathogenicity is in dispute. It is a pear-shaped organism, 13 to 24 μ long, with three anterior flagella and no undulating membrane (Fig. 219). There is a large round nucleus not well seen in unstained specimens; and anteriorly is a large, elongated, heavily outlined cystostome or "mouth," within which is a small, slender flagellum not easily seen. The cytoplasm contains numerous small food vacuoles. The posterior extremity is

[1] Bland, P. B., Goldstein, Leopold, and Wenrich, D. H.: Vaginal Trichomoniasis in the Pregnant Woman. A Clinical and Morphologic Study, Jour. Am. Med. Assn., *96:* 157–163 (Jan. 17), 1931.

[2] Powell, W. N.: *Trichomonas vaginalis* Donné 1836: Its Morphologic Characteristics, Mitosis and Specific Identity. Amer. Jour. Hygiene. *24:*145–169 (July), 1936.

projected into a narrow tail-like process. The usual habitat of *Chilomastix* is the large intestine.

The cyst is oval or, more often, pear shaped, and measures about 7.5 to 8.5 μ in length. The nucleus and the margins of the cystostome can be seen within it.

9. Genus Giardia.—Giardia lamblia (**Lamblia intestinalis**) is very common in the tropics and is a prevalent intestinal flagellate in this country. In a series of examinations of 2876 soldiers, as compiled by Kofoid, it was found 168 times. Other surveys place the incidence of infection among the general population as high as 12 per cent, while Maxcy found the incidence to be as high as 20 per

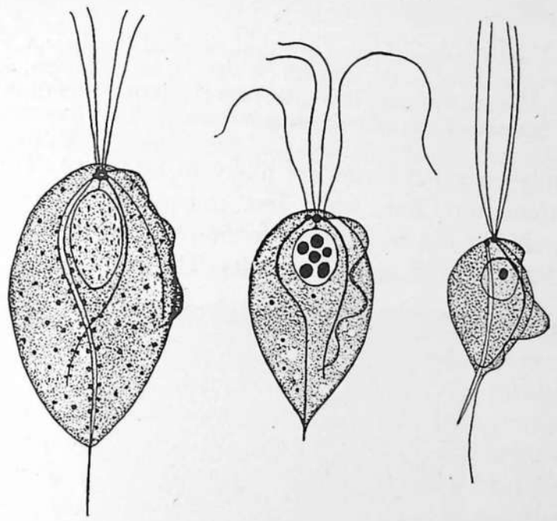


Fig. 220. *Trichomonas vaginalis, Trichomonas buccalis, Trichomonas hominis* (× 2000) (Powell).

cent in a group of children examined in Baltimore. However, Lynch found only 2.3 per cent in 1040 cases in four years' experience. The parasite is generally considered of little pathogenic importance, but appears capable of causing a chronic enterocolitis with mild diarrhea which is very resistant to treatment. Closely related species are frequent in the intestines of rats, mice, rabbits, and guinea-pigs.

Giardia lamblia is pear shaped, measures 12 to 20 μ in length, and has a depression on one side of the blunt end by which it attaches itself to the tops of the epithelial cells of the intestinal wall. Three pairs of flagella are arranged about the depression and one pair at the pointed end (Figs. 221 and 222). Two nuclei can be made out. The

cytoplasm does not contain food vacuoles. Its usual habitat is the upper part of the small intestine, especially the duodenum.

Giardia is disseminated in the encysted stage, and unless active diarrhea from the small intestine exists, or the stool is obtained by

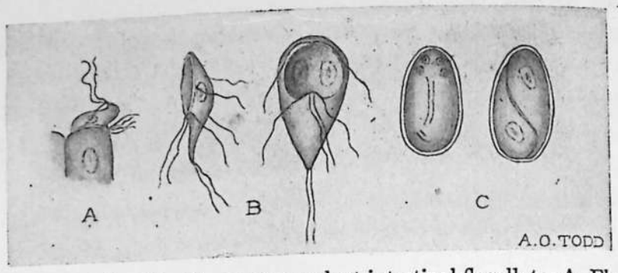


Fig. 221.—*Giardia lamblia*, the most prevalent intestinal flagellate: A, Flagellate form attached to the top of intestinal epithelial cell (× 500), B, flagellate form, side and ventral views (× 1000), C, cysts, frequently seen in the feces (× 1000). (After Chandler: A following Grassi and Schewiakoff, B and C following Wenyon.)

catharsis, only encysted forms are likely to be found. These may be extremely numerous and, while less conspicuous than the motile individuals, should not be difficult to find and identify even by one who is not familiar with protozoal cysts. The cysts are oval, measure

Fig. 222.—Vegetative form of *Giardia lamblia* in feces; photomicrograph (about × 800).

about 8 × 14 μ, have the internal structure shown in Fig. 221, and are surrounded by a fairly thick hyaline wall. Boeck found their longevity outside the body to be thirty-four days in water and their thermal death point 64° C.

Other Intestinal Flagellates.—The literature relating to the intestinal flagellates and their pathogenicity is much confused. Probably the only important members of the group in this country are the three described above. Other species which are very rarely found may be mentioned briefly. *Embadomonas intestinalis* has two anterior flagella and a definitely outlined cystostome, and its cyst resembles that of *Chilomastix*, but is only about 5 μ long. *Enteromonas hominis* usually has three anterior flagella, and a fourth which arises anteriorly, trails backward, and is adherent to the body. These two flagellates are small, 4 to 8 μ in length. *Tricercomonas intestinalis*, formerly described, is now, by good authorities, identified with *Enteromonas*. *Cercomonas*, with two anterior flagella, one free, one directed backward, attached to the body, and projecting posteriorly, is, contrary to the older belief which was due to confusion with *Chilomastix* and *Trichomonas*, very rare in fresh feces. *Bodo*, with a single nucleus and two anterior flagella, is also very rare. *Cercomonas* and *Bodo* do not live within the human body, but are to be classed as "coprozoic organisms" which reach the feces as contaminations and there find a favorable medium for growth.

Special methods of examining feces for flagellates and other protozoa are given on pages 488–491.

CLASS SPOROZOA

Subclass Telosporidia

All the members of this class are parasitic, but only a few have been observed in man, and only one genus, *Plasmodium*, is of much importance in human pathology. Propagation is by means of spores, and sporulation ends the life of the individual. In some species there is an alternation of generations, in one of which sexual processes appear. In such cases the male individual may be provided with flagella. Otherwise there are no special organs of locomotion.

1. Genus Eimeria.—Eimeria stiedae (Coccidium cuniculi).—This is a very common parasite of the rabbit, and has been much studied, but extremely few, if any, authentic cases of infection in man have been reported. The parasite, which when fully developed is ovoid in shape and measures about 30 to 50 μ in length and has a shell-like integument, develops within the epithelial cells of the bile passages. Its presence in rabbits causes the formation of whitish nodules, usually with caseous contents. On reaching adult size it divides into a number of spores or merozoites, which enter other epithelial cells and repeat the cycle. A sexual cycle which suggests that of the malarial parasite, but does not require an insect host, also occurs. By conjugation of microgamete with macrogamete a zygote is formed. This develops a definite membranous wall and thus becomes an *oocyst* which passes out with the feces. Its contents

then divide into a number of sporozoites. The cyst remains quiescent until it reaches the stomach of a new host, usually through contaminated food. Here the cyst wall is digested and the sporozoites are set free to travel to the liver and enter epithelial cells, where they initiate a new cycle.

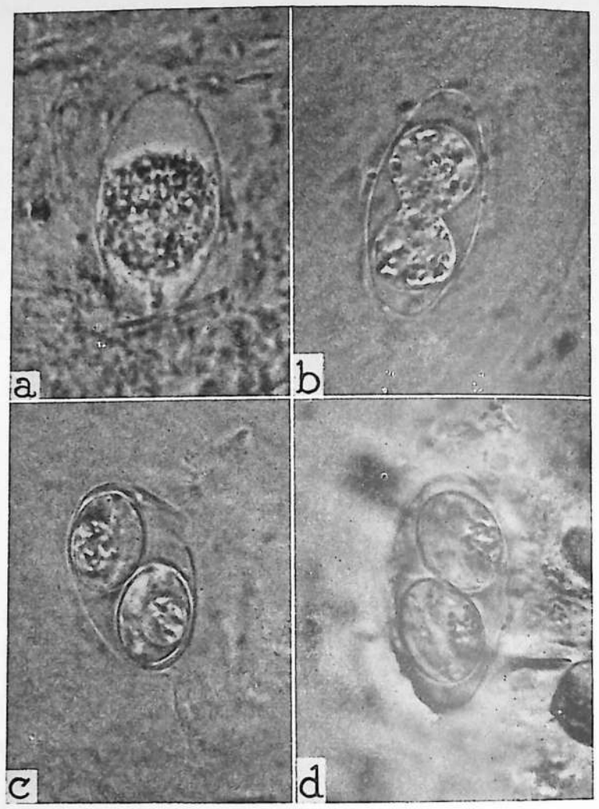


Fig. 223.—*Isospora hominis* Fantham 1917 (× 1000). *a*, Oocyst in stool at time of passage; *b*, beginning formation of two sporocysts; *c*, sporocysts formed (thirty-six hours); large residual mass; sporozoites not completely formed; oocyst wall ruptured by pressure; *d*, mature oocyst (fifty six hours). (Magath, in "The American Journal of Tropical Medicine," March, 1935. Williams and Wilkins Company, Publishers.)

A number of related species have in very rare instances been found in man. In most of these the oocyst was large and spherical.

2. Genus Isospora.—Isospora hominis.—This is closely related to the preceding and has a similar life history. It has been found in human feces. Apparently it causes no particular disturbance. Diagnosis depends upon the recognition of oocysts in the feces. These are

colorless, ovoid bodies, measuring about 14 by 28 μ, with a clear-cut definite wall usually with two or more layers. When they first pass out of the body the protoplasm is unsegmented and appears as a rounded granular mass which does not fill the cyst, as in Fig. 223. They might easily be mistaken for the eggs of some unknown worm. To gain an idea of their general appearance one may study the contents of the whitish nodules in the liver of a rabbit infested with the coccidium described above.

Isospora hominis has been reported chiefly in British soldiers coming from Gallipoli, Salonika, and the Balkans. The distribution of reported cases is shown in Fig. 224.

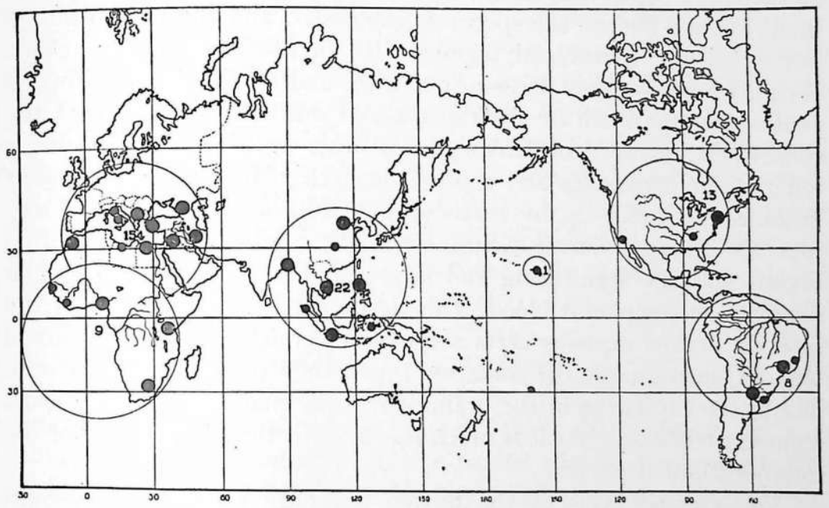


Fig. 224.—Geographical distribution of reported cases infested with *Isospora hominis*. (Magath, in "The American Journal of Tropical Medicine," March, 1935. Williams and Wilkins Company, Publishers.)

3. Genus Plasmodium.—This genus includes the malarial parasites. These protozoa belong to the Sporozoa (p. 523), order Hemosporidia, and family Plasmodiae, the members of which are parasites in the blood of a great variety of vertebrates. Three species, belonging to the genus *Plasmodium*, are associated with malarial fever in man: *Plasmodium vivax*, *P. malariae*, and *P. falciparum*, the parasites respectively of the tertian, quartan, and estivo-autumnal types of malaria. The life histories of the three are so similar that they may well be described together.

(1) **Life Histories.**—There are two cycles of development: One, the *asexual*, in the blood of man, and the other, the *sexual*, in the

intestinal tract of female mosquitoes of certain species of the genus *Anopheles*.

(*a*) *Asexual Cycle.*—The young organism enters the blood through the bite of the mosquito. It makes its way into a red corpuscle,[1] where it appears as a small, pale, "hyaline" body, which generally assumes the form of a ring. Later it loses its ringlike shape, increases in size, and exhibits ameboid movements. Within about eight hours dark brown granules, derived from the hemoglobin of the corpuscle, make their appearance within it. When it has reached its full size, almost filling the corpuscle and causing it to enlarge, in the case of the tertian parasite, smaller in the others, the pigment granules gather at the center or at one side; the organism divides into a number of small hyaline bodies, the spores or *merozoites;* and the red corpuscle bursts, setting spores and pigment free in the blood plasma. This is called segmentation. It coincides with, and by liberation of toxins causes the paroxysm of the disease. A considerable number of the spores are destroyed by leukocytes or other agencies; the remainder enter other corpuscles and repeat the cycle. Many of the pigment granules are taken up by leukocytes. In estivo-autumnal fever segmentation occurs almost exclusively in the capillaries of the internal organs and the segmenting and larger pigmented forms are seldom seen in the peripheral blood. This accumulation of parasites in the internal organs explains certain types of pernicious estivo-autumnal malaria, for example the comatose type, when the parasites accumulate in the capillaries of the brain. The other malarial parasites show a similar tendency, but it is much less marked than in the case of the estivo-autumnal parasite.

The asexual cycle of the tertian organism occupies forty-eight hours; of the quartan, seventy-two hours; of the estivo-autumnal, an indefinite time—usually twenty-four to forty-eight hours.

The parasites are thus present in the blood in great groups or broods, all the individuals of which reach maturity and segment at approximately the same time. This explains the regular recurrence of the paroxysms at intervals corresponding to the time occupied by the asexual cycle of the parasite. Not infrequently there is multiple infection, one group reaching maturity while the others are still young; but the presence of two groups which segment upon the

[1] In this section the malarial parasite is described, in accordance with the usual teaching, as living within the parasitized red corpuscle. The work of Mary Rowley-Lawson, however, tends to show that the parasite is extracellular throughout its whole existence; that it attaches itself to the external surface of the red corpuscle, but does not enter it; and that it migrates from corpuscle to corpuscle between paroxysms, destroying each cell before it abandons it.

same day is extremely rare. Fevers of longer intervals—six, eight, ten days—are probably due to the ability of the body, sometimes of itself, sometimes by aid of quinine, to resist the parasites, so that numbers sufficient to cause a paroxysm do not accumulate in the blood until after several repetitions of the asexual cycle. In estivo-autumnal fever the regular grouping, while usually present at first, is soon lost, thus causing "irregular malaria."

Bass has succeeded in cultivating the malarial parasite outside of the body.

(*b*) *Sexual Cycle.*—Besides the ameboid individuals which pass through the asexual cycle, there are present with them in the blood many individuals with sexual properties. These are called *gametocytes;* the males, *microgametocytes;* the females, *macrogametocytes.* Like the asexual forms they start as young parasites liberated from the sporulating parent. However, they grow more slowly and, when they reach the adult size, do not undergo segmentation, but remain inactive in the blood until taken up by a mosquito. Many of them are apparently extracellular, but stained preparations usually show them to be surrounded by the remains of a corpuscle. In tertian and quartan malaria they resemble the asexual individuals until a variable time after the blood leaves the body, when the male gametocyte sends out one or more flagella. In estivo-autumnal malaria the gametocytes take distinctive ovoid and crescentic forms, and are not difficult to recognize. These sexual forms are very resistant to quinine, and often persist in the blood long after the ameboid forms have been destroyed. Under ordinary conditions they are incapable of continuing the disease until they have passed through the cycle in the mosquito, but it seems probable that under certain unusual conditions the female gametocyte may, without fertilization, undergo further development and sporulate, thus starting an entirely new asexual cycle.

When a malarious person is bitten by a mosquito, the gametocytes are taken with the blood into its stomach. Here the male sends out one or more flagella known as microgametes. These break off and seek out the females, which have undergone a process of maturation and are now known as macrogametes, whom they fertilize much as the sperm fertilizes the ovum. The fertilized female is then a *zygote;* it becomes elongated and actively motile and finally penetrates the intestine and encysts on its outer wall. This "oocyst" grows enormously and projects into the body cavity of the mosquito as a conspicuous knob, easily seen in dissected mosquitoes with very low magnification. After about two weeks the oocyst rup-

tures, liberating many minute rods, or *sporozoites*, which have formed within it. These migrate to the salivary glands, and are carried into

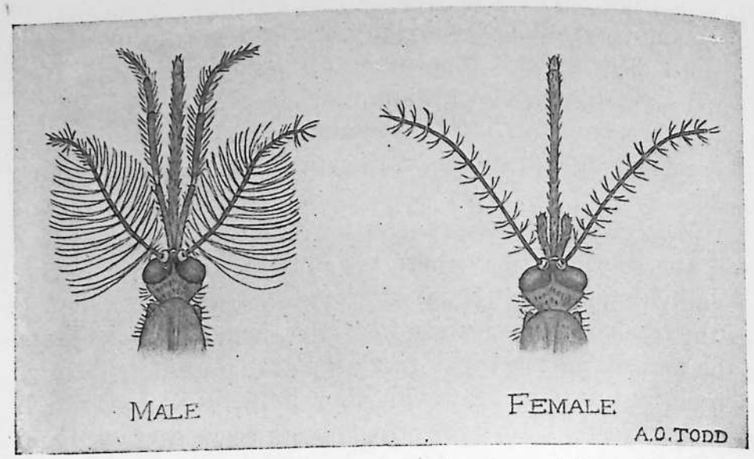


Fig. 225.—Heads of *Culex* (*Culex pipiens*) showing the straight proboscis, the jointed palpi, and external to these the hairy antennae. The male is distinguished from the female by the longer hairs on the antennae. Note that the palpi of the male are about as long as the proboscis, while those of the female are very much shorter (compare with Fig. 226).

the blood of the person whom the mosquito bites. Here they enter red corpuscles as young malarial parasites, and the majority pass

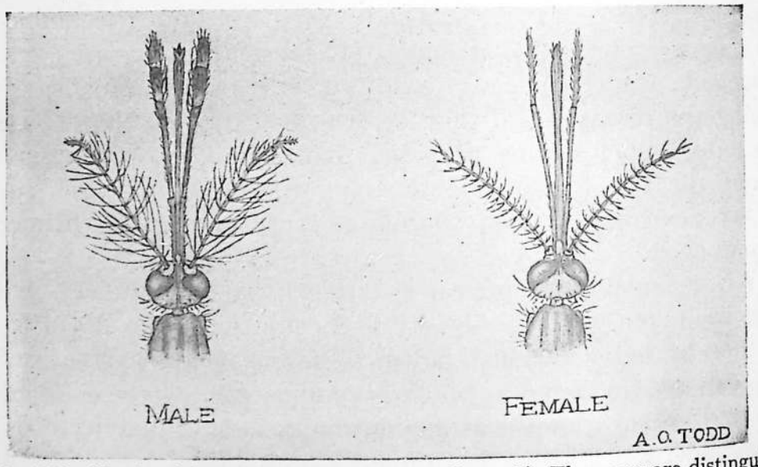


Fig. 226.—Heads of *Anopheles* (*Anopheles maculipennis*). The sexes are distinguished by the antennae as noted under Fig. 225. The palpi of *Anopheles* are nearly the same length as the proboscis in both sexes.

through the asexual cycle just described. Definite symptoms of malaria do not, however, appear until the parasites have multiplied

to a sufficient number—usually ten or twelve days in acute cases. Ross estimated that ordinarily about 150,000,000 parasites must be present in the body before symptoms are produced.

The sexual cycle can take place only within the body of the female of certain mosquitoes belonging to the genus *Anopheles*. The male does not bite. Absence of these mosquitoes from certain districts explains the absence of malaria. *Anopheles* is distinguished from other common mosquitoes, particularly the widely distributed genus *Culex*, by the relative lengths of proboscis and palpi (Figs. 225 and

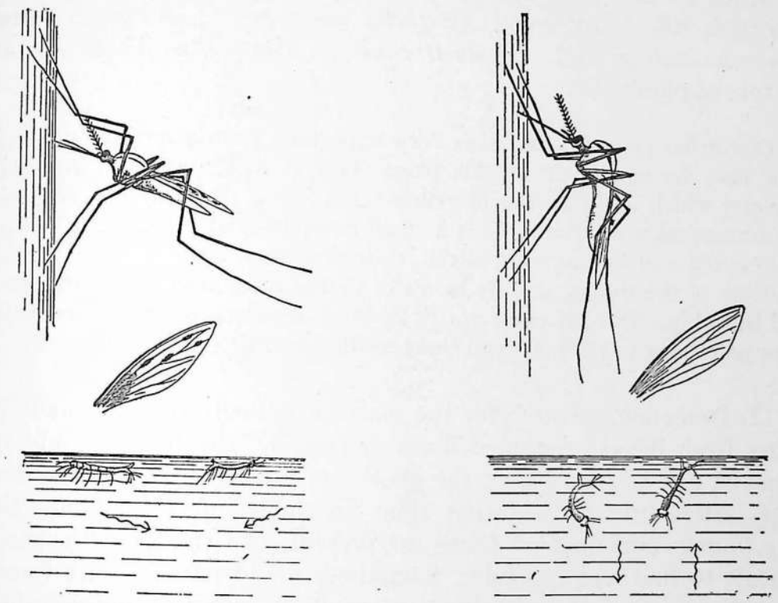


Fig. 227.—Showing, on the left, *Anopheles* in resting position, its dappled wing, and the position of its larvae in water; on the right, *Culex* in resting position, its plain wing, and the position of its larvae in water. The arrows indicate the directions taken by the larvae when the water is disturbed (Abbott).

226), which can be seen with a hand lens, by its attitude when resting, and by the dappled wing which characterizes most species of *Anopheles* (Fig. 227).

Anopheles is strictly nocturnal in its habits; it usually flies low, and rarely travels more than a few hundred yards from its breeding place, although it may be carried by winds. These facts explain certain peculiarities in malarial infection; thus, infection occurs practically only at night; it is most common near stagnant water, especially upon the side toward which the prevailing winds blow; and the

danger is greater when persons sleep upon or near the ground than in upper stories of buildings. Most of the mosquitoes die in the fall, but individuals frequently hibernate in warmed houses, and may bite during the winter. A mosquito becomes dangerous in eight to fourteen days after it bites a malarious person, and remains so throughout its life. Malaria is found most commonly in the southern states; however, it is found as far north as Minnesota. Sanford[1] reported a case of tertian malaria which was observed in southern Minnesota. The patient was two years of age and never had been out of the county in which he had been born. A number of sporadic cases have been reported. Riley[2] has found *Anopheles maculipennis*, *A. quadrimaculatus*, *A. walkeri* and *A. punctipennis* in Minnesota. These are all vectors of plasmodia.

One other mosquito which is very important from a medical point of view may be mentioned at this place. This is *Aedes aegypti* (*Stegomyia fasciata*) which is the carrier of yellow fever and is probably concerned in the transmission of dengue. It is a small mosquito and is distinguished by two curved and two straight silver white markings, suggesting a lyre, on the back of the thorax, and by bands of similar color around the abdomen and legs (Fig. 228). This and nearly all other members of the genus *Aedes* have palpi long in the male and short in the female, as in *Culex*.

(2) **Detection.**—Search for the malarial parasite may be made in either fresh blood or stained films. If possible, the blood should be obtained a few hours before the chill—not during it or within a few hours afterward, since at that time (in single infections) only the very young, unpigmented forms are present, and these are the most difficult to find and recognize. Sometimes many parasites are found in a microscopic field; sometimes, especially in estivo-autumnal infection, owing to accumulation in internal organs, careful search is required to find any, despite severe symptoms. In ordinary acute malaria, however, the parasites can usually be found within five to twenty minutes. Only in chronic cases and in "malaria carriers" is it necessary to resort to concentration methods. Quinine causes them rapidly to disappear from the peripheral blood, and few or none may be found after its administration. In the absence of organisms the presence of pigment granules within leukocytes—especially the endotheliocytes—may be taken as definite evidence of malaria. Pigmented

[1] Sanford, A. H.: Malaria in Northern States. St. Paul Med. Jour. *15*:83–86 (Jan.), 1913.

[2] Riley, W. A.: Indigenous Malaria and Its Vectors in Minnesota. Journal-Lancet. *59*:311–312 (July), 1939.

leukocytes (Plate XIV) are most numerous after a paroxysm and in chronic malaria.

(*a*) *In Fresh Unstained Blood.*—Obtain a small drop of blood from the finger or lobe of the ear. Touch the center of a cover glass to the top of the drop and quickly place it, blood side down, upon a slide. If the slide and cover be perfectly clean and the drop not too large, the blood will spread out so as to present only one layer of corpuscles. Search with an oil-immersion objective, using very subdued light. The

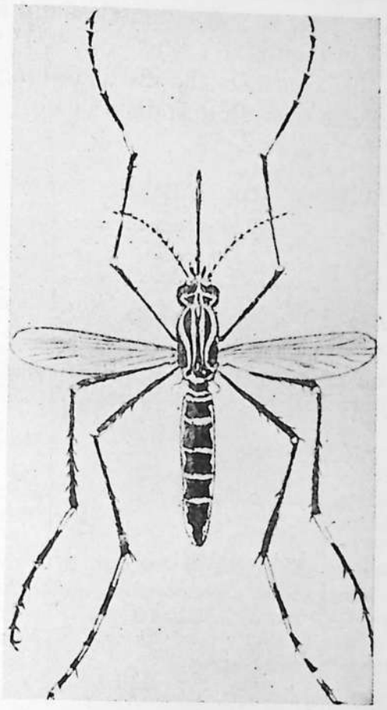


Fig. 228.—*Aedes aegypti* (*Stegomyia fasciata*), the yellow fever mosquito. Female. Note the lyre-like markings on the back of the thorax (Boyce, after Newstead).

preparation may be kept for many hours if ringed with vaselin or melted paraffin.

The young organisms appear as small, round, ringlike or irregular, colorless bodies within red corpuscles. The light spots caused by crenation and other changes in the corpuscles are frequently mistaken for them, but are generally more refractive or have more sharply defined edges. The older forms are larger colorless bodies containing granules of brown pigment. In the case of the tertian parasite,

these granules have active vibratory motion, which renders them conspicuous; and as the parasite itself is very pale, one may see only a large pale corpuscle in which fine pigment granules are dancing. Segmenting organisms, when typic, appear as rosets, often compared to daisies, the petals of which represent the segments, while the central brown portion represents the pigment. Tertian segmenting forms are less frequently typic than quartan. Flagellated forms are not seen until ten to twenty minutes after the blood has left the vessels. As Cabot suggests, one should, while searching, keep a sharp lookout for unusually large or pale corpuscles, and for anything which is brown or black or in motion.

The following table contrasts the distinguishing characteristics of the three species as seen in fresh unstained blood.

SPECIES OF THE MALARIAL PARASITE

Tertian.	Quartan.	Estivo-autumnal.
Asexual cycle, forty-eight hours.	Seventy-two hours.	Usually twenty-four to forty-eight hours.
Substance pale, transparent, comparable to hyaline tube-cast.	Highly refractive, comparable to waxy tube-cast.	Highly refractive.
Outline indistinct.	Distinct.	Distinct.
Ameboid motion active.	Sluggish.	Active.
Mature asexual form large; fills and often distends corpuscle.	Smaller.	Young forms, only, in peripheral blood.
Pigment-granules fine, brown, scattered throughout. Very active dancing motion.	Much coarser, darker in color, peripherally arranged. Motion slight.	Very few, minute, inactive. Distinctly pigmented forms seldom seen.
Segmenting body rarely assumes typical "daisy" form. 15 to 20 segments.	Usually typical "daisy." 6 to 12 segments.	Very rarely seen in peripheral blood.
Gametocytes resemble asexual forms.	Same as Tertian.	Appear in blood as distinctive ovoids and crescents.
Red corpuscles pale and swollen.	Generally darker than normal.	Dark, often bronzed.

(*b*) *In Stained Films.*—Recognition of the parasite, especially the young form, is much easier in films stained by Wright's or some similar stain than in fresh blood. The films must be thin and well stained. It is useless to search preparations in which the nuclei of leukocytes are not strongly colored.

PLATE XIV

MALARIAL PARASITES.

Wright's stain. × 1000 (1 mm. = 1 μ).

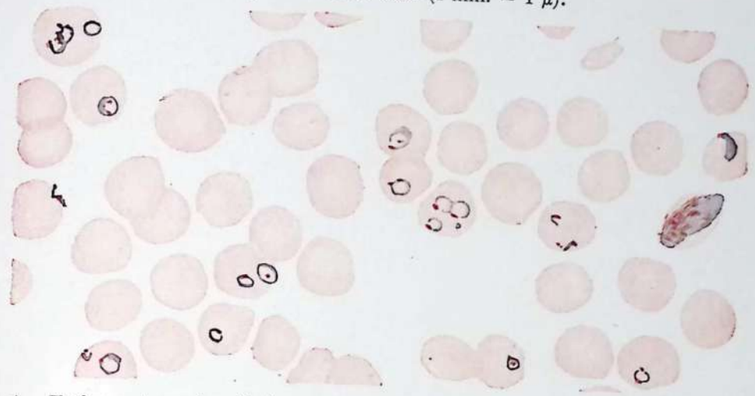


Fig. 1.—Estivo-autumnal malaria, exact reproduction of a portion of a field, showing an exceptionally large number of parasites.

Fig. 2.—Estivo-autumnal gametocytes.

Fig. 3.—Leukocytes with engulfed pigment.

Fig. 4.—Quartan parasites.

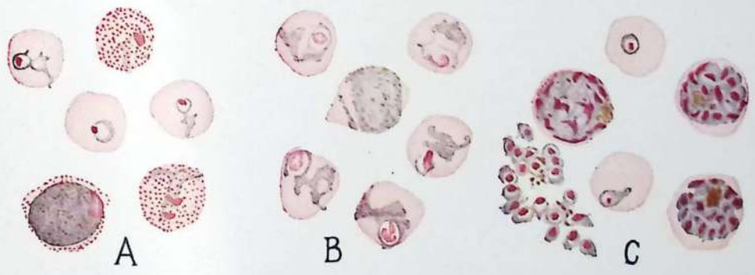


Fig. 5.—Tertian parasites. A, Eight hours after chill, showing malarial stippling, five young parasites, and one gametocyte, from two slides; B, twenty-four hours after chill, five half-grown parasites; one gametocyte; C, during chill, one presegmenter, two segmenters, a cluster of freshly liberated merozoites, and two very young parasites, from one slide. (J. W. Rennell, pinx.)

In films which are properly stained with Wright's or Giemsa's stain malarial parasites appear as follows:

The **young parasites** (Plate XIV, Figs. 1, 4, and 5, *a*) are small, round, ringlike or irregular, sky blue bodies, each with a very small, sharply defined, purplish-red chromatin mass. Many structures—deposits of stain, dirt, blood platelets lying upon red cells (Plate I and Fig. 155), and so forth—may simulate them, but should not deceive one who looks carefully for both the blue cytoplasm and the purplish-red chromatin. A platelet upon a red corpuscle is surrounded by a colorless zone rather than by a distinct blue body and there is no compact chromatin mass. The highly refractile colorless spots sometimes produced in red corpuscles by slow drying of the film are often mistaken for parasites by beginners. When a little out of focus these may show much color, owing to chromatic aberration of the objective. As quartan parasites grow a little older they tend to assume a straight or slightly curved, bandlike form which is fairly characteristic of this species. Young estivo-autumnal parasites commonly take the form of small, delicate, blue rings, each with one or two small purplish-red chromatin bodies upon its circumference. Their recognition is important because they may be the only form found in a given case. When young tertian parasites assume this form the ring is usually larger and thicker. Usually, it is the dotlike chromatin body which first attracts one's attention to the parasitized cell. In tertian malaria the fact that cells which harbor the parasites are somewhat larger and paler than their fellows is also helpful in attracting one's attention while searching. This may be evident as early as eight hours after the chill. No such enlargement of the red cells is noted in other forms of malaria.

Older tertian and **quartan parasites** (Plate XIV, Figs. 4 and 5, *b*) show larger, irregularly shaped, sky-blue bodies with more abundant, paler, and more reticular or granular chromatin, and contain brown granules of pigment, which, however, are less evident than in the living parasite The chromatin usually lies in a colorless area or "achromatic zone," and is sometimes so pale as to be difficult to see clearly. Not infrequently it appears to lie entirely outside of the cytoplasm. The pigment of the adult tertian parasite is usually fine and scattered uniformly through the cytoplasm. That of the quartan is coarser and more peripherally arranged. The quartan parasite is smaller than the tertian and does not enlarge the red corpuscle, which is often even smaller and more darkly colored than the surrounding unparasitized corpuscles. Very characteristic is its tendency to take the form of a broad band extending across the cor-

puscle. As was explained on page 526, the older asexual and the segmenting stages of the estivo-autumnal parasite rarely appear in the peripheral blood.

Typical **"segmenters"** (Plate XIV, Fig. 5, *c*) may present a ring of rounded segments or spores, each with a small, dotlike chromatin mass, but these regular forms are not often seen. With the tertian parasite, especially, the segments much more frequently form an irregular cluster. The pigment is collected near the center or at one side or is scattered among the segments.

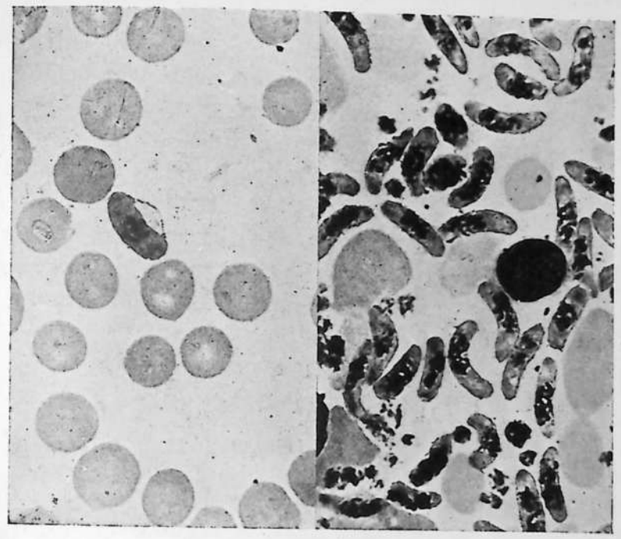
Fully grown tertian and quartan **gametocytes** (Plate XIV, Fig. 5, *a*, *b*) resemble the fully grown asexual forms in general appearance, but are more compact and less irregular in shape and contain more and larger pigment granules. The female (*macrogametocyte*) is generally the larger and has more compact chromatin, usually situated near the edge of the parasite, deeper blue cytoplasm, and more pigment. The male (*microgametocyte*) stains light blue or greenish. Its chromatin is pale and occupies a relatively large area, usually near the center. The crescentic and oval gametocytes of estivo-autumnal malaria (Plate XIV, Figs. 1 and 2) are easily identified. Their length is somewhat greater than the diameter of a red corpuscle. The macrogametocyte is usually thin and more or less pointed at the poles, and the chromatin is centrally placed and surrounded by pigment granules. The microgametocyte is generally paler and thicker, with blunt ends, while its chromatin and pigment are scattered throughout the middle third of the body. The remains of the red cell often form a narrow rim around them or fill the concavity of the crescent.

Method of Bass and Johns.—A method of concentrating plasmodia has been described by Bass and Johns. This method takes advantage of the fact that cells which contain parasites are lighter than normal cells and will therefore rise to the top of the sediment when blood is centrifugalized at 2500 revolutions per minute. The upper layers are skimmed off and transferred to smaller tubes and centrifugalized again. This process is repeated until there is a small bulk of sediment but many cells which contain parasites. These cells are placed on a slide and stained with Wright's stain. A decided advantage over other methods that have been described is that the parasites and erythrocytes are perfectly preserved and stain exactly as they do in ordinary smears (Fig. 229 and Plate XIV). However, the method is laborious and the thick film method may be preferred.

Barber and Komp[1] Thick Film Method.—The slides must be

[1] Barber, M. A., and Komp, W. H. W.: Method of Preparing and Examining Thick Films for the Diagnosis of Malaria, U. S. Pub. Health Rep., *44*:2330–2341 (Sept. 27), 1929.

perfectly clean and wiped with alcohol. Make a thin film of blood on one end of the slide. On the other end make a thick smear of blood, spreading the blood with a wooden applicator or a corner of another slide. Allow the blood to dry. Make a heavy mark between the two smears with a wax pencil. Stain the thin film with Wright's stain. The preparation may be used later for making a differential count or for studying the plasmodia in the erythrocytes, if any are found. Place the end of the slide, which contains the thick film, vertically in freshly diluted Giemsa's stain, which is prepared by adding 75 c.c. of neutral distilled water to 2 c.c. of stock stain. Distilled water should



A B

Fig. 229.—Estivo-autumnal malaria: Effect of concentration by the method of Bass and Johns: A, Direct smear, averaging one crescent in eight fields; B, blood of same patient concentrated. From slides prepared by F. M. Johns. Wright's stain (photographs, × 1000).

be brought to *p*H 7 by carefully adding dilute solution of sodium hydroxide. Test the *p*H after adding each portion of alkali to the distilled water. It is essential that there should be a slight amount of free alkali in the water for the proper working of Giemsa's stain. Komp,[1] Johnson,[2] and others have found that as a substitute for the stock solution of Giemsa's stain, the following stain works very satis-

[1] Komp, W. H. W.: Additional Notes on Preparation and Examination of Thick Blood Films for Malaria Diagnosis, U. S. Pub. Health Rep., *48*:875–884 (July 28), 1933.

[2] Johnson, F. B.: Diagnosis of Malaria by Thick Blood Film Method, South. Med. and Surg., *95*:185–187 (Apr.), 1933.

factorily: Dissolve 0.3 Gm. azure II eosin in 13 c.c. chemically pure anhydrous glycerin at 60° C., in a water bath; add 37 c.c. of chemically pure acetone-free methyl alcohol at 60° C. Handle the slide very gently, and allow it to stain for one and a half hours. Slides which show a central brown spot after staining have not been stained long enough, and should be restained. Then stand the slide vertically in water for from five to twenty minutes. Let the slide dry in the air without blotting, and without heating. Examine with an oil-immersion lens. The leukocytes will be stained, but the erythrocytes will be laked. The malarial organisms will have a deep red chromatin dot and blue-gray cytoplasm, the size and appearance depending upon the stage of development of the plasmodium. Their appearance can be studied in comparison with the thin film, which has been stained with Wright's stain, but the organisms will be found in far greater number in the thick film.

4. Genus Babesia.—The proper position of this genus is uncertain. It is placed among the flagellates by some. The chief member is *Babesia* (*Piroplasma*) *bigeminum*, the cause of Texas fever in cattle. It is a minute, pear-shaped organism, lying in pairs within the red blood corpuscles, and is transmitted by the tick, *Boophilus annulatus*. Other ticks are carriers of this and similar infections among cattle in other parts of the world than Texas.

CLASS INFUSORIA

Subclass Ciliata

The conspicuous feature of this class is the presence of cilia. These are hairlike appendages, which have a regular to-and-fro motion instead of the irregular lashing motion of flagella. They are also shorter and more numerous than flagella, and usually cover the greater part of the surface. Most infusoria are of fixed shape and contain two nuclei. Contractile and food vacuoles are also present. Encystment is common. Only one species is of medical interest. Certain ciliated structures, which have been described as infusoria, notably in sputum and nasal mucus, were probably ciliated body cells.

1. Genus Balantidium.—(1) **Balantidium coli.**—This parasite, formerly called *Paramoecium coli*, is an occasional inhabitant of the colon of man, where it penetrates into the mucous membrane and produces a diarrheal condition resembling amebic dysentery. Infection is most frequent among farmers, and in some cases has been

associated with the symptoms of pernicious anemia. It is an actively moving oval organism, about 60 to 100 μ long and 50 to 70 μ wide, is covered with cilia, which are arranged in longitudinal rows, giving a striated appearance, and contains a bean-shaped macronucleus, a globular micronucleus, two contractile vacuoles, and variously sized granules (Figs. 230 and 231). At the anterior end is a funnel-shaped mouth. The parasite is so large that it can hardly be overlooked if present upon the slide and still active.

Its ordinary habitat is the large intestine of the domestic pig, where it apparently causes no disturbance. It probably reaches man in the encysted condition.

(2) **Balantidium minutum** resembles *B. coli*, but is smaller,

Fig. 230.—*Balantidium coli* (about × 350) (after Eichhorst).

Fig. 231.—Photomicrograph of *Balantidium coli* in feces (about × 500).

measuring 20 to 30 by 15 to 20 μ. It has been found a few times in diarrheal stools.

PHYLUM PLATYHELMINTHES

The old phylum Vermidea has been subdivided into three phyla, those which are of interest here being the Platyhelminthes and Nemathelminthes, the flat worms and the round worms respectively. Of these, many species are parasitic in man and the higher animals. In some cases man is the regular host; in others the usual habitat is some one of the animals, and the occurrence of the worm in man is more or less accidental. Such are called *incidental parasites*. Only those worms that are found in man with sufficient frequency to be of medical interest are mentioned here.

The most important means of clinical diagnosis of infection by

either the flat worms or the round worms is the finding of ova. In many cases the ova are so characteristic that the finding of a single one will establish the diagnosis. In other cases they must be carefully studied and a considerable number measured. While ova from the same species will naturally vary somewhat, the average size of a dozen or more is pretty constant, probably as much so as is the case with different species of birds. The measurements given here are mainly those accepted by Stiles or Ward.

PHYLUM PLATYHELMINTHES

(Flat Worms)

Class **Trematoda**.—Flukes. Unsegmented, leaf-shaped, with alimentary tract.

Genus	*Species*
Fasciola.	*F. hepatica.*
Opisthorchis.	*Op. felineus.*
Clonorchis.	*C. sinensis.*
Fasciolopsis.	*F. buski.*
Paragonimus.	*P. westermanii.*
Schistosoma.	*S. haematobium.*
	S. mansoni.
	S. japonicum.

Class **Cestoda**.—Tapeworms. Segmented, ribbon-shaped, with no alimentary tract.

Genus	*Species*
Taenia.	*T. saginata.*
	T. solium.
	T. echinococcus.
Hymenolepis.	*H. nana.*
	H. dimininuta.
Dipylidium.	*D. caninum.*
Diphyllobothrium.	*D. latum.*

CLASS TREMATODA

The trematodes, commonly known as "flukes," are flat, unsegmented, generally tongue- or leaf-shaped worms. They are comparatively small, most species averaging between 5 and 15 mm. in length. Trematode infection is uncommon in this country and nearly all the cases are imported.

Most species have two radially striated sucking disks: An oral sucker surrounding the mouth at the anterior end, and a ventral sucker on the ventral surface of the anterior third of the body. The digestive tract is incomplete, without anus. The short esophagus divides into two intestines which pass backward in the lateral zones of the body and end as blind tubes. In some cases, for example, *F. hepatica*, the intestine gives off many lateral branches.

The nervous system consists merely of two ganglia with connecting fibers and several fine nerves which run posteriorly.

The excretory system includes numerous scattered excretory cells and a series of fine canals which unite to form an excretory duct which opens to the surface in an excretory pore situated dorsally or posteriorly.

Respiratory and circulatory systems are lacking.

Nearly all species are hermaphroditic, having the reproductive organs of both sexes. The male organs consist of two or more testes, usually situated near the center or back part of the body, and a spermatic duct which ends in a small cirrus or copulatory organ at the genital pore. This genital opening is generally situated near the ventral sucker. The female organs consist of an ovary, usually lying in front of the testes, a short oviduct, and a coiled tubular uterus, which opens to the surface at the genital pore. The oviduct receives the secretion from the numerous vitelline or yolk glands which lie along the lateral borders of the parasite. The junction of the oviduct and uterus is known as the ootype and receives the shell materials from neighboring shell glands. Most of the above-mentioned structures are shown in Figs. 232, 233, and 234.

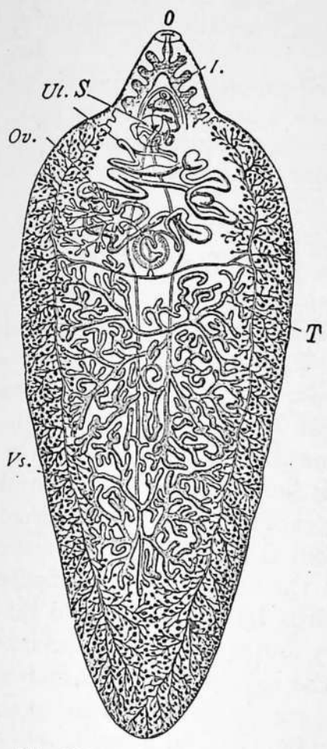


Fig. 232.—The liver fluke *Fasciola hepatica*, showing internal structure: *I.*, intestine; *Vs.*, vitelline glands; *Ov.*, ovary; *O*, oral aperture; *Ut*, uterus; *S*, ventral sucker; *T*, testes. The two vasa efferentia can be seen running upward in the midline. The branches of the intestine are shown only in the head-cone. Intermediate host is a water snail, *Limnaeus truncatulus* Müll. (After Claus.)

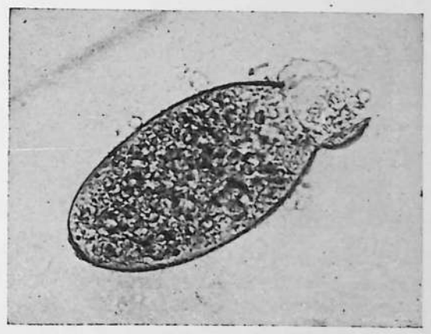


Fig. 233.—Egg of the liver fluke, *Fasciola hepatica*, with lid forced open by pressure on the cover glass (photograph, × 250).

The germinal cells are fertilized in the oviduct by spermatozoa derived from the same or another individual. These enter by way of the genital pore and uterine canal. After fertilization the yolk material is added, and

the egg then receives the shell substance and passes on into the uterus and thence to the genital pore. The diagnosis of fluke infection rests upon the finding of eggs. The number of eggs is enormous, sometimes running into the tens of thousands. The eggs of nearly all species are operculated (provided with a lid), the only important exception being the several species of the genus *Schistosoma*. This genus also differs from the other flukes in that the sexes are separate.

Development takes place by a complicated process of alternation of generations, the intermediate host being usually a snail, mussel, or crab. In some cases there are two intermediate hosts, parasitized in succession. The life history of *Fasciola hepatica* may be taken as typical of the group.

1. Genus Fasciola.—Fasciola hepatica.—The "liver fluke" inhabits the bile ducts of numerous herbivorous animals, especially sheep, where it is an important cause of disease. It brings about obstruction of the bile passages, with enlargement and degeneration of the liver—"liver rot." This fluke is readily obtained for study. The adult fluke is leaf shaped, the average size being about 2.8 by 1.2 cm. The anterior end projects like a beak (head-cone 3 to 4 mm. long—Fig. 232). The numerous small vitelline glands lie along the lateral and posterior borders, giving a granular appearance which in stained specimens sharply marks off this zone from the central field of the body. The central field, which has much the shape of the parasite itself, is occupied in its posterior two thirds by the branching testicular tubules and anteriorly by the ovary and coiled tubular uterus which is usually easily seen because of the eggs which it contains. Ova appear in the feces of the host. They are yellowish brown, oval, operculated, and measure about 130 to 140 by 75 to 90 μ.

The life history of *F. hepatica* is given in some detail because it is typical of the group. The eggs pass out with the feces of the host. After two or three weeks the embryo develops into a ciliated body resembling a ciliated infusorian, and called a *miracidium*. If the eggs reach water the miracidium escapes from the shell and swims about until it finds a certain water snail, *Limnaeus trunculatus* Müll., into which it penetrates and lodges in the pulmonary cavity. Here it changes into a cystlike structure, from the inner or germinal layer of which there develop a number of small wormlike organisms called *rediae*. These migrate to other parts of the snail's body and produce a second type of larvae called *cercariae*, which have much the form of minute tadpoles. Under certain conditions the rediae produce a second generation of rediae which, in turn, produce the cercariae. The cercariae leave the host, swim about in the water, and finally

attach themselves to submerged blades of grass, lose their tails, and become encysted. There they remain until taken into the digestive tract of a sheep or other appropriate host. Here they again become free and find their way up the bile passages to the liver, where they develop into adult flukes. In the case of certain other flukes (for example, the lung fluke) the cercariae do not encyst upon grass blades, but enter the bodies of certain aquatic animals (fish, crabs) and there encyst.

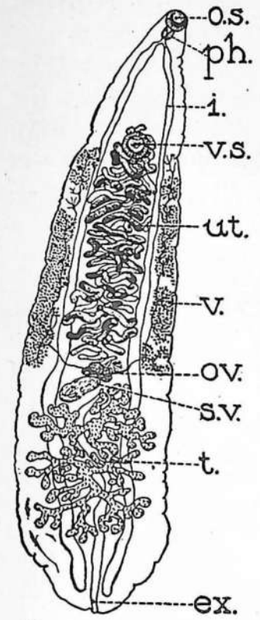


Fig. 234.—*Clonorchis sinensis*, showing internal structure: *o.s.*, Oral sucker; *ph.*, pharynx; *i.*, intestine; *v.s.*, ventral sucker; *ut.*, uterus; *v.*, vitellaria; *ov.*, ovary; *s.v.*, vesicula seminalis; *t.*, testis; *ex.*, excretory duct. *Bythinia striatula*, var. *japonica* is first intermediate host (× 6). (From Abt's Pediatrics.)

2. Genus Opisthorchis.—Opisthorchis felineus inhabits the gallbladder and bile ducts of the domestic cat and a few other animals. Infection in man has been repeatedly observed in Europe, and especially in Siberia. The body is flat, yellowish red in color, and almost transparent. It measures 8 to 11 by 1.5 to 2 mm. The eggs, which are found in the feces, are oval, with a well-defined operculum at the narrower end, and contain a ciliated embryo when deposited. They measure about 30 by 11 μ. Infection takes place through eating of insufficiently cooked fish. The life history is unknown, but

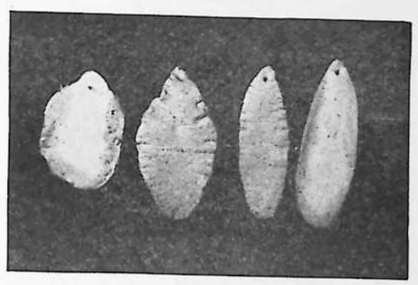


Fig. 235.—Photographs of *Fasciolopsis buski*, showing the appearance of fresh specimens (F. W. Goddard in The Jour. of Parasitology, June, 1919). Intermediate hosts, unknown species of either *Planorbis* or *Segmentina*, freshwater snails.

the last intermediate host is a fish (either *Idus melanotus*, *Leuciscus rutilus*, or *Tinca tinca*).

3. Genus Clonorchis.—Clonorchis sinensis (Fig. 234), like the preceding fluke, inhabits the gallbladder and bile ducts of domestic cats and dogs. It is, however, much more frequent in man, being a common and important parasite in certain parts of Japan and China.

The number present may be very great; over 4000 were counted in one case. The parasite resembles *Opisthorchis felineus* in shape and color. It is 10 to 14 mm. long and 2.5 to 4 mm. broad. The eggs have a sharply defined lid and measure 25 to 30 by 15 to 17 μ. When they appear in the feces they contain a ciliated embryo. The first larval stage is passed in a species of mollusk, *Bythinia striatula*, var. *japonica;* the second, in several species of fish, from which the larva passes to man when the fish are eaten without sufficient cooking.

4. Genus Fasciolopsis.—Fasciolopsis buski.—This fluke (Fig. 235) is parasitic in the duodenum of man, and is widespread in the East, notably in India, China, and Japan. A few imported cases have been reported in this country. When in considerable numbers it causes

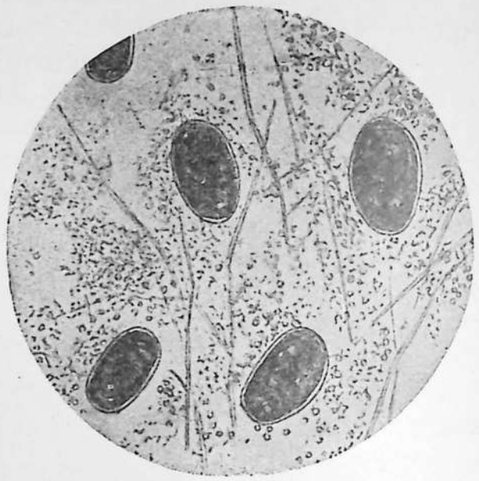


Fig. 236.—Sputum of man containing eggs of the lung fluke, about × 133 (after Manson).

a bloody diarrhea accompanied by fever. It is the largest of the flukes. The usual length is about 30 mm.; width, 10 to 12 mm.; thickness, 2 to 3 mm. The eggs are thin shelled, with granular contents, possess a minute operculum, and measure about 135 by 80 to 85 μ.

Barlow has worked out completely the life history of this fluke. Ninety-one days or more are required for the development of the adult fluke from the ovum. The cercariae after leaving the intermediate host encyst on water plants that are eaten raw by the Chinese. Carbon tetrachloride proved an effective anthelmintic in Barlow's experience.

5. Genus Paragonimus.—Paragonimus westermanii, called the "lung fluke," is also a common parasite of man in Japan, China, and Korea. It inhabits the lung, causing the formation of small cav-

ities. Moderate hemoptysis is the principal symptom. Ova are readily found in the sputum (Fig. 236); the worms themselves are seldom seen, except *postmortem.* The worms somewhat resemble a coffee bean in size and shape. They are faintly reddish brown in color, egg shaped, with the ventral surface flattened, and measure 8 to 10 by 4 to 6 mm. The ova are thin shelled, operculated, brownish yellow, and measure from 87 to 100 by 52 to 66 μ.

There are two intermediate hosts, a mollusk in which the cercariae are formed, and a fresh-water crab (a common article of food in Japan) in which they encyst. The encysted forms have also been found in fresh-water snails.

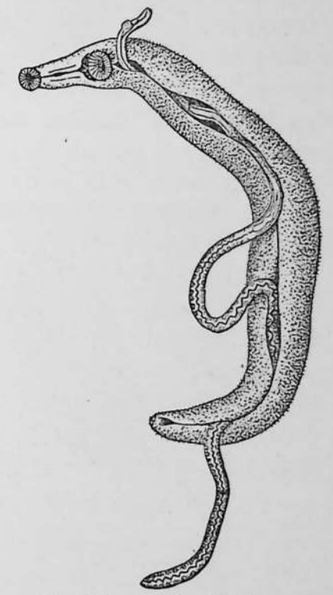


Fig. 237.—A blood fluke, *Schistosoma haematobium,* male and female (× 12) (after Looss). Intermediate hosts are snails, *Bullinus brocchi, Physopsis africana.*

According to Ward, three distinct species have been confused under the name *P. westermanii:* The original form, *P. westermanii,* found in the tiger; the American lung fluke, *P. hellicōtti,* thus far found only in cat, dog, and hog; and the Asiatic lung fluke of man, *P. ringeri,* described above.

6. Genus Schistosoma.—(1) Schistosoma haematobium.—This trematode, frequently called *Bilharzia haematobia,* is an extremely common cause of disease (bilharziasis or Egyptian hematuria) in Northern Africa, particularly in Egypt.

Unlike the other flukes, the sexes are separate. The male is 12 to 14 mm. long and 1 mm. broad. The body is flattened and the lateral edges curl ventrally, forming a longitudinal groove, in which the female lies (Fig. 237). The latter is cylindric in shape, about 20 mm. long and 0.25 mm. in diameter. The eggs are elongated ovals, about 120 to 190 μ long, and 50 to 73 μ broad, yellowish in color, and slightly transparent. They possess no lid such as characterizes the eggs of most of the trematodes, but are provided with a thorn-like spine which is placed at one end (Fig. 238). Within is a ciliated embryo.

In man the worm lives in the veins, particularly those of the bladder and rectum, leading to obstruction and inflammation. The eggs penetrate into the tissues and are present in abundance in the

mucosa of the bladder and rectum. They also appear in the urine and, less commonly, in the feces.

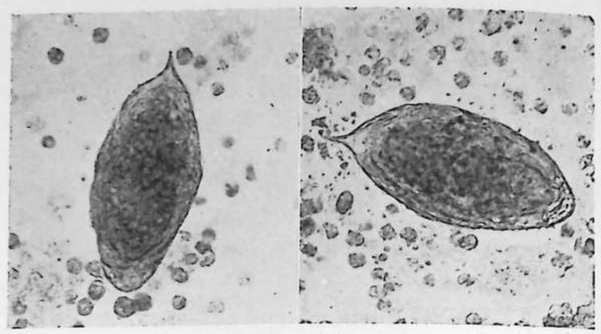


Fig. 238.—Ova of *Schistosoma haematobium* with pus corpuscles in urine (photographs, × 250).

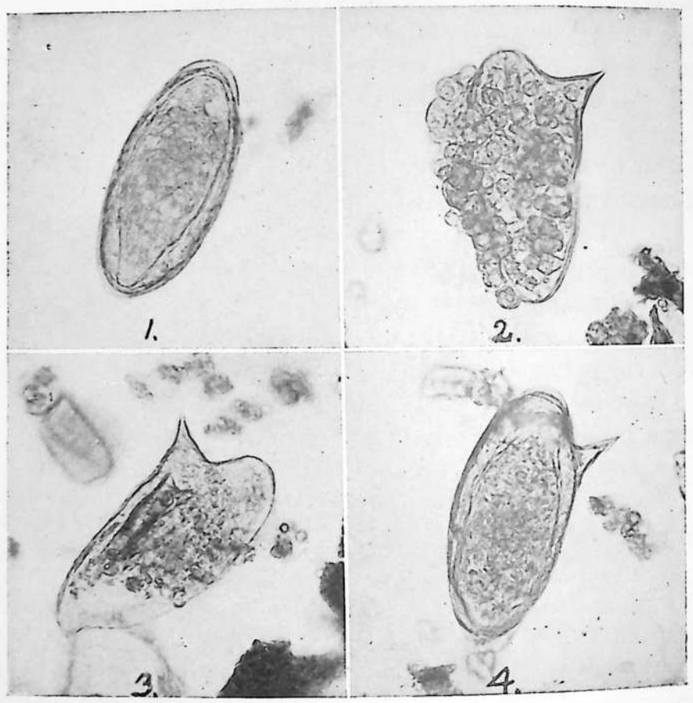


Fig. 239.—Ova of *Schistosoma mansoni:* 1, With spine out of focus; 2, in a clump of red blood cells; 3, apparently unfertilized; 4, usual appearance (photographs, × 250). Intermediate hosts are snails, *Planorbis boissyi*, or *Planorbis centrimentralis*.

The life history is similar to that given for *Fasciola hepatica*. A species of snail serves as intermediate host, and infestation of man

apparently takes place both by mouth and through the skin. The tadpole-like cercariae have forked tails.

(2) **Schistosoma mansoni.**—It has long been observed that schistosoma eggs in the urine have usually a terminal spine, while in the feces the lateral spine is more common. It is now known that the lateral-spined egg is that of a distinct species, to which the name *Schistosoma mansoni* has been given. It is found in Africa along with *Schistosoma haematobium*, but is especially prevalent in the West Indies and Central America. The adult worms closely resemble the male and female of *S. haematobium*. They inhabit the rectal and portal veins, and ova appear in the feces, where they are very easily recognized from their size and the characteristic spine (Fig. 239 and Plate XII). They are light yellow in color, measure 112 to 162 by 60 to 70 μ, and are provided with a clean-cut, sharply pointed spine, which is situated at the juncture of the last and third quarters of the egg, and is directed backward. Within the egg is a ciliated embryo (miracidium) which can be seen without difficulty. The life history is very similar to that of *S. haematobium*.

(3) **Schistosoma japonicum** resembles *S. haematobium* morphologically, but both the male and female are smaller. The ova, which appear in the feces, are ovoid, thin shelled, and without lid or spine. They average 83 by 62 μ in size, and contain a ciliated embryo. The worm inhabits the portal, and probably also other veins. A freshwater snail (some species of *Planorbis*) serves as intermediate host. The rice fields are often the place of infection.

Class Cestoda

The cestodes, or tapeworms, are very common parasites of both man and the animals. In the adult stage they consist of a linear series of flat, usually rectangular segments (proglottides), at one end of which is a smaller segment, the scolex or "head and neck," especially adapted by means of sucking disks and hooklets for attachment to the host. The series represents a colony, of which the scolex is ancestor. The proglottides are sexually complete hermaphroditic individuals which are derived from the scolex by budding, and the segment most distant from the scolex is thus the oldest. With the exception of the immature segments, near the scolex each contains a uterus filled with ova.

In general each proglottis (Fig. 240) contains the same reproductive organs with the same functions as have been described for the trematodes (p. 539). These are not found in the young segments adjacent to the scolex,

but are fully developed in the middle and latter part of the chain, the male organs maturing first. The testes generally consist of a great number of very small glands scattered throughout the segment. Their ducts unite to form a single vas deferens leading to the genital pore, which is situated either upon one lateral border of the segment or upon its flat surface.

The sperms from the same or another proglottis reach the genital pore and are carried by a tubular vagina to the oviduct which is situated in the posterior part of the proglottis. Here the germinal cells derived from the two ovaries are fertilized and receive their yolk material which is the secretion of numerous vitelline glands as in the case of the trematodes. The eggs pass into the ootype, where the shell substance is added, and thence on into the uterus. The uterus is either a convoluted tube which opens in

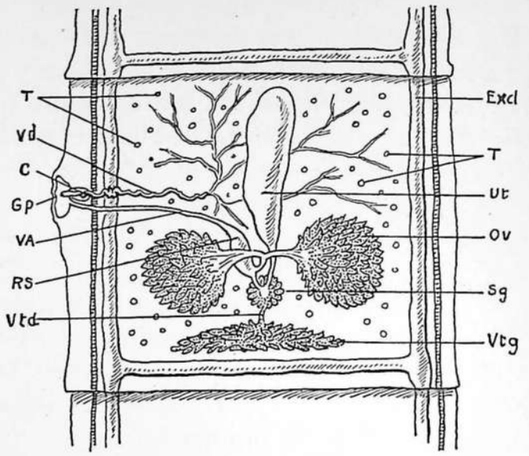


Fig. 240.—Diagram of the anatomy of a tapeworm (*Taenia saginata*): *T*, Testes; *Vd*, vas deferens; *C*, cirrus; *Gp*, genital pore; *Va*, vagina; *Rs*, receptaculum seminis; *Vtg*, vitelline glands; *Vtd*, vitelline duct; *Sg*, shell gland; *Ov*, ovaries; *Ut*, uterus, side branches not yet developed; at the sides are the two longitudinal nerves, also the excretory canals (*Excl*), which are connected by a transverse canal in each segment. (From Rivas' Human Parasitology.)

a birth pore (separate from the genital pore) upon a flat surface of the segment, usually anteriorly; or it is a closed sac which extends longitudinally in the midline, and as it fills with eggs sometimes comes to have lateral branches. In the latter case there is no birth pore and the eggs accumulate until the segment is packed with them, when the male organs and sometimes also the ovaries atrophy; the eggs are liberated only when the proglottis disintegrates.

The excretory apparatus consists of numerous scattered excretory cells with capillaries which empty into four excretory canals running the full length of the chain, two near each lateral border, and emptying posteriorly. In some species these communicate by a cross canal in each proglottis. Also running the length of the chain, near the lateral borders, are two or

more nerve cords derived from ganglia in the scolex. There is no digestive system, the parasite taking its nourishment by absorption through its surface. Circulatory and respiratory systems are also absent.

All the tapeworms pass a larval stage in the tissues of an intermediate host, which is rarely of the same species as that which harbors the adult worm. Within the ova which have developed in the proglottides of the adult worm, and which pass out with the feces of the host, there develop embryos, or *oncospheres*, each provided with three pairs of horny hooklets. When the egg is taken into the intestine of a suitable animal the oncosphere is liberated and penetrates to the muscles or viscera, and there, in the case of most of the tapeworms, forms a cyst in which develop usually one, but sometimes many, scolices, which are identical with the head of the adult worm. When the flesh containing this cystic stage is eaten without sufficient cooking to destroy the scolices the latter attach themselves to the intestinal wall and produce adult tapeworms by budding. The oncosphere of some of the tapeworms leaves the egg in the open and exists for a time as a free-living larva before entering the intermediate host.

Ordinarily only the adult stage occurs in man. In the case of *Taenia echinococcus* only the larval stage is found. *Taenia solium* may infest man in either stage, although the cystic stage is rare.

The large tapeworms, *Taenia saginata*, *T. solium*, and *Diphyllobothrium latum*, are distinguished from one another mainly by the structure of the scolex and of the uterus. The scolex should be studied with a low-power objective or a hand lens. The uterus is best seen by pressing the segment out between two glass slides.

Usually the patient picks out the main body of the worm and brings it in triumph, but too often the small head is lacking and one is left to guess whether it was lost with the feces. After a considerable portion of the worm has been expelled, some weeks or months may elapse before ova or segments again appear in the feces.

Since the head, or scolex, is the ancestor from which the worm is formed in the intestine, it is important, after giving a vermifuge, to make certain that the head has been passed with the worm. Should it remain, a new worm will develop. The technic described by Magath and Brown has proved most satisfactory at The Mayo Clinic. The patient is prepared by having him abstain from lunch and supper on the day previous to treatment, although black coffee or tea may be taken freely. At 6 P. M. he takes from 15 to 30 Gm. magnesium sulfate, and the following morning at 6 A. M. he takes the same dose. He is allowed no breakfast, and after his bowels have moved, he is given 30 c.c. of the following emulsion: Oleoresinae aspidii 3 Gm.; pulv. acaciae 4 Gm.; aqua dist. q. s. ad 30 c.c. One hour later he is

given a second 30 c.c. of the same emulsion. Two hours later he is given 30 Gm. of magnesium sulfate; two hours after this a large soapsuds enema is given. All stools are saved in a container and taken, together with the stool passed before the administration of the drug, directly to the laboratory. To make sure of finding the head the entire quantity of feces is passed through a coarse sieve (Fig. 241).

The principal tapeworms found in man belong to the genera Taenia, Hymenolepis, and Diphyllobothrium.

1. Genus Taenia.—(1) **Taenia saginata.**—This, the beef tapeworm, is the common tapeworm of the United States, and is widely

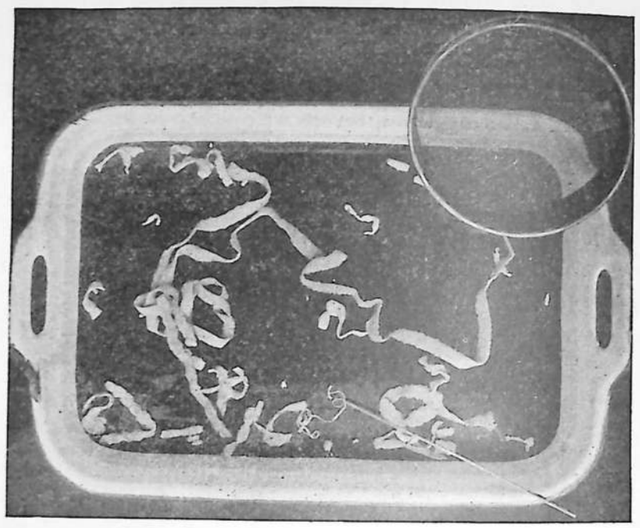


Fig. 241.—Apparatus used for recovery of the heads of tapeworms. The sieve is a No. 20 mesh. The enamel pan is painted black with asphalt paint. The wooden applicator points to the head which is relatively much smaller than the segments usually seen by the patient.

distributed over the world. Its length is generally about 4 to 8 meters. The scolex is about the size of a large pin-head (1.5 to 2 mm. in diameter), and is surrounded by four sucking disks, but has no hooklets (Fig. 242). The neck is about 1 mm. wide. The terminal segments, which become detached and appear in the feces, measure about 18 to 20 mm. long by 4 to 7 mm. wide. The genital pore lies at the top of an elevation upon one of the margins of the segment, and the side upon which it is situated alternates in adjoining segments. The uterus extends along the midline of the segment and gives off twenty to thirty branches upon each side (Fig. 252, *a*). There is no birth pore. Malformations of the segments are frequent.

The larval stage is passed in the muscles of various animals, especially cattle. It rarely or never occurs in man, hence there is little or no danger of infection from examining feces.

The scolex is ingested with the meat, its capsule is dissolved by the digestive juices, and it attaches itself to the intestinal wall by means of its suckers. It then develops into the mature worm, which may grow very rapidly, even as many as ten segments being formed in a day.

Diagnosis rests upon the finding of segments, which usually come away singly, or of ova, in the feces. Since there is no birth pore the ova appear only when ripe segments disintegrate in the intestine, which, however, is a common occurrence. They are spheric or ovoid, yellow to brown in color, and have a thick, radially striated cortex

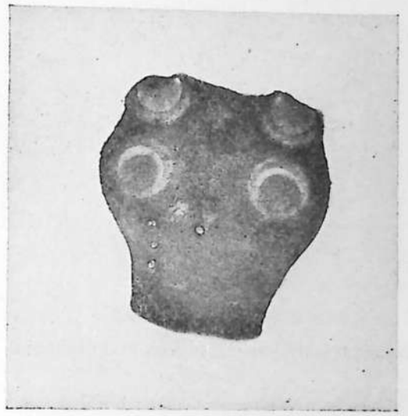


Fig. 242.—Head of *Taenia saginata*, showing four sucking disks (× 15).

which is commonly called the shell, although strictly speaking it is not a true egg shell since it is produced by the embryo (Fig. 243 and Plate XII). Within them the six hooklets of the embryo (oncosphere) can usually be made out as three pairs of parallel lines. The size of the ova varies from 20 to 30 μ wide and 30 to 40 μ long. Surrounding the egg, particularly when pressed out from the worm, is sometimes seen a broad transparent slightly granular zone called the vitelline membrane. Vegetable cells, which are generally present in the feces, are often mistaken for these ova, although there is seldom any great resemblance.

(2) **Taenia solium,** the pork tapeworm, is very rare in this country. It is usually much shorter than *Taenia saginata*. The scolex is about 0.6 to 1 mm. wide, is surrounded by four sucking disks,

and has a projection, or rostellum, with a double row of horny hooklets, usually twenty-six to twenty-eight in number (Fig. 244). The terminal segments measure about 5 to 6 by 10 to 12 mm. The uterus has only seven to fourteen branches on each side (Fig. 252, *b*).

The cysticercus stage occurs ordinarily in the muscles of the pig, but is occasionally seen in man, most frequently affecting the brain and eye (*Cysticercus cellulosae*). There is, therefore, danger of infection from handling feces.

The ova so closely resemble those of *Taenia saginata* as to be practically indistinguishable. They average about 31 to 36 μ in diameter and are usually spheric (Plate XII).

(3) **Taenia echinococcus.**—The mature form of this tapeworm inhabits the intestines of the dog and wolf, never of man. The larvae develop in cattle and sheep ordinarily, but are sometimes found in

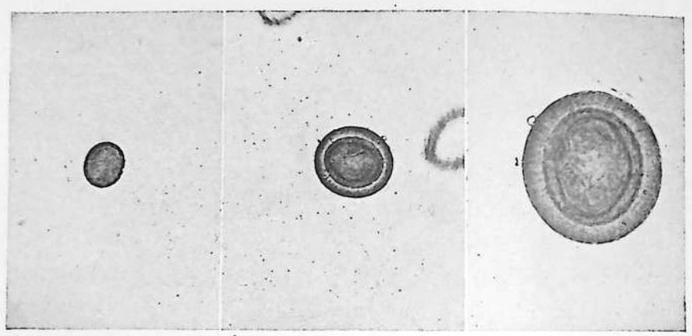


Fig. 243.—Eggs of *Taenia saginata*, magnifications 100, 250, and 500 diameters (photographs).

man, where they give rise to echinococcus or "hydatid" disease. The condition is unusual in North America, but is not infrequent in Central Europe, and is common in Greece, Iceland, Australia, and South America.

The adult parasite is 2.5 to 5 mm. long, and consists of only four segments (Fig. 245). It contains many ova, which resemble those of *Taenia saginata*. When the ova reach the digestive tract of man the embryos are set free and find their way to the liver, lung, or other organ, where they develop into cysts, thus losing their identity. The cysts grow very slowly, and after many years may attain the size of a child's head. Other cysts, called "daughter cysts," are formed within these. The cyst wall is made up of two layers, from the inner of which (the so-called "brood membrane") there develop larvae which are identical with the head, or scolex, of the mature parasite. These are ovoid structures 0.2 to 0.3 mm. long. Each has four lateral suckers

and a rostellum surmounted by a double circular row of horny hooklets. The rostellum with its hooklets is frequently invaginated into the body.

Diagnosis of echinococcus disease may be made in several ways:

(*a*) The complement-fixation method is reliable. The best antigen is cyst fluid, filtered through cotton and preserved with 0.5 per cent phenol, and the dose used in the test is one third or one fourth the anticomplementary unit. It keeps well, but just before use it should be heated at 60° C. for from fifteen to thirty minutes to reduce anticomplementary activity. Fluid from cysts in sheep can probably be obtained from the abattoirs of the large packing houses by special arrangement. Adequate controls should be run, including a strongly positive syphilitic serum.

Fig. 244.—Head of *Taenia solium* (Mosler and Peiper).

Fig. 245.—*Taenia echinococcus*, enlarged (Mosler and Peiper).

(*b*) The precipitin test is described on pages 662 to 665.

(*c*) The cutaneous reaction, using cyst fluid applied upon the skin, as in von Pirquet's test, or intradermally, as in the Mantoux test (Casoni test) is also reliable if adequate control tests be made. The reaction may appear within a few minutes; it is characterized by a large wheal, with pseudopod formation, and a zone of erythema. Rose and Culbertson[1] have reported that antigens prepared from a number of different cestodes give positive skin reactions in Echinococcus disease similar to the Casoni test. These antigens also can be used for the complement-fixation test.

[1] Rose, H. M., and Culbertson, J. T.: A Diagnosis of Echinococcus (Hydatid) Disease' Jour. Am. Med. Assn., *115*:594–598 (Aug. 24), 1940.

Culbertson, J. T., and Rose, H. M.: Further Observations on Skin Reactions to Antigens from Heterologous Cestodes in Echinococcus Disease, Jour. Clin. Invest., *20*:249–254 (May), 1941.

(*d*) Microscopic examination of the cyst fluid obtained at operation or autopsy is decisive. It is, however, unwise to make an exploratory puncture because of the danger of leakage of the fluid into the tissues or peritoneal cavity. This might induce anaphylactic shock or, at least, spread the disease by implantation. The cyst fluid is

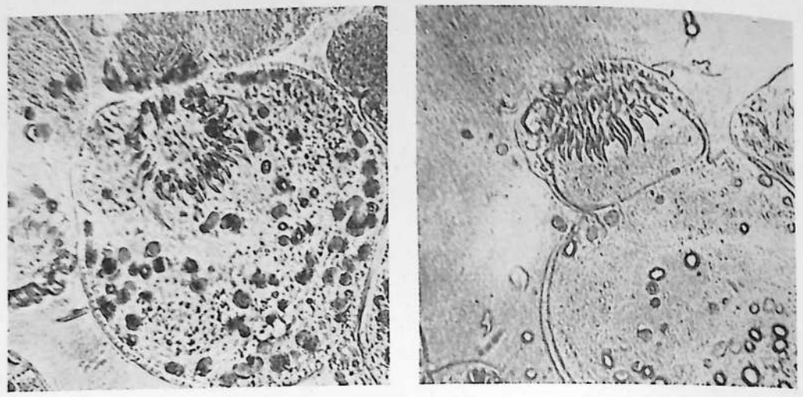
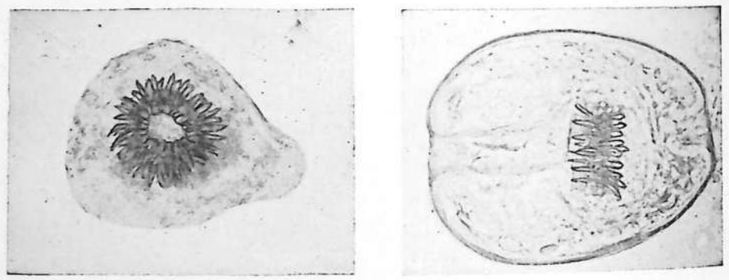


Fig. 246.—Scolices and free hooklets of *Taenia echinococcus* from hepatic cyst (photographs about × 300).

clear, between 1.009 and 1.015 in specific gravity, and contains a notable amount of sodium chloride, but no albumin. Positive identification of the fluid depends upon detection of scolices, free hooklets which have fallen off from degenerated scolices, or particles of cyst



A B

Fig. 247.—Scolices of *Taenia echinococcus* from an hepatic cyst: A, Portion of a degenerated scolex showing circle of hooklets; B, a well-preserved scolex with crown of hooklets invaginated, a common appearance (photographs × 250).

wall which are characteristically laminated, and usually have curled edges. The lamination is best seen at the torn edge of the membrane. All of these structures can be found in fluid from the cysts or, less frequently, in the sputum or the urine, when the disease involves the lung or kidney (Figs. 78, 246, and 247). In such material the scolices

are usually much degenerated, and many of them have entirely lost the hooklets. The scolices and hooklets are readily found with the 16-mm. objective. The latter require a high-power eyepiece, owing to their pale color and small size; but, when they are found, their appearance is striking and characteristic (Fig. 246). Their length is about 25 to 40 μ.

The statistics of Vegas and Cranwell, also of Greenway, of more than 2000 cases in South America show more than 75 per cent localization in the liver. Magath's report of cases in the United States since 1902 showed localization in the liver in 69 cases, in the peritoneum in 10, in the lungs in 4, in the kidneys in 4, in the omentum in 4, and cysts elsewhere in 13; a total of 104 organs affected in 93 cases. Although a few cases have been reported in which the disease affected natives of the United States, in 95 per cent of the cases observed in this country the patients have been foreign born.

2. Genus Hymenolepis.—(1) **Hymenolepis nana,** the dwarf tapeworm (Figs. 248 and 249), is 1 to 4.5 cm. in length and 0.4 to 0.7 mm. in breadth at the widest part. The head is about 0.3 mm. broad and has a retractable rostellum with a crown of twenty-four to thirty hooklets. Its shape is generally described as globular, but depends somewhat upon the condition of the worm when killed. The segments number 150 to 200. There is no birth pore, and the eggs escape through disintegration of the segments.

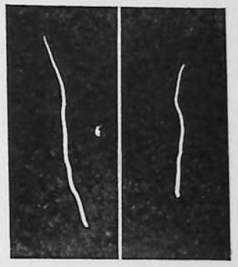


Fig. 248.—Dwarf tapeworm (*Hymenolepis nana*), adults. From photographs. Natural size.

Diagnosis must, in general, depend upon the discovery of ova in the feces, since the worms themselves are usually partly disintegrated when they leave the body and are recognized with difficulty. The ova are colorless, semitransparent, nearly spheric, and contain an embryo surrounded by two distinct membranous walls, between which is a broad zone of gelatinous substance (Fig. 250). The outer membrane is about 35 and 45 μ in its short and long diameters. The inner averages about 22 by 28 μ, and at each pole has a slight projection provided with indistinct filamentous processes, which may lie between the two membranes in such a way as sometimes to simulate a third membrane. The embryo, of which only the three pairs of hooklets are clearly seen, fills the space within the inner wall.

The worm is common in Europe and America, and is probably the most common of all the tapeworms of man in the United States.

It is most frequent in children, particularly in orphanages, and is generally present in large numbers, producing considerable digestive and nervous disturbance. Its small size, the fact that it is more or

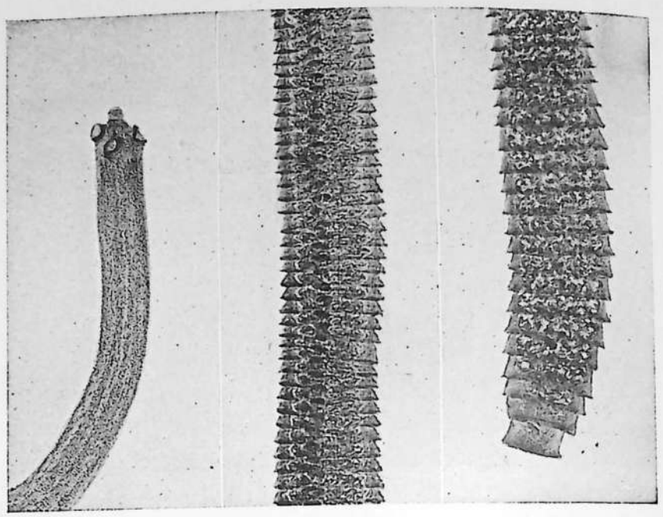


Fig. 249.—Dwarf tapeworm (*Hymenolepis nana*), head, middle segments, and terminal segments. Note the protruded rostellum and the three suckers. From stained and mounted specimens (photographs × 30).

less disintegrated in the intestine, and, especially, the infrequency of routine microscopic examination of the feces account for the failure to recognize its prevalence in the past. Now that attention has been

Fig. 250.—Ova of *Hymenolepis nana* in feces. The egg to the right was compressed by pressure upon the cover glass (photographs). The figure at the left is magnified 250 diameters; the other two, 500 diameters.

directed to it undigested banana fibers in the diarrheal stools of children have been very frequently mistaken for it (see Fig. 203).

The mode of dissemination is not definitely known. It is presumed that the ova are transmitted to man through contamination of food by excrement of rats and mice. A similar dwarf tapeworm which is now believed to be identical with *H. nana* is a very common parasite of rats. Apparently both larval and adult stages occur in the intestine of the same host. A larval stage has also been found in certain insects.

(2) **Hymenolepis diminuta** is a common intestinal parasite of rats. A few cases of infection in man have been reported in America. The parasite measures 20 to 60 cm. in length, is very narrow, and is composed of 600 to 1300 segments. The scolex lacks hooklets. The ova resemble those of *H. nana*, but the outer shell is thicker and sometimes radially striated, and the filamentous processes between the two membranes are lacking. The egg is 56 to 80 μ in diameter, and the inner shell, which contains a six-hooked embryo, measures about 24 by 40 μ.

3. Genus Dipylidium.—Dipylidium caninum, sometimes called *Taenia elliptica*, is a very common tapeworm of dogs and cats. Its length is 15 to 35 cm. The head, globular in shape, is armed with hooklets. Terminal segments are shaped like cucumber seeds, 8 to 11 mm. long and 1.5 to 3 mm. broad. Ova are spheric, 43 to 50 μ in diameter, and thin shelled. They contain a six-hooked embryo, 32 to 36 μ in diameter. The eggs are grouped in packets of eight to fifteen, and are usually passed from the bowel within the proglottides.

The intermediate host is the flea or louse. Infection of human beings is rare, and is mostly confined to children, who are probably infected from getting lice or fleas of dogs or cats into their mouths.

4. Genus Diphyllobothrium.—Diphyllobothrium latum (Dibothriocephalus latus), the fish tapeworm, sometimes reaches 12 meters in length, although it is generally not more than one half or one third as long. When several worms are present they are much shorter, often only 1.5 to 2 meters. The head is a flattened ovoid, about 1 mm. broad and 1.5 mm. long. It is unprovided with either suckers or hooklets, but has two longitudinal grooves which serve the same purpose (Fig. 251). The length of the segments is generally less than their breadth, mature segments measuring about 3 by 10 or 12 mm. The uterus, which is situated in the center of the segment, is roset shaped (Fig. 252, *c*) and brown or black in color. The ova are discharged through a birth canal, and are not retained until the proglottid disintegrates, as is the case with tapeworms of the family *Taeniidae*. The number of segments sometimes exceeds 3000. As a rule, they do not appear in the feces singly, but in chains of considerable length.

The ova are usually easily found in the feces without resort to concentration methods, although they may be scarce or absent for a short time after passage of a considerable portion of the worm. The

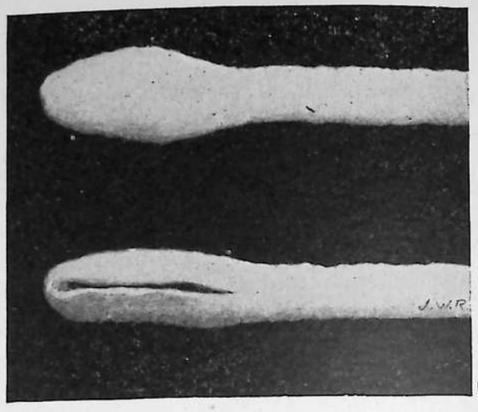
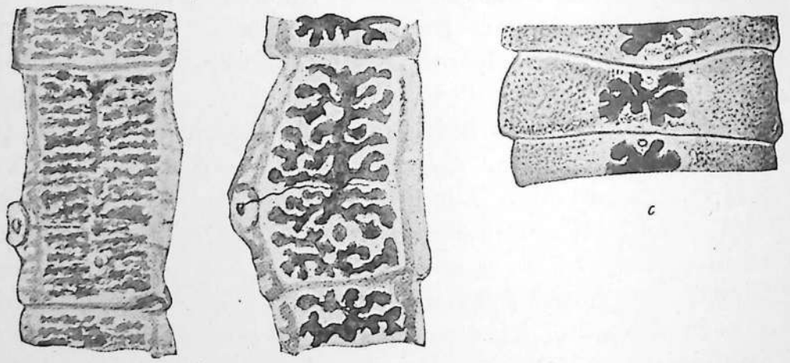


Fig. 251.—Head of the fish tapeworm, *Diphyllobothrium latum*. Above, flat side; below, edge showing characteristic groove (× 12).

eggs measure about 45 by 65 μ, are brown in color, and are filled with small spherules. The shell is thin and has a small hinged lid at one end. As the eggs appear in the feces the lid is not easily seen, but it



a *b* *c*

Fig. 252.—Segments of the three large tapeworms of man, showing arrangement of uterus: *a*, *Taenia saginata; b*, *Taenia solium; c*, *Diphyllobothrium latum* (× 5). (From P. J. Cammidge, The Feces of Children and Adults.)

may be demonstrated by sufficient pressure upon the cover glass to force it open (Fig. 253). The only other operculated eggs met with in man are those of the fluke worms. Magath has called attention to a very small projection or localized thickening of the shell at the end

opposite the lid, which is helpful in distinguishing this egg from that of the flukes. This may be seen, although not clearly, in Plate XII.

When the eggs reach water a ciliated embryo develops and hatches in about two weeks. This swims about until it finds a minute crustacean, cyclops, which is the first intermediate host. The crustaceans serve as food for fish, and the larvae thus reach the second intermediate host. They do not form cysts, but live in the muscles and certain organs of the fish as wormlike structures (plerocercoids) which may grow to a length of 2 or 3 cm. These are the infective larvae, and are found in various fish, notably the pike, burbot, grayling, and certain trout. Infection of man prevails only in regions where these fish are found. It is very common in Japan and in various countries of Europe, especially Ireland and the countries on the Baltic. Many cases of

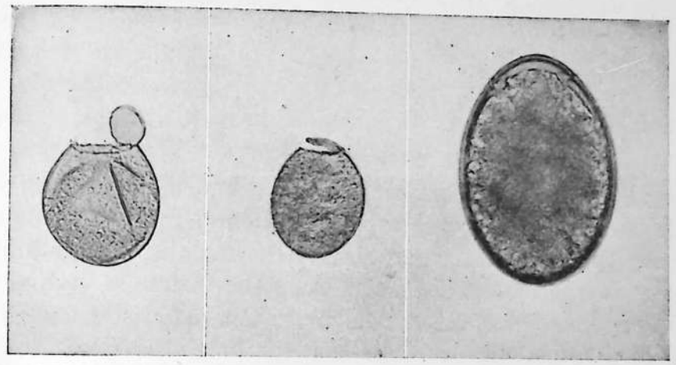


Fig. 253.—Ova of *Diphyllobothrium latum* (photographs × 250 and 500). The lids were forced open by pressure upon the cover glass.

infection have been reported in this country, a few of which were undoubtedly acquired here. Any locality in which cyclops and the favorable fish are native becomes a possible center of dissemination if the worm be introduced by infected immigrants. The longevity of the worm in the intestine is difficult to determine. Riley has reported a case in which the worm existed for at least thirteen years.

Diphyllobothrium latum is interesting clinically because in many cases it causes a very severe grade of anemia, which is indistinguishable from pernicious anemia. Why it should produce anemia in some cases and not in others is not altogether clear. There is apparently some factor other than the mere presence of the worm. In a case of Todd's in which six worms with a combined length of 75 feet were present there was no appreciable anemia. The anemia is probably connected with absorption of hemolytic poisons from segments disin-

tegrating in the intestine and may be absent when the segments are habitually passed intact. Eosinophilia may exist, but is not usual.

PHYLUM NEMATHELMINTHES
(Round Worms)

CLASS **Nematoda.**—Unsegmented, cylindric or fusiform, with alimentary tract.

Genus	*Species*
Anguillula.	*A. aceti.*
Ascaris.	*A. lumbricoides.*
Enterobius.	*E. vermicularis.*
Filaria.	*F. bancrofti.*
	F. philippinensis.
	F. perstans.
	F. diurna.
	F. medinensis.
Ancylostoma.	*A. duodenale.*
Necator.	*N. americanus.*
Strongyloides.	*S. stercoralis.*
Trichinella.	*T. spiralis.*
Trichuris.	*T. trichiura.*

CLASS NEMATODA

The nematodes, or round worms, are cylindric or fusiform worms, varying in length, according to species, from 1 mm. to 40 or 80 cm. As a rule, the sexes are separate, and the male is smaller and more slender than the female. In a few cases the female is viviparous; in most cases she deposits ova which are characteristic, so that the finding of a single egg may establish the diagnosis. The life history in some cases is simple; in others complicated. It will be dealt with in the descriptions of the several species. In general, the young are different from the adult, and must pass a certain larval stage of development before again reaching a host. An intermediate host is, however, necessary with only a few species.

The digestive tract of the nematodes is complete. The mouth is at the tip of the anterior end and is frequently surrounded by thick lips or papillae. The esophagus is a thick muscular tube with radially arranged fibers. At the posterior end there is a bulbous expansion; or there may be a constriction in the middle with an anterior and a posterior expansion (Fig. 259). The intestine is a straight thin-walled tube, ending in an anus near the tip of the posterior end of the worm.

The excretory system is drained by two lateral canals which usually unite anteriorly and end in an excretory pore on the ventral surface near the mouth. The nervous system consists of a nerve ring at the head end, a dorsal and a ventral nerve cord, and circular bridges connecting the cords. Vascular and respiratory systems are lacking.

The generative organs of the male consist of a single sinuous tube which is divisible into testis, spermatic duct, seminal vesicle, and ejaculatory duct. The last opens into the terminal portion of the rectum, which is known as the cloaca. Within the cloaca are one or two spicules or copulatory organs which may be projected and retracted. The generative organs of the female usually consist of two extensively coiled tubules, each divisible into ovary, oviduct, and uterus. These unite to form a short vagina which opens upon the ventral surface of the body posteriorly or near the middle.

Between the internal organs and the ectoderm is a cavity containing lymph.

1. Genus Anguillula.—Anguillula aceti.—This worm, commonly called the "vinegar eel," is usually present in vinegar. A drop of the vinegar, particularly of the sediment, will frequently show great numbers, all in active motion: Males, about 1 or 1.5 mm. long; females, somewhat larger and frequently containing several coiled embryos; and young, of all sizes up to the adult (Fig. 79, p. 159).

The vinegar eel is never parasitic, but is occasionally met with as a contamination in the urine (p. 159), and has there been mistaken for the larva of filaria or strongyloides.

2. Genus Ascaris.—Ascaris lumbricoides.—The female is 20 to 40 cm. long and about 5 mm. thick; the male, from 15 to 17 cm. long and 3 mm. thick. They taper to a blunt point anteriorly and posteriorly (Fig. 254). Their color is reddish or light brown.

At the anterior tip are three small papilla-like lips which can easily be seen with a hand lens. The posterior end of the male curls ventrally, and the cloaca near the posterior tip is provided with two copulatory spicules about 2 mm. long. The vulva of the female is situated on the ventral surface at the junction of the anterior and middle thirds of the body.

The common round worm of the hog, *Ascaris suilla*, is identical morphologically with *A. lumbricoides*, although somewhat smaller, and since it is easily obtained at slaughter houses serves well for class study. The interesting precipitin experiments of Schwartz[1] suggest that the two parasites are identical biologically. The similar but much smaller ascarids so frequently present in cats (*Belascaris cati*) and dogs (*Toxascaris canis*), and commonly known as "stomach worms," have been reported some ten or twelve times in children. They produced slight or no symptoms.

Ascaris lumbricoides is the common "round worm" so frequently found in children. Its habitat is the small intestine. Usually several

[1] Schwartz, B.: The Biological Relationships of Ascarids, Jour. Parasitology, *6*:115–123 (Mar.), 1920.

individuals are present and sometimes many, even 200 or more. Symptoms—nervous and gastro-intestinal—may or may not be evident. When numerous the worms may form abdominal tumors of considerable size, or even completely block the intestine. Adults are much less susceptible to infection than are children.

The diagnosis is made by detection of the worms or their ova in the feces. The worms (Fig. 254) seldom appear except as a result of anthelmintic treatment. The eggs are generally numerous. As a rule, microscopic examination of the feces shows one to several upon every slide, even when no more than one laying female is present in the intestine. Typical fertilized eggs are easily recognized. They

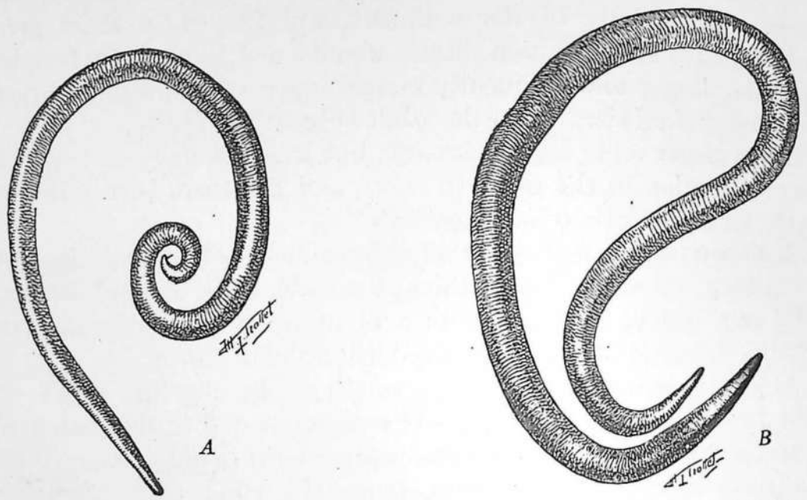


Fig. 254.—The common "round worm" *Ascaris lumbricoides*, natural size: *A*, Male; *B*, female. (After Brumpt.)

are elliptic, measuring about 45 to 50 by 60 to 75 μ, are yellow to brown in color, and have an unsegmented protoplasm. There is usually a crescentic clear space at each pole, between the contents and the shell (Fig. 255). The shell is moderately thick and smooth, and is covered with an irregular albuminous coating which gives it a roughly mammillated or sculptured surface (Fig. 256). Sometimes this coating is lost and the surface of the shell is then smooth. When only females are present in the intestine, and occasionally at other times, one finds unfertilized eggs. These are generally more elongated, have a thinner shell, and are filled with coarse granular contents which obliterate the crescentic clear spaces at the ends. Many are roughly globular and some are so extremely irregular in outline

(Fig. 257) as to bear little resemblance to an egg. Such unfertilized ova have doubtless many times passed unrecognized even by clinical microscopists of some experience.

The life history of *A. lumbricoides* has been worked out in detail by Stewart, Ransom and Foster, and Yoshida, and is more complicated than

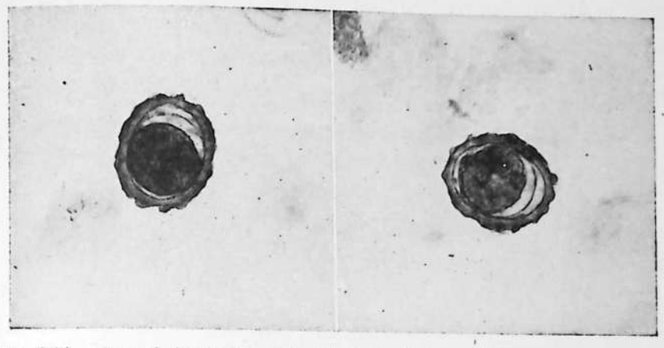


Fig. 255.—Ova of *Ascaris lumbricoides* in fresh feces (photographs × 250).

was formerly supposed. The eggs pass out of the host's intestine in the unsegmented form described above. Segmentation of the germ cell and development of the embryo take place in the open. Usually by the end of thirty days, not less than eighteen days even under most favorable conditions, the egg comes to contain a wormlike embryo, which remains without further development until taken into the intestine of an appropriate host.

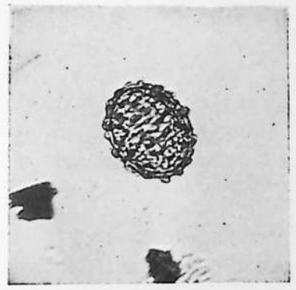


Fig. 256.—Egg of *Ascaris lumbricoides*, surface view (photograph × 250).

The eggs are extremely resistant and in the temperate zone may pass the winter without injury. Formaldehyde and certain other germicides have little injurious effect: Apparently they harden the albuminous envelop and fail to penetrate. In the 5 per cent formalin used for preserving feces for class demonstration many of the eggs continue slowly to segment, and some fully mature and remain alive even for a year or more. Phenol and the cresol preparations are most effective in destroying the eggs.

Unripe eggs are not infective. If ingested they pass harmlessly through the intestine. When, upon the other hand, matured eggs are ingested, the embryos leave the shells within twelve to eighteen hours. They then migrate to the lungs chiefly by way of the blood vessels, but also by boring through the tissues, piercing the intestinal wall and the diaphragm in their course, and penetrating into the lung from the pleural cavity. In experimental animals stray larvae can be found in the peritoneal cavity, and in the liver, spleen, and other abdominal organs. During migration the larvae undergo a process of development and grow from a length of 0.25 mm. when in the intestine to 1.5 or 2 mm. in the lung. Finally they appear in the trachea,

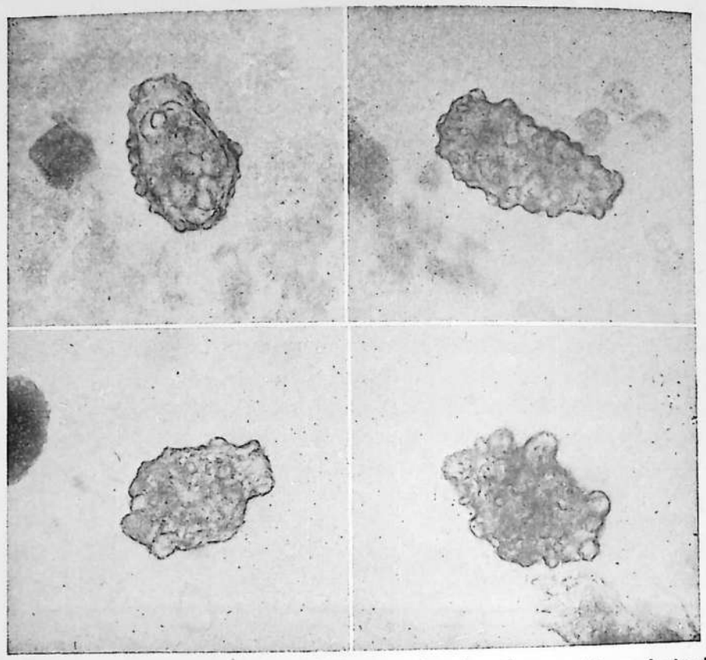


Fig. 257.—Unfertilized ova of *Ascaris lumbricoides*, showing the great irregularity in shape. Some of these eggs are extremely difficult to identify (photographs × 250).

migrate (or are carried in mucus) to the mouth, thence down the esophagus to the stomach, and on to the small intestine, where each grows into the familiar adult ascarid. In experimental animals the cycle of migration, from the intestine and back to it again, is completed in eight days or more. The abdominal organs are apparently not injured by the passage of the larvae, but the lungs show considerable hemorrhage. This has been suggested by Ransom as a possible important cause of pulmonary disease, even pneumonia, in children.

3. Genus Enterobius.—Enterobius vermicularis (Oxyuris vermicularis).—It is the well-known "threadworm" or "pinworm" which

matures in the small intestine and cecum and in the adult stage inhabits the colon and rectum, especially of young children. Its presence should be suspected in all unexplained cases of pruritus ani. The female is about 9 to 12 mm. long; the male, about 3 to 5 mm. (Fig. 258).

The cuticle is transversely striated. At each side of the head is a thin, transversely striated, cuticular expansion, which is usually prominent (Fig. 259). The bulbous esophagus can generally be clearly seen. The posterior end of the male is curled ventrally and near the tip is provided with a single copulatory spicule. The posterior end of the female tapers to form a straight, sharply pointed tail. The vulva is situated at about the junction of the anterior and middle thirds of the body.

The worms are not infrequently found in the feces, particularly after a copious enema; the ova, rarely. The latter are best found by scraping the skin with a dull knife at the margin of the anus, where they are deposited by the female, who wanders out from the rectum for this purpose, thus producing the troublesome itching. They are colorless and asymmetrically oval with one flattened side, are about 50 μ long by 16 to 25 μ wide, have a moderately thin, double-contoured shell, and when deposited contain a partially developed embryo (Fig. 260, and Plate XII). The diagnosis is best made by giving a purgative or a copious enema and searching the stool for the adult worms. It is essential to examine the stool by the method given on page 473, best in a large Petri dish over a dark background placed some distance below. Unless the water be perfectly clear the very small male pinworms are almost certain to be overlooked (Fig. 261).

Fig. 258.—*Enterobius vermicularis*, male and female, natural size (after Heller).

Infection takes place through swallowing the ova. Auto-infection is likely to occur in children; the ova cling to the fingers after scratching and are thus carried to the mouth. This is the greatest hindrance to successful treatment. Diagnosis can sometimes be made by finding the ova in the dirt beneath the fingernails.

Hall[1] has devised a cellophane-tipped, anal swab which is very useful for obtaining material to be examined for pinworm eggs. This is known as the NIH swab (Fig. 262). For the diagnosis of pinworm infestation it is best to make at least four swabs early in the morning

[1] Hall, M. C.: Studies on Oxyuriasis. I. Types of Anal Swabs and Scrapes, with a Description of an Improved Type of Swab, Amer. Jour. Trop. Med., *17*:445–453 (May), 1937

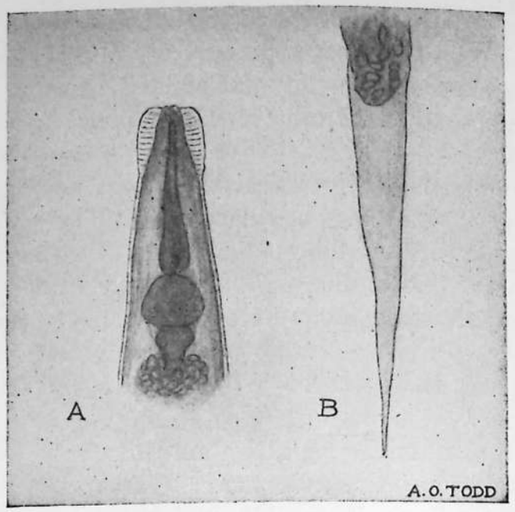
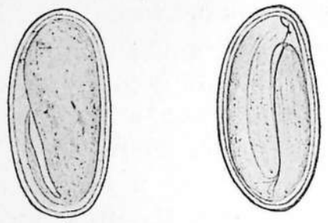


Fig. 259.—Head and tail of female "pinworm," *Enterobius vermicularis:* A, Head, showing the two cuticular appendages, and the beginning of the esophagus with its bulbous expansions; B, tail showing sharply pointed tip (× 35). Compare with Fig. 267.



a *b*

Fig. 260.—Eggs of *Enterobius vermicularis: a,* Freshly deposited, with tadpole-like embryo; *b,* twelve hours after deposition, with nematode-like embryo (× 500). (After Fantham, Stephens, and Theobald.)

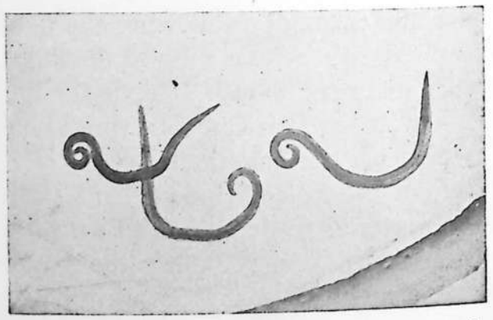


Fig. 261.—Three male pinworms (*Enterobius vermicularis*). Note the curled tail (photograph × 10).

on different days, and preferably not on consecutive days, before a negative finding is accepted as evidence of the absence of pinworms.

Kofoid and White have reported the egg of an unknown nematode evidently related to *Enterobius vermicularis* in the feces of 429 recruits among 140,000 examined at Camp Travis, Texas. These men came from 22 states. Adults or larvae were not seen, and the name *Oxyuris incognita* was tentatively given. Sandground more recently showed that these ova are those

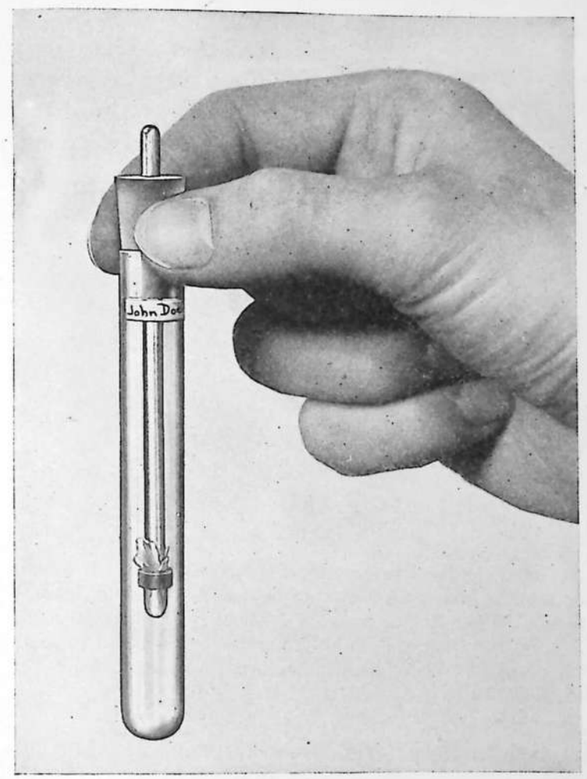


Fig. 262.—NIH anal swab (Eli Lilly and Co.).

of the free-living root nematode, *Heterodera radicicola*. These ova may be ingested with vegetable salads, and pass unchanged through the alimentary tract. They are larger than those of any known human intestinal nematode, ranging up to 68 by 133 μ, and averaging about 40 by 95 μ.

4. Genus Filaria.—(1) **Filaria bancrofti.**—The adults are thread-like worms, the male about 4 cm., the female about 8 cm. long. They live in pairs in the lymphatic channels and glands, especially those

of the pelvis and groin, and often occur in such numbers as to obstruct the flow of lymph. This is the most common cause of elephantiasis and chyluria. Infection is very common in tropical and subtropical countries, where in some regions as high as 50 per cent of the natives harbor microfilariae. Even as far north as Charleston, S. C., Johnson has found over 19 per cent of infection among the poorer classes. Of these, only one fourth showed any symptoms referable to the filariae. Surveys in other parts of the southern states have shown much lower percentages of infection.

Fig. 263.—Filarial larva in blood (photograph × 300). The larva is contracted, hence appears somewhat less slender than is usual. (From Beattie and Dickson's A Text-book of General Pathology, by kind permission of William Heinemann, Publisher.)

The female is viviparous, and produces vast numbers of larvae, which appear in the circulating blood. These are conveniently called

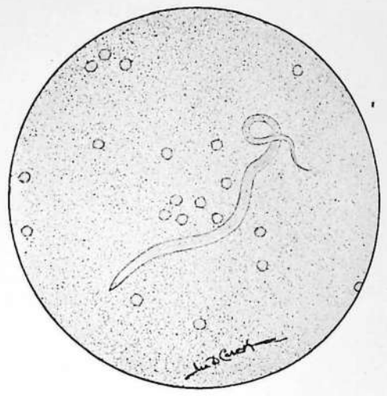


Fig. 264.—Larva of *Filaria bancrofti* in chylous hydrocele fluid; length, 300 μ; width, 8 μ. The very transparent sheath is not shown. A number of red blood corpuscles also appear (studied through courtesy of Dr. S. D. Van Meter).

microfilariae; the name *Filaria sanguinis hominis* which was formerly applied to them is incorrect, since they do not constitute a species but are merely larval forms. These larvae are slender, being about as wide as a red corpuscle and 0.2 to 0.4 mm. long (Fig. 263), and are very active, although, owing to the fact that they are inclosed in a loose transparent sheath, they do not move about from place to place. They are found in the peripheral blood chiefly at night, being usually easily demonstrable by 8 o'clock and reaching their maximum number—which may be enormous—about 2 A. M. By a concentration method a few can usually be demonstrated during the day. Take about 1 c.c. of blood from a vein and place it in 5 c.c. of 2 per cent

acetic acid. Mix well and centrifugalize. Spread the sediment, which is not abundant, on slides and examine in the moist state. The larvae will not be active as they are in fresh smears. The slide may be dried, fixed, and stained with hematoxylin. In the case of a medical student from Puerto Rico with no symptoms, Smith and de Rivas found 30 microfilariae in a cubic centimeter of blood at 4 P. M., and 6500 in a cubic centimeter at 2 A. M. If the patient change his time of sleeping, they will appear during the day. The periodicity is apparently dependent primarily upon the state of the capillaries and secondarily upon the motility of the species.

Infection is carried by certain species of mosquito, mostly belonging to the genus *Culex*, which act as intermediate host.

Diagnosis rests upon detection of larvae in the blood, but the number of larvae found bears little relation to the severity of the symptoms, since the symptoms are largely mechanical and depend upon the localization of the adults within the body.

The larvae are sometimes found in urine and in chylous fluids from the serous cavities. Their motion is then usually less active than when in blood. That shown in Fig. 264 was alive sixty hours after removal of the fluid. Larvae were present in the blood of the same patient.

A number of other filariae whose larvae appear in the blood are known, some of them only in the larval stage. Among these are *Filaria philippinensis* and *F. perstans*, which exhibit no periodicity, and *F. loa*, whose larvae appear in the blood during the day. The adult of the last named is especially frequent in the orbit and beneath the conjunctiva.

(2) **Filaria medinensis,** the "guinea worm," is a very interesting and important worm of Africa and Southern Asia. It has been thought to be the "fiery serpent" which molested the Children of Israel in the Wilderness.

The larva probably enters the body through the skin or gastrointestinal tract. It wanders about in the subcutaneous tissues until maturity, producing slight, if any, symptoms. The male, which is very rarely seen, is only about 4 cm. long. It dies soon after the female is impregnated. The adult female is a very slender, yellowish worm, from 50 to 80 cm. long, its appearance somewhat suggesting a catgut suture. When gestation is complete the greater part of the female's body consists of a uterus filled with embryos. The female then travels to the feet or ankles of the host and there causes the formation of a red nodule, and, finally, an ulcer, from the center of which her head protrudes. Through this great numbers of larvae are

discharged whenever it comes in contact with water. Little damage is done unless the worm is pulled out, when the larvae are set free in the tissues and cause serious disturbances.

When discharged the larvae seek out a small crustacean, cyclops, which serves as intermediate host.

5. Ancylostoma duodenale and Necator americanus.—These, the Old and New World hookworms, respectively, are among the more harmful of the animal parasites. They inhabit the small intestine often in great numbers, and commonly produce an anemia which is often severe and sometimes fatal. The presence of a few, however, may cause no appreciable disturbance.

The anemia is probably due to (*a*) abstraction of blood by the worms and hemorrhage from the bleeding points left when the worms change position in the intestine, the continued bleeding being due to a secretion of the buccal glands which hinders coagulation; (*b*) toxic secretions of the parasites, and (*c*) secondary microbic infection. In well-marked cases the red cells average 2,500,000 to 3,000,000 in each cubic millimeter, hemoglobin 6 to 8 Gm. (35 to 50 per cent of normal) in each 100 c.c. of blood, color index low. Eosinophilia of 10 to 25 per cent is the rule, but may be absent. Charcot-Leyden crystals are often present in the feces.

Ancylostoma duodenale (Fig. 265) is common in Southern Europe and in Egypt, and is not infrequently found in America.

The body is cylindric, reddish in color, and the head is bent sharply dorsally.

There is a well-marked buccal cavity which carries three pairs of hooklike teeth, two pairs situated on the ventral side and one pair dorsally (Fig. 266). The female is 12 to 15 mm. long, and the tail is bluntly pointed. The vulva is situated at the junction of the middle and posterior thirds of the body. The male is 8 to 10 mm. long and the posterior end is expanded into an umbrella-like pouch, the caudal or copulatory bursa, which is supported by a number of stiff ribs or "rays" whose mode of branching is helpful in determining the species. Through the opening of this bursa project (unless retracted) two very slender hairlike copulatory spicules which can be seen only with a lens (Fig. 267).

The eggs are oval and have a thin, smooth, transparent shell. As they appear in the feces the protoplasm is divided into 2, 4, 8, or more rounded segments. They measure 32 to 38 by 52 to 61 μ.

Necator americanus is very common in central and southern Africa and in subtropical America including the southern part of the United States and the West Indies. Extensive surveys among rural

school children by the plain smear method in eleven southern states in 1920–1923 showed 27.8 per cent infected. In Puerto Rico 90 per cent of the rural population was infected until active measures were taken to combat the disease. Isolated cases, probably imported, have been seen in most of the northern states.

Necator americanus is smaller than *A. duodenale*, the male being 7 to 9 mm. long, the female 9 to 11 mm. The four ventral hooklike teeth are replaced by two well-developed semilunar chitinous plates, and the dorsal pair of teeth by two poorly developed plates (Fig. 266). Within the buccal cavity, projecting from the dorsal wall, is a prominent, conical, toothlike structure which forms the outlet for the head gland, and is more prominent than is its analogue in *A. duodenale*. The caudal bursa of the male is similar to that of *A. duodenale*, but there is a different arrangement of the rays. The copulatory spicules unite at the tips into a barbed point, which when seen is very helpful in determining the species (Fig. 267). The vulva of the female is situated anterior to the middle of the body, and can readily be located with a low power of the microscope.

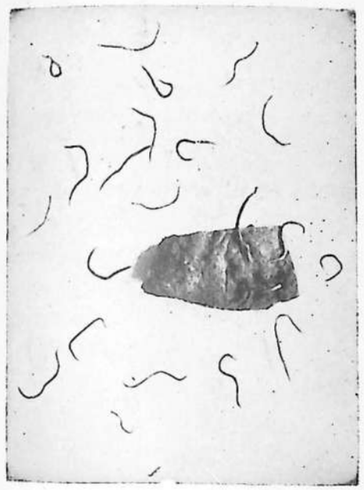


Fig. 265.—Hookworm (*Ancylostoma duodenale*), life size. Shows some worms adherent to a bit of intestinal mucosa and some free (from Jefferys and Maxwell).

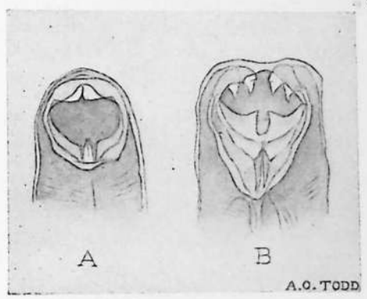


Fig. 266.—Heads of hookworms showing mouth parts: A, *Necator americanus;* B, *Ancylostoma duodenale.* (After E. R. Stitt.) As the head of the hookworm is sharply curved backward, the upper part of the figure represents the ventral surface.

The ova (Fig. 268) very closely resemble those of *Ancylostoma duodenale*, but are larger, 36 to 45 by 64 to 76 μ.

The life history of the two species is probably the same. The ova pass out with the feces, and, under favorable conditions of warmth and moisture, develop an embryo which hatches within a few days.

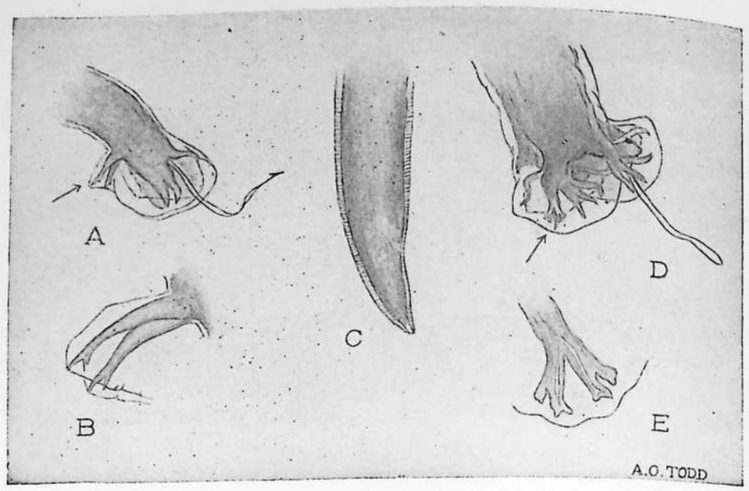


Fig. 267.—Tails of hookworms: A, *Necator americanus*, male, showing copulatory bursa and spicules with barb (× 35); B, enlarged drawing of the rays indicated by an arrow in A; C, tail of female (× 35); D, *Ancylostoma duodenale*, male, showing copulatory bursa and spicules without barb (× 35); E, enlarged drawing of the rays indicated by an arrow in D. (A, B, D, E after E. R. Stitt.)

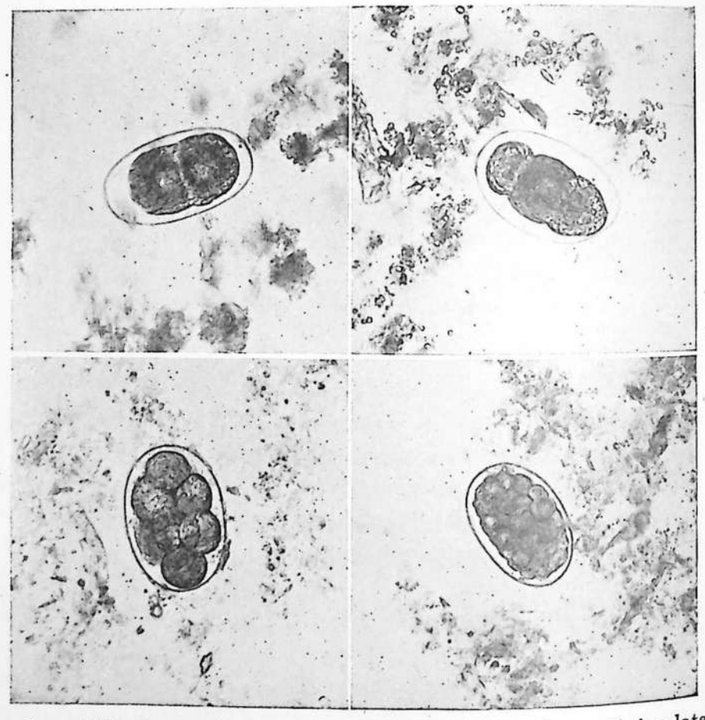


Fig. 268.—Ova of *Necator americanus* in feces. The egg showing three cells is a lateral view of a four-cell stage (photographs × 250).

The resulting larvae pass through a stage of development in warm moist earth, moulting twice, and growing to a length of 0.5 to 0.6 mm. They are then ready to infect a new host. Cort and Payne found that their life in the soil does not exceed three to six weeks, and that they do not migrate more than 4 inches from the spot where they are deposited. In some cases they probably reach the host's intestine by way of the mouth, with food or water, but the usual route is probably by way of the skin, as established by Looss. When moist earth containing the larvae comes in contact with the skin they penetrate into the subcutaneous tissues. This is favored by retention of mud between the toes of those who go barefooted. When the larvae are abundant a dermatitis is induced ("ground itch"). From the subcutaneous tissue they pass by way of lymph and blood streams to the lungs. Here they make their way into the smaller bronchi, are carried by the bronchial mucus to the pharynx, and are swallowed. They thus ultimately reach the small intestine, where they develop into mature worms.

The diagnosis of hookworm infection usually rests upon detection of ova in the feces. The worms themselves seldom appear except after a vermifuge. A small portion of the feces, diluted with water if necessary, is placed upon a slide and the larger particles removed. The material is covered and searched with a 16-mm. objective. A higher power may rarely be necessary to identify an egg, but should not be used as a finder. The eggs (Fig. 268 and Plate XII) are nearly always typical, showing a thin, but very distinct shell, a clear zone, and a finely granular segmented protoplasm. A light spot, representing the nucleus, can usually be made out in each segment. After having once been seen the eggs are not easily mistaken.

In heavy infections they may be found in nearly every microscopic field; in most cases, even when so mild as to cause no symptoms, they can be found on the first slide examined. It is seldom necessary to search more than half a dozen slides. From the estimate of Dock and Bass it seems probable that ova will average at least one to the slide if ten or more laying females are present in the intestine. Very old females may fail to produce eggs. When they are scarce, some method of sedimenting the feces should be tried (p. 492).

Pepper's method of concentration is simple, but is not applicable to ova other than those of the hookworm. It is best first to sediment the feces. A layer of the diluted feces is placed on a slide and allowed to remain for some minutes. The slide is then gently immersed in water. The ova, which have settled to the bottom, cling to the glass

and are not washed away as is other material. This may be repeated several times and numerous eggs collected.

Stoll's method of counting eggs in feces is recommended by Cort as the only accurate means of compiling statistical evidence in preliminary surveys, evaluating effects of treatment, or measuring the results of control measures. The technic is as follows:

1. Feces in their container are balanced on the scales together with a spatula, and 3 Gm. weighed, by difference, into a large-sized test tube or centrifuge tube graduated at 45 c.c.
2. Decinormal NaOH is poured in to the 45-c.c. mark.
3. Ten small (3 mm.) glass beads are added, the tube rubber-stoppered, and the mixture vigorously shaken for one minute.
4. Exactly 0.15 c.c. is pipeted with an accurate pipet on to a 2 x 3-inch slide, and covered with a 22 x 40-mm. No. 2 cover glass.

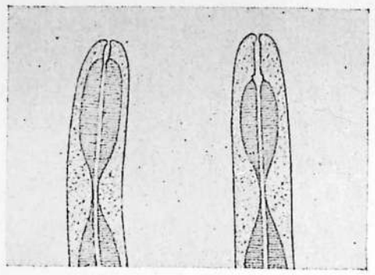


Fig. 269.—Diagram showing the difference in length of the mouth cavities of the larvae of A, *Strongyloides*, and B, *Necator*. The heavily shaded portions represent the bulbous esophagus.

5. A mechanical stage is used and all the eggs on a slide are counted with low power.
6. The number of eggs in each gram of feces is found by multiplying the count by 100.

Two counts are made from different 3-Gm. samples and averaged. The diluted material must be removed from the tube immediately after shaking.

Hookworm larvae are not found in fresh feces, but may hatch within twenty-four to forty-eight hours after the stool is passed. They are then easily mistaken for the larvae of *Strongyloides stercoralis*, but can be distinguished by the depth of the mouth cavity, which is about equal to the diameter of the larva at the posterior end of the cavity. In *S. stercoralis* the mouth is about one half as deep (Fig. 269). The size of the genital anlage is also important.

Cultivation of the larvae may be resorted to for diagnosis, and is said to be more effective than the search for eggs even by concentration methods. The Baermann apparatus as modified by Cort[1] and his co-workers is especially convenient. This consists of an ordinary 8-inch glass funnel supported on a ring stand. The tip of the funnel is covered with a short piece of rubber tubing which is closed by pinching tightly with a Hoffman clamp. The funnel is nearly filled with water heated to 115° F., and a piece of wire screen is so placed that it dips below the surface of the water. Over this and dipping just below the surface of the water is placed a small towel. In the center of the cloth and in the pool of water is placed the infested soil or feces. The larvae grow out in a few days at room temperature and sink to the tip of the funnel. They can be readily drawn off in a drop of water on to a microscope slide by slightly loosening the Hoffman clamp, or a few cubic centimeters may be drawn off into a centrifuge tube and the larvae sedimented by centrifugalization.

Occult blood can always be detected in the feces when hookworms are present in the intestine in any considerable number.

6. Genus Strongyloides.—Strongyloides stercoralis.—Infection with this worm is by no means so rare in this country as the few clinical reports would indicate. It is apparently widespread in the southern states, and is very common in subtropical countries, notably in Italy and in Southern China. It is possible that the parasite may cause a mild catarrhal enteritis, although most authorities regard it as harmless. It is extremely resistant to treatment.

The adult worm, which is probably a hermaphrodite, in which the male organs atrophy early or possibly a female reproducing by parthenogenesis, is about 2 mm. long, and inhabits the upper portion of the small intestine, but neither it nor the ova appear in the stool unless an active diarrhea exists. Ordinarily ova hatch in the intestines, and when infection is heavy larvae can be found in the feces in enormous numbers. They have also been found in the duodenum. These are the "rhabditiform larvae," which measure 250 to 500 μ by 15 to 24 μ, according to their age. Just back of the middle of the body is a conspicuous oval structure, the genital anlage, about 30 μ long (Fig. 270). This and the length of the mouth cavity are important in distinguishing these larvae from those of the hookworm in which the genital anlage is very small and inconspicuous. These larvae are actively motile, with a striking "wiggling" motion, and, when the stool is

[1] Cort, W. W., Ackert, J. E., Augustine, D. L., and Payne, Florence K.: Investigations on the Control of Hookworm Disease, II. The Description of an Apparatus for Isolating Infective Hookworm Larvae from Soil, Am. Jour. Hyg., *2*:1–16 (Jan.), 1922.

solid, are best found by making a small depression in the fecal mass, filling it with water, and keeping in a warm place (preferably an incubator) for twelve to twenty-four hours. The larvae will collect in the water, and can be easily found by transferring a drop to a slide and examining with a 16-mm. objective. The inexperienced worker should *make sure that the worms move*, or he may be misled by the vegetable hairs which are generally present in the feces. Certain of these hairs (notably those from the skin of a peach) closely resemble small worms (p. 481).

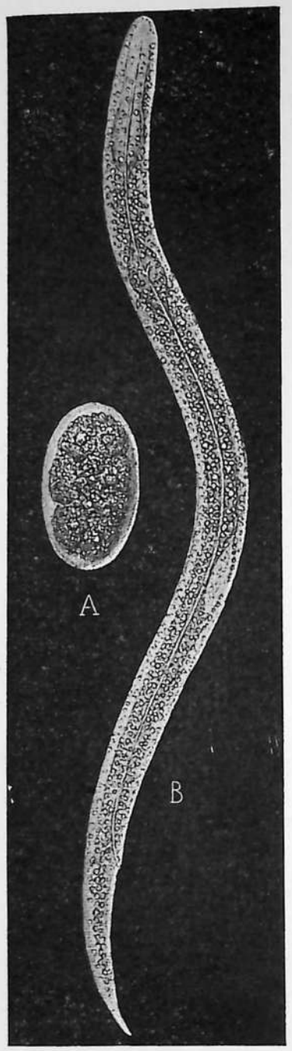


Fig. 270.—A, Egg of *Strongyloides stercoralis* (parasitic mother worm) found in stools of a case of chronic diarrhea; B, rhabditiform larva of *Strongyloides stercoralis* from the stools. (William Sydney Thayer in Journal of Experimental Medicine.)

Outside the body the rhabditiform larvae develop into a free-living, sexually differential generation. The young of this generation are the more slender "filariform larvae" which constitute the infective form. Direct transformation of rhabditiform into filariform larvae also occurs. Infection takes place by ingestion or by way of the skin.

Sandground has reported the finding of *Rhabditis hominis* Kobayashi in stools sent to him for examination supposedly containing *Strongyloides stercoralis*. This worm, which greatly resembles *S. stercoralis*, is not a parasite, but a free-living coprophagous species that may be introduced into the feces, after leaving the body, by contamination with soil, or by filth flies.

7. Genus Trichinella.—Trichinella spiralis.—This is a very small worm—adult males, 1.5 to 1.6 by 0.04 mm.; females, 3 to 4 by 0.06 mm. Infection in man occurs from eating of pork which contains encysted larvae and is insufficiently cooked. Ordinary "curing" of pork does not kill them. According to Winn heating to 55° C. for fifteen minutes for each pound of meat is sufficient to kill all larvae. Six days' refrigeration at 0° F. (−17.7° C.) is also effective. Protection against infection must be secured through such measures as these; meat inspection is of little value unless every part of the carcass be examined, and this, of course, is impracticable. When the larvae reach the stomach the capsule surrounding them is digested away, and they grow to maturity in the small intestine. Soon after copulation the males die, and the females penetrate into the mucous membrane, where they live for about six weeks, giving birth to great numbers of young, averaging as high as 1500 from a single female. The larvae migrate to the striated muscles, chiefly near the tendinous insertions, where they grow to a length of about 0.8 mm., and finally become encysted. In this condition they may remain alive and capable of development for twelve years or longer.

Trichinella is widespread throughout the world and is more abundant in the United States than the reported cases of human infection would lead one to expect. It is capable of living in many animals, but is most common in the pig and the rat. Rats when once infected continue the infection through cannibalism. A convenient means of finding whether the parasite is common in a given community is to examine a series of slaughter-house rats.

Fig. 271.—Rhabditiform larva of *Strongyloides stercoralis* in feces (photograph × 150).

Except during the acute stage trichiniasis is generally accompanied by a marked eosinophilia (p. 278). The diagnosis is made by teasing out upon a slide a bit of muscle, obtained in man preferably from the pectoralis major, the outer head of the gastrocnemius, the insertion of the deltoid, or the lower portion of the biceps. In the case of rats the diaphragm, which is the most likely site, is pressed out between two glass slides. The coiled larvae can easily be seen with a 16-mm. objective (Fig. 272). The larvae can usually be found in the spinal fluid and the blood before they have reached their final resting place in the

muscles as demonstrated by Herrick and Janeway.[1] At this stage they are about 125 μ long and 6 μ broad. During the diarrheal stage adults may be present in the feces, and can sometimes be found by diluting with water, decanting several times, and examining the sedi-

Fig. 272.—*Trichinella spiralis* in a bit of human muscle teased and mounted upon a slide (photograph and diaphragm nearly closed, × 125).

Fig. 273.—Whipworms (*Trichuris trichiura*): A, Females; B, males. The posterior portion of the male is usually coiled as is shown at the right. Photographs of mounted specimens. Natural size.

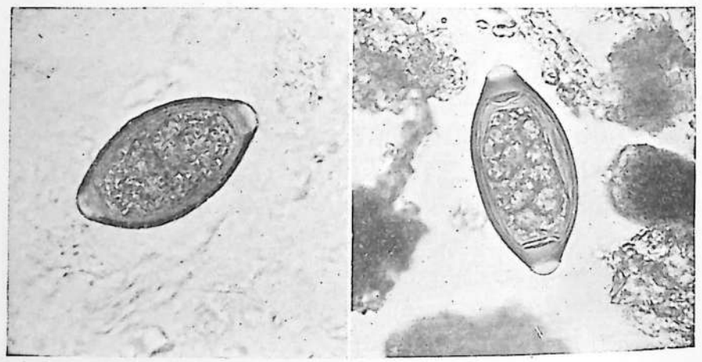


Fig. 274.—Ova of *Trichuris trichiura* in feces (photographs × 500).

ment in a very thin layer in clean water with a hand lens. The skin test described on page 823 has been found useful in the diagnosis of

[1] Herrick, W. W., and Janeway, T. C.: Demonstration of the Trichinella spiralis in the Circulating Blood in Man, Arch. Int. Med., *3*:263–266, 1909.

trichiniasis. The extract for performing this test may be obtained from biological supply houses. The results of the skin test are of importance in the decision on the advisability of performing a biopsy.

8. Genus Trichuris.—Trichuris trichiura (Trichocephalus dispar).—This, the "whipworm," is 3.5 to 5 cm. long. Its anterior portion is slender and threadlike, while the posterior portion is thicker (Fig. 273). It is widely distributed geographically, and is one of the most common of intestinal parasites in this country. It lives in the large intestine, especially the cecum, with its slender extremity embedded in the mucous membrane. Whipworms do not, as a rule, produce any symptoms, although gastro-intestinal disturbances, nervous symptoms, and anemia have at times been ascribed to them. They, as well as many other intestinal parasites, are probably an important factor in the etiology of appendicitis, typhoid fever, and other intestinal infections. The damage which they do to the mucous membrane favors bacterial invasion. They are extremely refractory to anthelmintic treatment.

The number present is usually small. The worms themselves are rarely found in the feces. The ova, which are not often abundant, are easily recognized with the 16-mm. objective. Although they are comparatively small, their appearance is striking. They are brown, ovoid in shape, 50 to 54 μ long by about 23 μ wide, and have a button-like projection at each end (Fig. 274).

The eggs are said (Castellani) to require eighteen months in the open before the embryo is fully developed. Not until then are they infective. They reach the host through contaminated food and water.

PHYLUM ARTHROPODA

The arthropoda are of medical interest chiefly because they are among the most important agents for the spread of disease. Many are simple carriers and disseminators of bacteria, as fleas carry plague bacilli, and flies, typhoid bacilli. Others serve as the necessary hosts for a part of the life cycle of important animal parasites of which they are thus the sole "vectors," as certain mosquitoes are the vectors of the malarial parasites.

Only a few of the arthropoda are directly parasitic, and these are unimportant, so that only brief descriptions need be given in the following pages. They belong to the classes *Arachnida* and *Insecta*.

Class Arachnida

The class includes spiders, mites, and ticks. In general the body is divided into two regions, cephalothorax and abdomen. Antennae

are absent. The two orders to which the parasitic forms belong have become considerably modified from the type. In the order *Acarina*, comprising mites and ticks, "the body is an unsegmented sac to which is attached a movable capitulum, or false head, bearing the mouth parts" (Root). In the order *Linguatulida* or "tongue worms" the body is ringed, elongated and wormlike, and legs are absent in the adult.

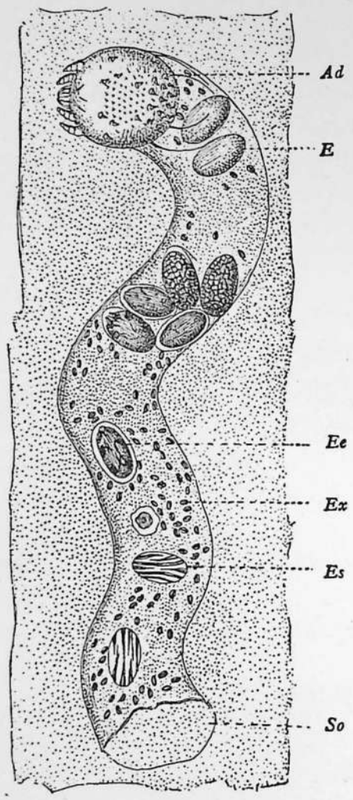


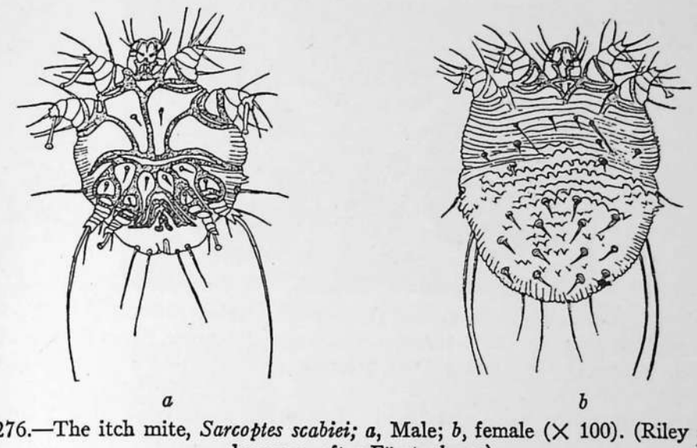
Fig. 275.—*Sarcoptes scabiei.* Diagram of a subcutaneous burrow: *Adf*, Adult female; *E*, eggs; *Ee*, embryo egg; *Ex*, excrement; *Es*, egg shell; *So*, skin orifice. (After Railliet in Brumpt.)

The most important of the parasitic mites is the well-known "itch mite," *Sarcoptes scabiei.* Its tunnels in the skin can be seen with a good magnifying glass as lines 2 to 4 mm. long, often black from the presence of dirt or the excrement and eggs of the parasite (Fig. 275). The parasite and its eggs can often be found by scraping the burrow with a small scalpel, and mounting the scrapings in water on a slide. To see them well the low power of the microscope is required. The parasite is round or oval and set with bristles (Fig. 276). The males average about 170 by 220 μ, females about 300 by 400 μ. Related species cause mange in dogs and cats, and may very rarely attack man.

Another mite, *Demodex folliculorum*, the "face insect," lives in the hair follicles and sebaceous glands of a large proportion of human beings, where it produces no disturbance beyond mild irritation. The parasites can be found by pressing out the contents of the glands and examining the material with a low power of the microscope by very subdued light (Fig. 277). The male measures about 40 by 300 μ, while the female is a little longer. A related parasite causes an obstinate type of mange in dogs.

In many regions the larger members of the order *Acarina*, the ticks, are a source of annoyance, but they are of little medical importance, save as agents for the transmission of such diseases as relapsing fever, tularemia and Rocky Mountain spotted fever.

In the western part of the United States, adults are usually affected more often than children. Probably because of their occupations they are more exposed to infected ticks. In the eastern part of the United States children are more frequently affected. Rocky Mountain spotted



a *b*

Fig. 276.—The itch mite, *Sarcoptes scabiei; a*, Male; *b*, female (× 100). (Riley and Johannsen, after Fürstenberg.)

fever is carried chiefly by the wood tick, *Dermacentor andersoni* (Fig. 278). The tick that transmits Rickettsia in the eastern states is the common eastern dog tick, *Dermacentor variabilis*. There are a number of species of *Ornithodorus* that may transmit relapsing fever. *Ornitho-*

Fig. 277.—The "face insect" *Demodex folliculorum* (× 100); *Kt.*, Biting jaws. (After R. Blanchard in Brumpt.)

dorus parkeri (Fig. 279) is found over a large range of territory in the West and is known to have transmitted this disease (see page 512).

There are a number of mites, the six-legged larvae of which parasitize insects and small animals ordinarily, but which, when the opportunity offers, may attack man, burrowing into the skin or entering

the sweat glands, and thus causing inflammation and intense itching. These are very common in some parts of the United States, and are popularly known as mites, red bugs, chiggers, and so forth. Most of them are just visible to the unaided eye.

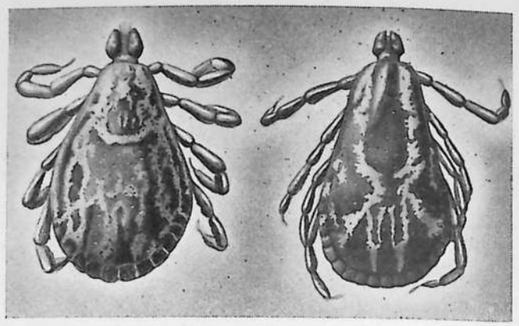


Fig. 278.—*Dermacentor andersoni* and *Dermacentor variabilis;* vectors of Rocky Mountain spotted fever rickettsii. (By permission of the copyright owner, Sharp & Dohme, Inc., as originally presented in their May, 1942, Seminar.)

One other parasitic arachnid, *Linguatula serrata*, the "tongue worm," requires mention since a few cases of human infection have been reported from Europe, the Panama Canal Zone, and Brazil; it

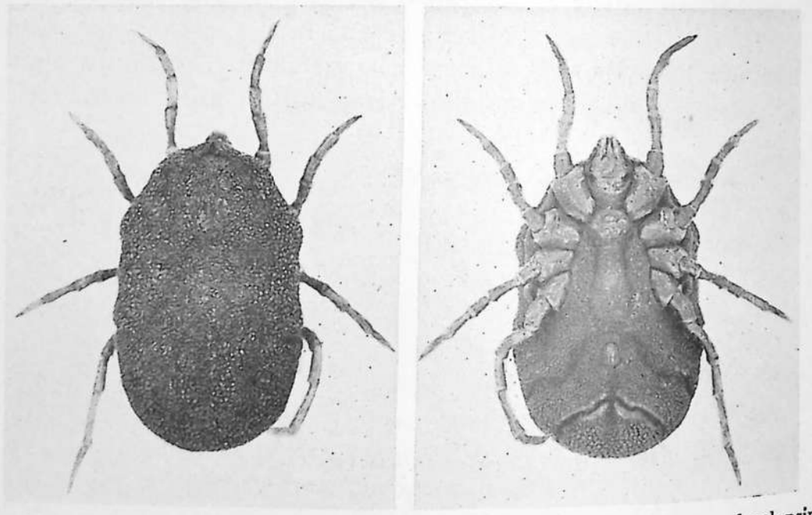


Fig. 279.—*Ornithodorus parkeri.* (Cooley.) Ticks of this genus are vectors of relapsing fever Borrelia. (Courtesy of R. A. Cooley.)

may prove to be more common than has been recognized. This and related species are not rare in dogs, horses, goats, and certain birds. Man may be parasitized by either the adult or larval stage.

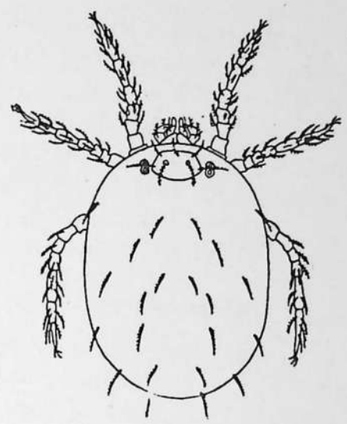


Fig. 280.—The North American chigger, *Trombicula irritans* (larva, × 100). (Ewing, A Manual of External Parasites, Charles C. Thomas, Publisher.)

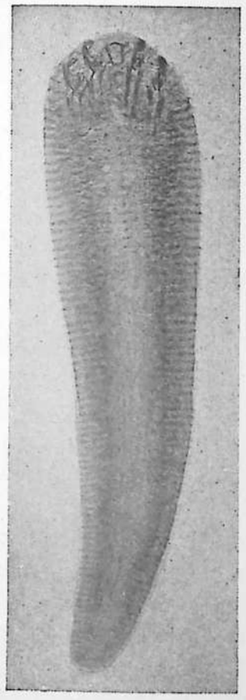


Fig. 281.—Larva of *Linguatula serrata.* (De Faria and Travassos.)

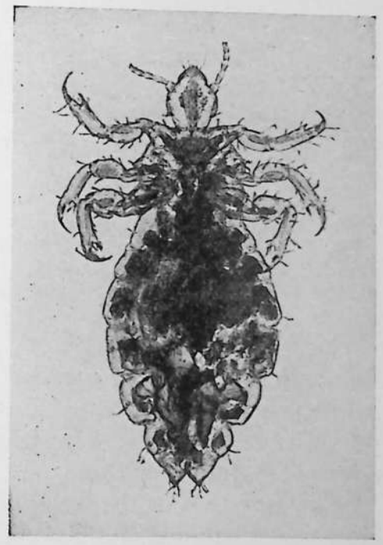


Fig. 282.—Body louse (*Pediculus vestimenti*), female (× 15). The male is a little smaller and the posterior end of the abdomen has no notch. The body louse is distinguished from the head louse by its larger size and by the relative widths of thorax and abdomen.

The adults are wormlike, without legs, and live in the nasal passages attached to the mucous membrane. The female, which is several

times as large as the male, attains a length of 8 to 10 cm. The eggs are carried out with the nasal discharges and ultimately reach another host where the larvae hatch out and soon encyst in the internal organs, particularly the liver. After a period of development the larvae again become free, and in this stage usually reach the feces, where, because of their serrations, they may be mistaken for minute tapeworms (Fig. 281). They are white and measure about 4 to 6.5 mm. long and 0.9 to 1.5 mm. broad at the widest (anterior) part.

Class Insecta

In adult members of this class the body is divided into three distinct regions, head, thorax, and abdomen; and the head bears one pair of antennae. There are three pairs of legs, all attached to the

Fig. 283.—Head louse (*Pediculus capitis*), male (× 15). The female is a little larger and the posterior end of the abdomen is notched.

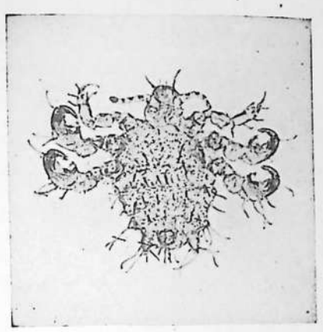


Fig. 284.—Pubic louse (*Phthirius pubis*) (× 15).

thorax. Typically there are one or two pairs of wings, but in most parasitic species the wings have been lost with the adoption of the parasitic mode of life. Parasitic insects include lice, fleas, and the larvae of certain flies.

The structure of the fleas, lice, and similar small insects may be more easily studied if they are made more or less transparent by soaking them for a day or two in 20 per cent solution of sodium hydroxide.

The lice are wingless and are flattened dorsoventrally. There are many species, all of which are closely confined to their particular host species. Only three infest man: The body louse, *Pediculus vestimenti;* the head louse, *Pediculus capitis;* and the pubic louse, *Phthirius pubis.* These are sufficiently described by the accompanying pictures, and their legends (Figs. 282–284). The head louse and the pubic louse

attach their eggs to hairs, where they are commonly known as "nits" (Fig. 285). The body louse lives in the clothing, and seeks the body only at the time of feeding. Its eggs are cemented to the clothing, chiefly in the folds and seams.

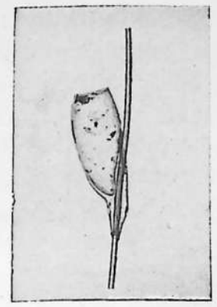


Fig. 285.—Empty egg of *Pediculus capitis* cemented to a hair. Before hatching the egg is covered with a cap or lid (photograph × 15).

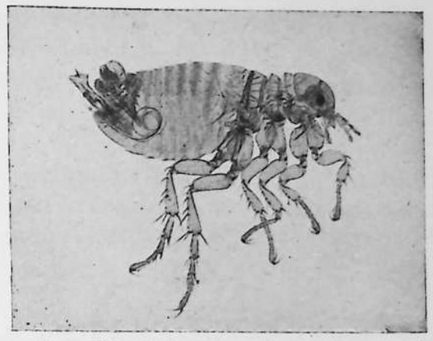


Fig. 286.—The flea, *Pulex irritans*, male. Note the absence of "combs" (photograph × 15).

The fleas are reddish brown, wingless, and flattened laterally. Their shape permits them to move rapidly along the hairs. While each species has its favorite host, the fleas are not strictly confined

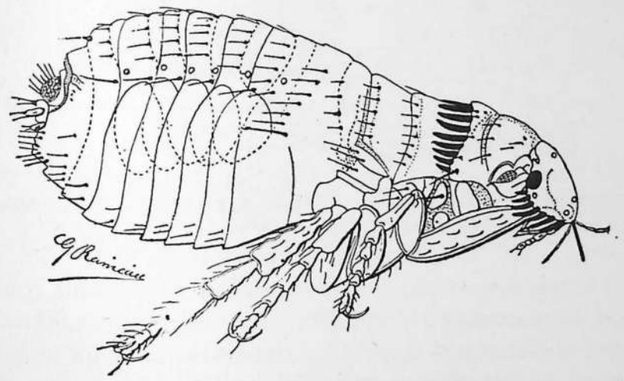


Fig. 287.—*Ctenocephalus canis*, female (× 25). (After Brumpt.) Note the conspicuous "combs" along inferior border of head and on prothorax.

Fig. 288.—Head of *Ceratophyllus*. Note "combs" only on prothorax. (After Brumpt.)

to particular host species as are the lice. The best-known human flea is *Pulex irritans* (Fig. 286). A dog flea, *Ctenocephalus canis* (Fig. 287), also infests man in certain regions. The two chief rat fleas are *Ceratophyllus fasciatus* (Fig. 288) and *Xenopsylla cheopis*. The latter

is the rat flea of India, and is, therefore, the more concerned in the transmission of plague. It closely resembles *Pulex irritans*, being without combs, but is more yellow.

The fleas mentioned live on the surface of the body. A very small flea, *Tunga* (*Dermatophilus*) *penetrans*, which burrows into the skin, is common in tropical and subtropical America and Africa. This is popularly known as the chiga flea or "chigger," but should not be confused with the larval mites which are called "chiggers" in the United States (p. 580).

A number of flies, including the common house fly, may deposit their ova in wounds or in such of the body cavities as they can reach,

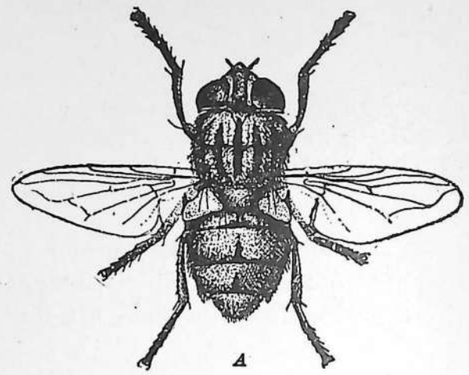


Fig. 289.—*Chrysomyia macellaria*, screw-worm fly: *A*, Adult; *B*, maggot (× 3). (*A*, After Castellani and Chalmers; *B*, after Blanchard.)

and the resulting maggots may cause intense irritation. To this condition the general term *myiasis* is applied. Ova may be swallowed with the food, and the maggots appear in the feces. In some cases ova are deposited in the tonsillar crypts, with resulting formation of abscesses. A few cases in which larvae have been found in the urinary passages have been reported. The physician should be able to recognize these as maggots, but the exact determination of species is a matter for the trained entomologist.

Probably the most important form of myiasis is infection with the "screw worm," the larva of *Chrysomyia macellaria* (Fig. 289), which is not rare in some parts of the United States, particularly

west of the Mississippi. The ova are most commonly deposited in the nasal passages, and the larvae, which may be present in great numbers, burrow through the soft parts, cartilage, and even bone, always with serious and often with fatal results.

Cutaneous myiasis, in which fly larvae develop in the skin, producing boil-like nodules similar to "bots" or "warbles" in animals, is not rare in tropical America, and a few imported cases have been seen in this country. The best known is the larva of the fly *Dermatobia hominis*, ver macaque of the tropics (Fig. 290), which is transferred to man by mosquitoes, and possibly by ticks, to which the fly affixes its eggs.

Among insects which are not parasitic, but are extremely important as vectors of disease, are mosquitoes (p. 529), the tsetse fly (p.

Fig. 290.—Larva of *Dermatobia hominis* (× 3).

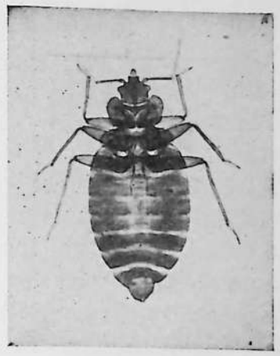


Fig. 291.—The common bedbug, *Cimex lectularius*, male (× 5). In the female the posterior end of the abdomen is more rounded. (Cleared with sodium hydroxide to bring out the structure more clearly.)

515), the South American house bug or "cone nose," *Triatoma* (p. 516), and the well-known bedbug, *Cimex lectularius* (Fig. 291).

Arachnidism, "Black Widow" Spider Poisoning.—Though not an animal parasite, the notorious "black widow" spider is enough of a menace that the laboratory worker should be able to recognize the only spider found throughout the United States that is definitely known to be poisonous. *Latrodectes mactans* (Fig. 292) is black, about the size and shape of a shoe-button, and on the ventral surface, it has a characteristic red or yellow marking which is shaped like an hour-glass. The female is the spider which usually is seen, as she has the habit of eating her mate, hence her sobriquet of "black widow." Her web is tough and slovenly; it extends in a haphazard manner

in rock gardens and outhouses, in contrast to the beautiful geometric designs which are spread by the harmless spiders, the so-called "orb spinners." Hiding in some nearby crevice, the spider emerges when this net is disturbed and the victim is conscious of a stinging bite, followed by severe pains which become progressively worse for an hour. Boardlike rigidity of the abdominal muscles, excruciating pains, and an elevation in temperature may lead to a faulty diagnosis of peritonitis. Arachnidism is self-limited and usually sub

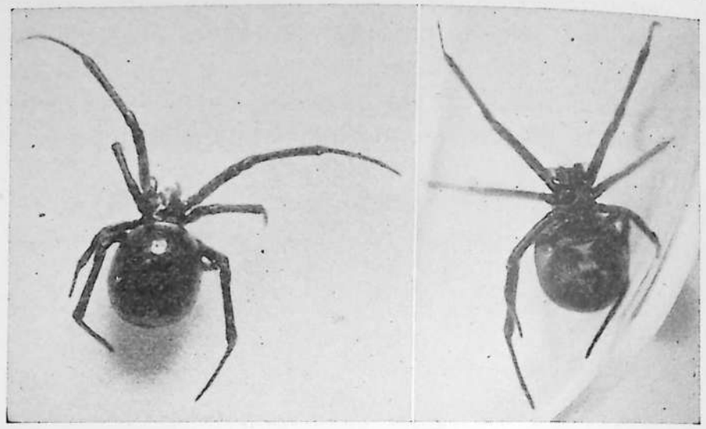


Fig. 292.—Black widow spider, *Latrodectes mactans*.

sides in a few days, although death occurs occasionally in from fourteen to thirty-two hours after the bite. While patients are usually bitten when the spider is disturbed near its web, cases have been reported in which the patient was bitten while in bed. A very comprehensive study of arachnidism may be found in Bogen's[1] articles on the subject.

[1] Bogen, Emil: Dangers of Spider Bites, Hygeia, *11*:621–623 (July), 1933. Poisonous Spider Bites, Newer Developments in Our Knowledge of Arachnidism, Ann. Int. Med., *6*:375–388 (Sept), 1932. Arachnidism, Spider Poisoning. Report of a Typical Case, Arch. Int. Med., *38*:623–632 (Nov.), 1926.

CHAPTER VIII

PUS, PUNCTURE FLUIDS, ANIMAL INOCULATION

PUS

Pus contains much granular débris and numerous more or less disintegrated cells, the great majority being polymorphonuclear leukocytes—so-called "pus corpuscles." Eosinophilic leukocytes are common in gonorrheal pus and in asthmatic sputum. Examination of pus is directed chiefly to detection of bacteria.

When very few bacteria are present cultural methods, which are outlined in Chapter XI, must be resorted to. When considerable numbers are present, they can be detected and often identified in cover-glass smears. Several smears should be made, dried, and fixed, as described on page 779.

One of these should be stained with a bacterial stain. Löffler's methylene blue and Pappenheim's pyronine-methyl-green are especially satisfactory for this purpose. These stains are applied for one-half minute to two minutes or longer, without heating; the preparation is rinsed in water, dried, mounted, and examined with an oil-immersion lens. Another smear should be stained by Gram's method (p. 780). These will give information concerning all bacteria which may be present in any considerable numbers, and frequently no other procedure will be necessary for their identification.

As a control of treatment of infected wounds it is desirable to determine approximately the number of bacteria in the pus from day to day. For this purpose a loopful of the pus or discharge from the worst-looking part of the wound is spread on a slide, dried, fixed, and stained with Löffler's methylene blue, carbol thionin, or other appropriate stain. The film is examined with the low power and an area selected in which the pus cells just touch, but do not overlap. This area is then studied with the oil-immersion (1.9 mm.) objective and 10 × eyepiece. The average number of bacteria per field is counted and recorded, and the predominant type, whether streptococcus, staphylococcus, or bacillus, is noted. From 5 to 20 fields must be counted to get a reliable average. As the wound improves the number of bacteria per field should fall to 1 or 2, when, in the Carrel method of treatment, the wound may be closed.

The most common pus-producing organisms are **staphylococci** and **streptococci.** They are both cocci, or spheres, their average di-

ameter being about 0.7 μ. Staphylococci are commonly grouped in clusters, often compared to bunches of grapes (Fig. 293). There are several varieties which can be distinguished only in cultures. Streptococci are arranged side by side, forming chains of variable length (Fig. 294). Sometimes there are only three or four individuals in a chain; sometimes a chain is so long as to extend across several microscopic fields. Streptococci are more virulent than staphylococci, and are less commonly met. Both are gram-positive, but both may at times be relatively gram-negative when inclosed within pus corpuscles (degenerating forms). Their culture characteristics are given on pages 790 and 791.

Should bacteria resembling **pneumococci** be found, Rosenow's or Smith's method for capsules (pp. 53, 54) should be tried. When these

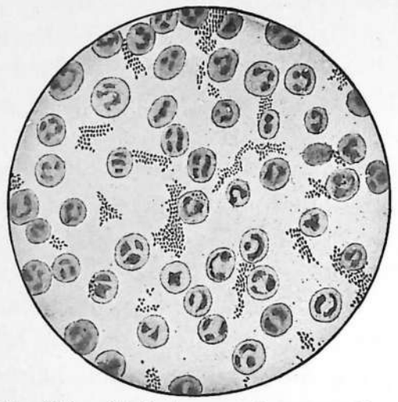


Fig. 293.—*Staphylococcus pyogenes albus* from an abscess of the parotid gland. (Jakob.)

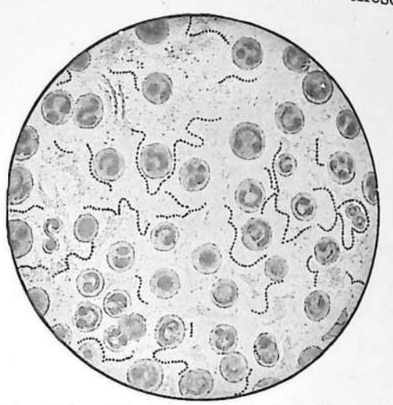


Fig. 294.—*Streptococcus pyogenes* from case of empyema. (Jakob.)

are not available, capsules can usually be shown by the method of Hiss. The dried and fixed smear is covered with a stain composed of 5 c.c. saturated alcoholic solution gentian violet and 95 c.c. distilled water, and heated until steam rises. The preparation is then washed with 20 per cent solution of copper sulfate, dried, and mounted in Canada balsam.

Pneumococci (Fig. 295), while most common in connection with the lung and pleural cavity, may give rise to inflammation in many locations (p. 52). When they form short chains, demonstration of the capsule or cultural methods may be necessary to distinguish them from streptococci. The presence of a capsule is extremely significant, but is not always conclusive, since streptococci may very rarely show capsules. Cultural methods and the technic of determining the types of pneumococci are given on pages 792 and 793.

If tuberculosis be suspected, the smears should be stained by one of the methods for the **tubercle bacillus** (pp. 45–49), or guinea-pigs may be inoculated. The bacilli are generally difficult to find in pus, and bacteria-free pus would suggest tuberculosis.

Gonococci when typical can usually be identified with sufficient certainty for clinical purposes in the smear stained with Löffler's methylene blue or, much better, with Gram's stain. They are ovoid or coffee-bean-shaped cocci which lie in pairs with their flat surfaces together. They lie for the most part within pus cells, an occasional cell being filled with them, while the surrounding cells contain few or none (Fig. 296). Their intracellular position and their appearance in clusters are very important points in their identification. While a few are often found outside of cells, one should hesitate to accept them as gonococci unless further search reveals intracellular organisms. It is usually difficult to find gonococci when many other bacteria are present, even though the pus is primarily of gonorrheal origin. Whenever the identity of the organism is at all questionable, Gram's method should be tried, but it should be remembered that staphylococci, streptococci, and pneumococci are sometimes relatively gram-negative when lying intracellularly. In rare instances it may be necessary to resort to cultures. The gonococcus is distinguished by its failure to grow upon ordinary media (p. 797), but grows readily on chocolate-blood-agar in an atmosphere containing added carbon dioxide.

Fig. 295.—*Diplococcus pneumoniae* from ulcer of cornea (oil-immersion objective). Study through courtesy of Dr. C. A. Oliver (Boston).

Gonococci are generally easily found in pus from untreated, acute and subacute gonorrheal inflammations—conjunctivitis, urethritis—but are found with difficulty in pus from chronic inflammations and abscesses, and in urinary sediments.

In the urine gonococci are most likely to be present in the well-known "gonorrheal threads" or "floaters," which consist of strands of mucus with entangled pus corpuscles, and are suggestive of chronic gonorrhea, but are by no means diagnostic of it. These are fished out with a platinum wire, spread upon slides, fixed, and stained. When floaters are absent it may be necessary to examine the sediment obtained by thorough centrifugation. In order to remove urea, which

prevents proper drying of the smear, the sediment may be washed once with water or physiologic saline. Smears should be thin and quickly dried in order that the pus corpuscles may be as well preserved as possible. Very often the pus cells are so shrunken that the contained gonococci are difficult to recognize. There is likewise difficulty in finding gonococci in vaginal discharges unless comparatively pure pus from the suspected lesion can be obtained; otherwise the organisms sought are to a great extent lost among the myriads of bacteria and the epithelial and pus cells of the leukorrheal discharge. Also, it should be borne in mind that the female genitals frequently

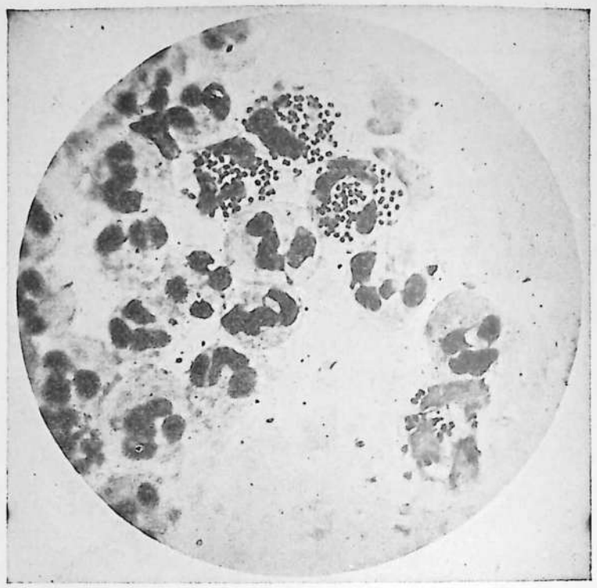


Fig. 296.—Gonococci in urethral pus (× 1000).

harbor a nonpathogenic gram-negative diplococcus which closely resembles the gonococcus.

PERITONEAL, PLEURAL, AND PERICARDIAL FLUIDS

The serous cavities contain very little fluid normally, but considerable quantities are frequently present as a result of pathologic conditions. The pathologic fluids are classed as transudates and exudates.

Transudates are noninflammatory in origin. Their color is light yellow or greenish yellow, and they may be clear, slightly cloudy, or opalescent. They contain only a few cells, and less than 2.5 per

cent of albumin, and do not coagulate spontaneously. The specific gravity is below 1.018. Micro-organisms are seldom present.

Exudates are of inflammatory origin. They are richer in cells and albumin, and tend to coagulate upon standing. The specific gravity is above 1.018. The amount of albumin may be estimated by Kingsbury's, or Esbach's method, after diluting the fluid, if much albumin be present. A mucin-like substance, called serosomucin, is likewise found in exudates. It is detected by acidifying with a few drops of 5 per cent acetic acid, when a white cloudy precipitate results. This reaction is very helpful in distinguishing between transudates and exudates, although some transudates give a slight turbidity with acetic acid. Bacteria are generally present and often numerous. When none are found in stained smears or cultures, tuberculosis is to be suspected, and animal inoculation should be resorted to.

Exudates are usually classed as serous, serofibrinous, seropurulent, purulent, putrid, and hemorrhagic, which terms require no explanation. In addition, chylous and chyloid exudates are occasionally met, particularly in the peritoneal cavity. In the chylous form the milkiness is due mainly to the presence of minute fat droplets, and is the result of rupture of a lymph vessel, usually from obstruction of the thoracic duct. Chyloid exudates are milky, chiefly from proteins in suspension or fine débris from broken-down cells. These exudates are most frequently seen in carcinoma and tuberculosis of the peritoneum. Blankenhorn believes that all milky effusions are, in reality, chylous, although in some (chyloid, pseudochylous) the fat is so finely divided as to take on some of the properties of colloids.

Cytodiagnosis.—This is diagnosis from a differential count of the cells in a transudate or exudate, particularly one of pleural or peritoneal origin.

A tube of the fresh fluid, obtained by aspiration and, preferably, mixed at once with a little citrated salt solution to prevent clotting, is centrifugalized for at least five minutes; the supernatant liquid is poured off, and smears are made from the sediment and dried in the air. The fluid must be very fresh, and the smears must be thin and quickly dried, otherwise the cells will be small and shrunken and hence difficult to identify. The smears are then stained with Wright's blood stain, which has preferably been previously diluted with one third its volume of pure methyl alcohol. They are examined with an oil-immersion objective.

Predominance of *polymorphonuclear leukocytes* (pus corpuscles) points to an acute infectious process (Fig. 297). These cells are the neutrophils of the blood. Eosinophils and mast cells are rare. In thin

smears they are easily recognized, the cytoplasmic granules often staining characteristically with polychrome-methylene-blue-eosin

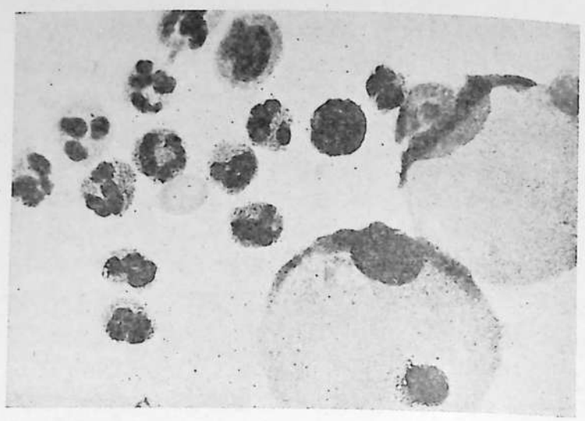


Fig. 297.—Cytodiagnosis. Polymorphonuclear leukocytes and swollen endothelial cells from acute infectious nontuberculous pleuritis. (Percy Musgrave; photo by L. S. Brown.)

stains. In thick smears, upon the other hand, they are often small and shrunken, and may be identified with difficulty, being easily mistaken for lymphocytes.

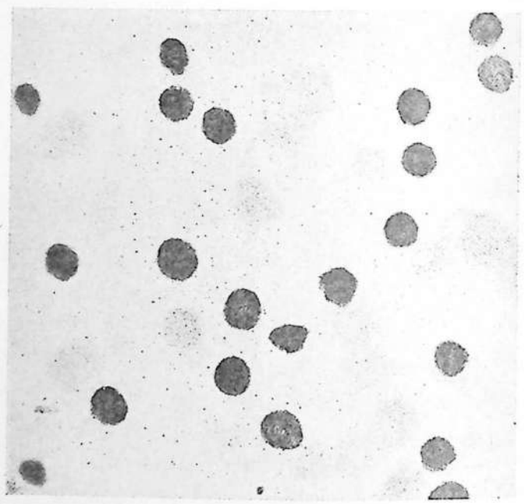


Fig. 298.—Cytodiagnosis. Lymphoid cells from pleural fluid; case of tuberculous pleuritis. (Percy Musgrave; photo by L. S. Brown.)

A large number, or even a preponderance, of *eosinophils* is seen in about 1 to 5 per cent of pleural effusions. The significance is uncertain. Some of these effusions have followed artificial pneumothorax, some others have been of tuberculous origin.

Predominance of *lymphocytes* (Fig. 298) generally signifies tuberculosis. They are the same as found in the blood. The cytoplasm is usually scanty, is often ragged, and sometimes is apparently absent entirely. Tuberculous pleurisy due to direct extension from the lung may give excess of neutrophils owing to mixed infection.

Predominance of *mesothelial cells*, few cells of any kind being present, indicates a transudate (Fig. 299). These cells are large, with relatively abundant cytoplasm, and contain one, sometimes two, round or oval, palely staining nuclei. Mesothelial cells generally predominate in carcinoma, but are accompanied by considerable numbers of lymphocytes and red blood corpuscles. Cancer cells cannot be

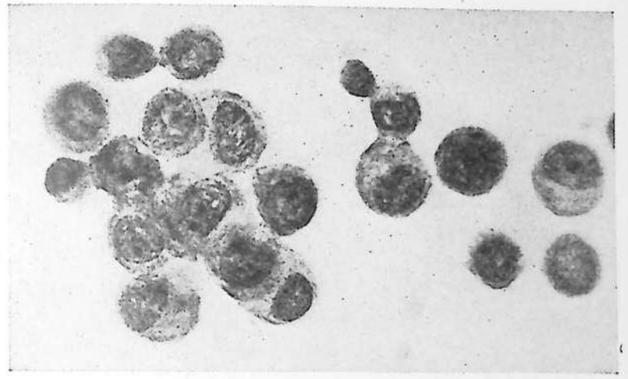


Fig. 299.—Cytodiagnosis. Mesothelial cells from transudate or mechanical effusion. (Percy Musgrave; photo by L. S. Brown.)

recognized as such, although the presence of mitotic figures would suggest malignant disease.

Mandelbaum's Cytodiagnostic Method for Effusions.—Many years ago Mandelbaum reported a cytodiagnostic method which he later redescribed.[1] It also has been recommended by Zemansky,[2] Goldman,[3] and others. The technic is briefly as follows: Place the fluid in a large Erlenmeyer flask in the icebox for twelve to eighteen hours. Decant the supernatant fluid and pour the sedi-

[1] Mandelbaum, F. S.: The Diagnosis of Malignant Tumors by Paraffin Sections of Centrifuged Exudates, Jour. Lab. and Clin. Med., *2*:580 (May), 1917.

[2] Zemansky, A. P.: The Examination of Fluids for Tumor Cells; Analysis of 113 Cases Checked Against Subsequent Examination of Tissue, Am. Jour. Med. Sci., *175*: 489–504 (Apr.), 1928.

[3] Goldman, Alfred: Cytology of Serous Effusions, with Special Reference to Tumor Cells, Arch. Surg., *19*:1672–1678 (Dec.), 1929.

ment into a 50-c.c. centrifuge tube which has a tapered bottom. Centrifugalize at moderate speed for twenty minutes. Decant the supernatant fluid, and harden the sediment with 10 per cent formalin solution for twenty-four hours. Treat the fixed sediment as tissue; run it through the regular alcohol solutions, embed it in paraffin, and section it with a microtome as if it were tissue. For a description of this technic, the student must consult a textbook on histologic methods. Cut the tissue from above downward so as to include all of the cellular elements, which may not lie in either the top or bottom layers. Stain with hematoxylin or iron hematoxylin. According to the advocates of this method, bits of malignant tissue may be definitely demonstrated in effusions by this technic in a large percentage of cases.

CEREBROSPINAL FLUID

Cerebrospinal fluid is obtained by lumbar puncture between the third and fourth lumbar vertebrae with a flexible needle having a length of about 10 cm. and a bore of from 1 to 1.5 mm., and provided with a stylet. For children a shorter needle is used. The needle and stylet are sterilized by boiling, and the site of the puncture is prepared as described for venous puncture. A local anesthetic is usually used, the skin and subcutaneous tissue being infiltrated with sterile 1 per cent solution of eucaine or novocain. The patient sits upon a stool and bends forward as much as possible, or lies upon his left side at the edge of a bed with knees drawn up and back bent. The site for the puncture is found by running the fingers along the spinous processes of the vertebrae until the "soft spot" between the third and fourth lumbar spines is found. This lies on or just above a line joining the crests of the ilia. The needle is inserted in the midline and pushed directly forward with a quick thrust until it reaches the tough spinous ligaments, when it may be pushed more slowly. A sudden cessation of resistance indicates that it has entered the spinal canal. The stylet is then removed, and the fluid should flow at once. Should it fail to do so, it may usually be started by inserting the needle a little farther or pulling it out a trifle, or by inserting the stylet to dislodge anything that may have clogged the needle, or by having the patient take a deep breath. The pressure under which the fluid appears should be noted. Normally it drips from the needle and the number of drops a minute may be counted. When under high pressure it spurts out. The best method for estimating and recording the pressure is with an Ayer manometer[1] (Fig. 300). Response to pressure on the

[1] To be obtained from Codman and Shurtleff, Incorporated, Boston.

jugulars is noted at once with this apparatus. In this way a "block" in the spinal canal can be detected.

The fluid is best collected in three sterile test tubes. One tube receives the first drops, which are usually blood tinged and unfit for examination. The second tube receives the greater part of the fluid withdrawn. The third should contain a trace of powdered potassium oxalate to prevent clotting. This portion of the fluid is reserved for the cytologic examination. Ordinarily not over 6 to 8 c.c. of fluid should be withdrawn at one time. It is best for the patient to remain in bed for some hours after the puncture.

Examination of the fluid obtained by lumbar puncture has of recent years become a very important aid in diagnosis, particularly in syphilitic conditions of the nervous system The routine exam-

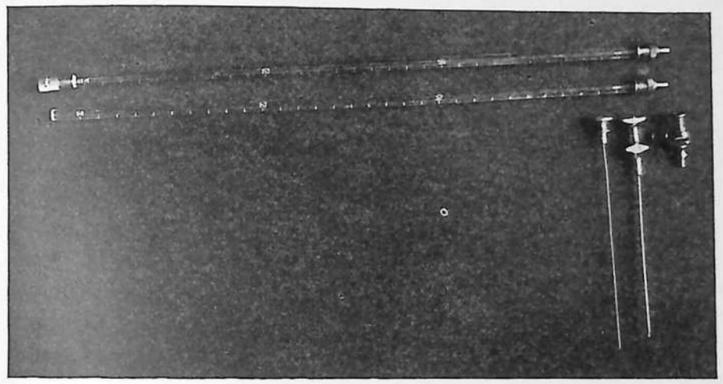


Fig. 300.—Ayer manometer. One part may be fitted into the other, thus making a water manometer either 30 or 60 cm. long. Stopcock and spinal puncture needle are also shown.

ination in these cases includes: 1, Macroscopic examination; 2, chemical test for globulin; 3, colloidal gold or mastic test; 4, cytology: (*a*) total cell count, (*b*) differential cell count; 5, Wassermann test. When acute infections are in question, the turbidity, bacteriology, and cytology are of chief importance.

1. Macroscopic Examination.—The amount obtainable varies from a few drops to 100 c.c. Normally the fluid is clear and limpid, resembling water. The reaction is alkaline. The specific gravity is 1.003 to 1.008. Not infrequently it is tinged with fresh blood from a punctured vessel. This should not be confused with the dull red or brown color which is seen in hemorrhagic conditions, like intraventricular and subdural hemorrhage and hemorrhagic meningitis. When the bleeding is extensive and recent it may give the appearance of practically pure blood.

A condition in which the spinal fluid exhibits a clear yellow color (xanthochromia), usually with rapid and marked coagulation, large amounts of globulin, and no blood pigments by chemical tests (**Froin's syndrome**) has been described. It is generally the result of compression by a tumor of the cord or vertebrae with interruption to the flow of cerebrospinal fluid and formation of a pocket of greater or less extent in which the fluid stagnates.

In acute meningitis the fluid may exhibit varying degrees of cloudiness, from slight turbidity to almost pure pus. In the less acute stage of the epidemic form it is sometimes quite clear. It is also clear in tuberculous meningitis.

After standing for twelve to twenty-four hours the fluid will often coagulate. This occurs especially in the various forms of meningitis, rarely in noninflammatory conditions. In tuberculosis the coagulum is usually very delicate and cobweb-like, and is not easily seen.

2. Chemical Examination.—Only a few constituents are of clinical importance.

(1) **Proteins.**—Traces are present normally. A notable increase occurs in acute inflammations, in tuberculous meningitis, and in syphilitic and parasyphilitic affections. The chief protein is globulin, and this is the only one considered in clinical work. The three tests for globulin which follow are positive in 93 to 95 per cent of all cases of paresis, and are, therefore, an important diagnostic consideration. When acute inflammation and tuberculous meningitis are excluded, they run practically parallel with the Wassermann reaction when the latter is applied to the spinal fluid. There may, however, be slight increase of globulin in lethargic encephalitis, poliomyelitis, and other conditions. The globulin test is valueless when applied to fluid containing blood, owing to the presence of serum globulin.

Noguchi's Butyric Acid Test.—In a small test tube take 1 to 2 c.c. of the fluid and 5 c.c. of a 10 per cent solution of butyric acid in normal salt solution. The original test calls for one tenth these quantities, but they are too small for convenient manipulation. Heat to boiling, and immediately add 1 c.c. of normal sodium hydroxide solution, and boil again for a few seconds. A positive reaction, corresponding to a pathologic amount of globulin, varies from a distinct cloudiness to a heavy flocculent precipitate which generally appears within twenty minutes, but may be delayed for two hours. A slight opalescence may be seen in normal fluids.

Ammonium Sulfate Test.—Globulin is precipitated by strong solutions of ammonium sulfate. Ross and Jones apply the test after the manner of the ring tests for albumin in the urine. In a test tube or horismascope take a few cubic centimeters of a completely saturated solution of ammonium sulfate and overlay with the suspected fluid. In the presence of an excess of globulin, a clear-cut, thin, grayish-white ring appears at the zone of contact of the two fluids within a few seconds. Under normal conditions a ring may appear within five minutes to three hours. This test appears to be fully as reliable as the butyric acid test.

Pandy's test is said to be more definite and more sensitive. The reagent consists of a saturated aqueous solution of phenol crystals, made by adding 10 Gm. of the crystals to about 100 c.c. of water, and keeping in an incubator with frequent shaking for several days. To 1 c.c. of the reagent add 1 drop of the cerebrospinal fluid. A bluish-white cloud indicates an abnormal amount of globulin.

Quantitative Method for Protein.—The Kingsbury, Clark, Williams, and Post[1] method for the quantitative determination of albumin in urine, which is mentioned on page 95, is a very satisfactory method for determining the total protein in the spinal fluid. Place 1 c.c. of spinal fluid in a tube measuring $\frac{1}{2}$ by 4 inches. Add 3 c.c. of 3 per cent sulfosalicylic acid, and mix thoroughly. Allow the mixture to stand for five minutes and compare the degree of turbidity with that of the permanent standard tubes. A Clark lamp[2] (Fig. 301) is convenient for comparing turbidities. The standard tubes are filled with clear gelatin mixed with varying amounts of *formazin*, which represents the turbidity produced by mixing 3 per cent sulfosalicylic acid with known amounts of protein in serum. The standards will last for some years, although they should be checked from time to time for accuracy. They may be prepared according to the method which

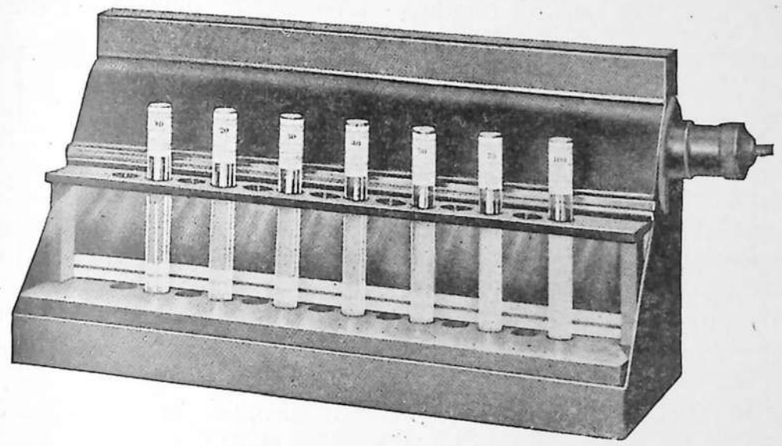


Fig. 301.—The Clark lamp. (Courtesy of Dr. Charles P. Clark, Newark, N. J.)

[1] Kingsbury, F. B., Clark, C. P., Williams, Gertrude, and Post, Anna L.: The Rapid Determination of Albumin in Urine, Jour. Lab. and Clin. Med., *11*:981–989 (July), 1926.

[2] The Clark lamp may be obtained from Dr. Charles P. Clark, Mutual Benefit Life Insurance Company, Newark, New Jersey.

has been described by the originators of the method, but can be procured more conveniently from the Fales Chemical Company, New York. The normal amount of protein in spinal fluid is usually about 30 mg. per 100 c.c.

This method is sufficiently accurate for clinical use. If a more accurate method is desired, the relatively simple technic designed by Johnston and Gibson[1] may be employed. This is a colorimetric method which employs Folin-Ciocaltieu reagent and a tyrosine standard.

(2) **Colloidal Gold Test.**—Lange's colloidal gold test, introduced in 1912 and now very widely used, consists in mixing cerebrospinal fluid in certain proportions with a colloidal solution of gold. Normal cerebrospinal fluid causes no change in color. Fluids from cases of syphilis and certain pathologic conditions of the nervous system induce changes in the color of the gold solution from red to purple, deep blue, pale blue, or colorless. Moreover, the dilution at which

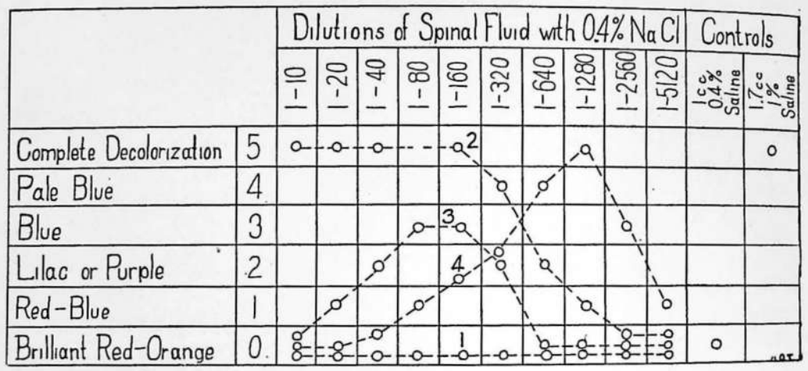


Fig. 302.—Types of reactions in colloidal gold test: 1, Normal cerebrospinal fluid, no reaction; 2, paretic type; 3, luetic or tabetic type; 4, meningitic type.

the maximum color change occurs is more or less characteristic of the different pathologic conditions. The typical "curves" are shown in Fig. 302. The test gives its most consistent and valuable results in cases of general paresis. In lethargic encephalitis and poliomyelitis atypical curves in the tabetic zone have been observed.

The exact explanation of the test is not yet wholly clear, but it is undoubtedly dependent upon the presence of a globulin.

The test itself is relatively simple, and any difficulty may be attributed to imperfectly cleaned utensils or to a faulty reagent, the preparation of which is time consuming and uncertain. The reagent can be purchased ready prepared, or is easily prepared by the Borowskaja method.

[1] Johnston, G. W., and Gibson, R. B.: The Determination of Blood Plasma and Spinal Fluid Proteins, Amer. Jour. Clin. Path. (tech. suppl.), *8*:22–31 (Jan.), 1938.

It must be absolutely transparent and of a brilliant salmon or orange-red color with no trace of blue; it must be neutral to alizarin red on the day it is used; a 5-c.c. sample must be completely decolorized in one hour when added to 1.7 c.c. of 1 per cent sodium chloride; it must give a typical paretic curve with a known paretic spinal fluid, and must not give a reaction beyond "red blue" with a normal spinal fluid.

Borowskaja Modification.[1]—This very simple method for making a stable colloidal gold solution has been found satisfactory. Add 1 c.c. of 1 per cent solution of gold chloride (Merck's "Blue Label") to 95 c.c. of distilled water. Heat to 90° C., on an electric hot plate and add 5 c.c. of 1 per cent solution of sodium citrate (Merck's "Blue Label"). Boil for from one to three minutes.

Technic of Test.—Arrange a series of twelve clean test tubes in a rack. Place 1.8 c.c. fresh sterile 0.4 per cent solution of sodium chloride in the first test tube and 1 c.c. in each of the others except the twelfth. In the twelfth tube place 1.7 c.c. of sterile 1 per cent sodium chloride. To the first tube add 0.2 c.c. of the spinal fluid, which must be free from any trace of blood. Mix well by sucking the fluid up into the pipet and expelling it, and then transfer 1 c.c. to the second tube. Mix and transfer 1 c.c. to the third tube, repeating this down the row to the tenth tube and discarding the last 1-c.c. portion. This leaves the eleventh and twelfth tubes with salt solution only to serve as controls. To each of these twelve tubes add 5 c.c. of the colloidal gold solution. Let stand at room temperature for an hour or longer, at the end of which time, in the case of a positive reaction, the solution in some of the tubes will have changed from red to purple, deep blue, pale blue, or colorless. In the case of normal fluids no change will occur. The fluid in the eleventh and twelfth tubes which serve as controls should be orange-red and colorless, respectively. The results are usually charted, as shown in Fig. 302, in which each column represents a tube. For the purpose of brevity the colors may be indicated by the corresponding numbers, which are placed in the same order as the tubes. Thus the "paretic reaction" in Fig. 302 may be expressed as 5555542100. Felton's suggestion that the type of reaction is best indicated by using the terms Zone I, Zone II, Zone III is used in many laboratories for reporting the "paretic," "tabetic," or "meningitic" curves.

Boerner and Lukens[2] have described a modification of the original technic, which is more economical in the use of the gold sol. Place 1.8 c.c. of 0.4 per cent sodium chloride solution in the first tube and only 0.5 c.c. of this solution in the other tubes. Add 0.2 c.c. of spinal fluid to the first tube. Mix, discard 1 c.c., and transfer 0.5 c.c. to the second tube. Mix and transfer 0.5 c.c. of the mixture to the next tube, and so on to the last tube, discarding 0.5 c.c. from this tube after mixing the fluids. Then add only 2.5 c.c. of colloidal gold solution to all of the tubes.

[1] Borowskaja, D. P.: Zur Methodik der Goldsolbereitung. Zeitschr. f. Immunitätsforsch. u. exper. Ther., *82*:178–182, 1934. Abstract, Davidsohn, I., Arch. Path., *19*: 449 (Mar.), 1935.

[2] Boerner, Fred, and Lukens, Marguerite: A Modification of the Lange Colloidal Gold Test, Jour. Lab. and Clin. Med., *19*:1007–1008 (June), 1934.

(3) **Mastic Test.**—Because of the many difficulties in the way of preparing satisfactory and uniform colloidal gold solutions, the mastic test has been proposed as a substitute for the gold test. The reagent is inexpensive and easily made, and the test is easily carried out. Results appear to parallel those obtained with colloidal gold, being almost uniformly positive in paresis, cerebrospinal syphilis, and tabes; but there is much less definite differentiation of the various types of reaction. Complete precipitation of the mastic corresponds to complete decolorization of the colloidal gold solution; while partial precipitation of the mastic corresponds roughly to the purple or blue of the colloidal gold test. The method which follows is that used by Cutting.

Preparation of Solutions.—(*a*) *Mastic Solution.*—Make a stock solution by completely dissolving 10 Gm. of gum mastic, U. S. P., in 100 c.c. of absolute alcohol and filter. To 2 c.c. of this stock solution add 18 c.c. of absolute alcohol, mix well, and pour rapidly into 80 c.c. of distilled water.

(*b*) *Alkaline-saline Solution.*—Make a 1.25 per cent solution of sodium chloride (C. P.) in distilled water, and to each 99 c.c. of this solution add 1 c.c. of a 0.5 per cent solution of potassium carbonate in distilled water.

Technic of Test.—Arrange a series of six small test tubes. In the first place 1.5 c.c. of the alkaline-saline solution and in each of the others place 1 c.c. To the first tube add 0.5 c.c. of the spinal fluid, which must be completely free from blood. Mix by sucking the fluid up into the pipet and expelling it, and transfer 1 c.c. to the second tube. Again, mix and transfer 1 c.c. to the third tube and continue down the line to the fifth tube, discarding the 1-c.c. portion which is removed from this, and leaving the sixth tube with alkaline saline solution alone to serve as a control. Finally add 1 c.c. of the mastic solution to each tube. Mix well and set aside at room temperature for twelve to twenty-four hours, or in the incubator for six to twelve hours. Tubes in which the reaction is complete will show a heavy precipitate with clear supernatant fluid (Fig. 303).

(4) **Colloidal Benzoin Test.**—This test, devised by Guillain, Laroche, and Lechelle, is similar in many respects to the mastic test. It is not specific for neurosyphilis, but does give practically the same results as the more complicated colloidal gold test.

Reagents.—(*a*) *Benzoin Solution.*—Sumatra benzoin resin, 1 Gm.; absolute alcohol, 10 c.c. After forty-eight hours filter off the clear supernatant fluid. Keep in a tightly stoppered bottle. This is a stock solution from which the colloidal solution which is used in the test is freshly prepared each day as follows:

Add 0.3 c.c. of the stock benzoin solution, drop by drop with constant

shaking, to 20 c.c. of doubly distilled water. Heat to 35° C. in a water bath with constant shaking.

(*b*) *Salt Solution.*—Make 0.01 per cent sodium chloride in doubly distilled water.

Technic of Test.—Set up in a rack sixteen small test tubes (75 by 10 mm., or 85 by 13 mm.). In the first tube place 0.25 c.c. of salt solution (*b*); in the second tube 0.5 c.c.; in the third 1.5 c.c., and in each of the remaining tubes 1 c.c. Next add cerebrospinal fluid: 0.75 c.c. to the first tube; 0.5 c.c. to the second and third tubes. From tube three 1 c.c. of the thoroughly mixed dilution of spinal fluid is transferred to the fourth tube, and so on, until the fifteenth tube is reached, from which, after mixing, 1 c.c. is discarded. The sixteenth tube is used for control. The dilutions thus range from 3 parts in 4 in the first tube to 1 in 16,384 in the fifteenth tube. Finally, add 1 c.c. of the benzoin suspension (*a*) to each tube, and

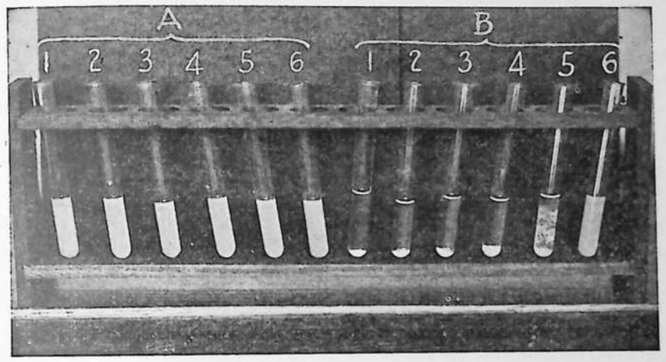


Fig. 303.—The mastic reaction in cerebrospinal fluid: *A*, From a case of dementia praecox, negative; *B*, from a case of paresis, positive. (Courtesy of Jas. A. Cutting.)

mix by shaking. The tubes are allowed to stand for from eighteen to twenty-four hours, preferably in the refrigerator.

The reaction will vary from no change in the mixture to complete precipitation of the benzoin, with absolute clearing of the supernatant fluid. The degree of reaction in each tube is reported: 0, no precipitation; 1, slight precipitation, with partial clearing; 2, more than half precipitated, fluid still cloudy; 3, complete precipitation, water-clear fluid. A curve may be plotted, or the figures representing the degree of reaction may be set down for each tube. Precipitation in the first six tubes indicates cerebral involvement, the first, or paretic zone; precipitation beginning with the seventh tube indicates involvement of the meninges, or spinal cord, the second, or meningeal zone. The test is not as sensitive as is the Lange colloidal gold method, and is not so definite in its reaction in multiple sclerosis.

(5) **Sugar.**—The normal cerebrospinal fluid contains about 0.04 to 0.07 per cent of dextrose, or roughly, about one half as much as

the blood. This is sufficient to give a distinct reaction with Benedict's test (p. 97), but it is well to use twice as much of the cerebrospinal fluid as is recommended for the urine. This reduction of copper is diminished or absent in certain forms of meningitis, probably owing to destruction of dextrose by the bacteria. From a study of a series of cases Jacob finds that: (1) No reduction of copper occurs in pyogenic meningitis (pneumococcus, streptococcus) or in acute meningococcic meningitis; (2) reduction occurs, but may be diminished in tuberculosis and in the more chronic cases of meningococcic meningitis; (3) reduction is normal in poliomyelitis. Increased production occurs in diabetes mellitus, and a moderate increase is sometimes observed in lethargic encephalitis.

Quantitative estimation may be carried out by the methods already given for blood sugar. It is advisable to make also a blood-sugar determination on a sample of the patient's blood which has been obtained by venipuncture at the same time that the spinal puncture has been made. These determinations should be carried out as promptly as possible after withdrawal, for accurate comparison. For the method of Folin and Wu (p. 391) dilute 1 volume of spinal fluid with 4 volumes of water and use in place of the protein-free blood filtrate. There must, of course, be a corresponding change in the calculation. Since 2 c.c. of the diluted spinal fluid represents 0.4 c.c. of the original amount, the formula on page 392 becomes

$$\frac{\text{Reading of the Standard}}{\text{Reading of the Unknown}} \times \text{D} \times 250 = \text{mg. of sugar in 100 c.c. of spinal fluid.}$$

(6) **Antimeningococcus-serum Test.**—Vincent and other French investigators have developed the following test, which they believe to be specific for epidemic cerebrospinal meningitis:

To 1 c.c. of the spinal fluid, which has been cleared by thorough centrifugation, are added a few drops of antimeningococcus serum. The tube, along with a control tube of the untreated fluid, is then placed in an incubator at 52° C. for a few hours. A positive reaction consists in the appearance of a white cloud. The present view is that the test has some value, but cannot be wholly relied upon. Sometimes a positive reaction occurs in other forms of meningitis. Sometimes both tubes become cloudy during the incubation.

(7) **Tryptophan Test.**—Lichtenberg[1] reported favorably on the use of Aiello's simple test for tryptophan in spinal fluid in the diagnosis of tuberculous meningitis.

[1] Lichtenberg, H. H.: The Tryptophan Test in Tuberculous Meningitis, Am. Jour. Dis. Child., *43*:32–39 (Jan.), 1932.

Reagents Required.—(*a*) Concentrated hydrochloric acid.

(*b*) Two per cent solution of formalin. This is made by diluting formalin 1 to 20 with distilled water.

(*c*) A 0.06 per cent solution of sodium nitrite.

Method.—1. Place 2 or 3 c.c. of spinal fluid in a large test tube.

2. Add 15 to 18 c.c. of concentrated hydrochloric acid (*a*), and 2 or 3 drops of 2 per cent formaldehyde solution (*b*). Shake the tube, and allow the mixture to stand for from four to five minutes.

3. Add carefully, to form a supernatant layer, 1 to 2 c.c. of 0.06 per cent sodium nitrite solution (*c*), and allow the mixture to stand for from two to three minutes.

In tuberculous meningitis a violet ring develops at the juncture of the first and second layers; this is the positive tryptophan reaction. A negative reaction is shown by a brown ring, or the absence of any colored ring.

(8) **Levinson's Test for Tuberculous Meningitis.**—This test, which was described by Levinson[1] many years ago, has been widely used. It is based upon the principle that the ratio between the alkaloidal precipitate formed by sulfosalicylic acid and the metallic precipitate formed by mercuric chloride may be greatly altered from the normal in cases of tuberculous meningitis. The test is indicative of this condition but is not specifically diagnostic.

Reagents.—(*a*) One per cent solution of mercuric chloride. (*b*) Three per cent solution of sulfosalicylic acid.

Method.—1. Place 1 c.c. of spinal fluid in each of two small test tubes.

2. Add 1 c.c. of 1 per cent solution of mercuric chloride (*a*) to the spinal fluid in one tube.

3. Add 1 c.c. of 3 per cent solution of sulfosalicylic acid (*b*) to the spinal fluid in the other tube.

4. Shake the tubes, stopper, and allow them to stand at room temperature for twenty-four hours.

5. Measure the height of the precipitate in millimeters.

The *normal* amount of precipitate is very slight; the sulfosalicylic acid precipitate is heavy, compact, and starts to form at once, while the mercuric chloride precipitate is light and feathery, and forms slowly. In cases of *tuberculous meningitis* the mercuric chloride precipitate is three times as great as that formed with sulfosalicylic acid, while in cases of *suppurative meningitis* a reverse ratio may result, and the heavy acid precipitate may be two or three times as great as that formed by the mercuric chloride.

[1] Association for Research in Nervous and Mental Diseases: The Human Cerebrospinal Fluid, New York, Paul B. Hoeber, Inc., Vol. 4, 1924, 568 pp.

3. Microscopic Examination.—This consists in a study of the bacteria, and of the number and kind of cells.

(1) **Bacteria.**—*Tubercle bacilli* can be found in the great majority of cases of tuberculous meningitis. The delicate coagulum which forms when the fluid is allowed to stand in a cool place for twelve to twenty-four hours will entangle any bacilli which may be present. This clot may be removed, spread upon slides, and stained by one of the methods already given (pp. 45–49). Still better is a method proposed by Sheo-Nan Cheer: To a portion of the spinal fluid in a centrifuge tube add with constant, gentle shaking, one third to one half its volume of 95 per cent alcohol and centrifugalize thoroughly at high speed. Just enough alcohol should be added to precipitate a slight cloud of proteins which will carry the tubercle bacilli to the

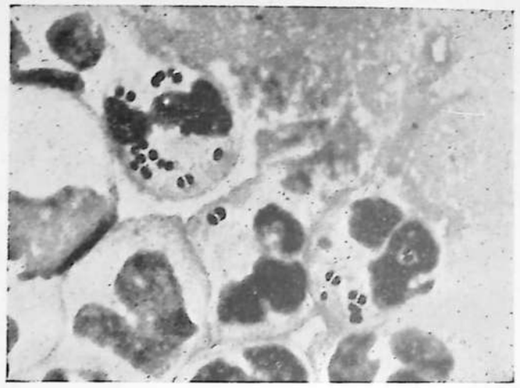


Fig. 304.—Meningococci in cerebrospinal fluid from a case of epidemic spinal meningitis. Gram's method and carbolfuchsin (photograph × 1500).

bottom. If the fluid gives no precipitate, a trace of egg albumin should be added. It may be necessary to examine a considerable number of smears. In doubtful cases inoculation of guinea-pigs must be resorted to.

The *Diplococcus intracellularis meningitidis* or *meningococcus* is the cause of epidemic cerebrospinal fever, and can be detected in the cerebrospinal fluid of most cases, especially those which run an acute course. Cover-glass smears from the sediment should be stained by a simple bacterial stain and by Gram's method. The meningococcus is an intracellular diplococcus which often cannot be distinguished from the gonococcus in stained smears (Fig. 304). It also decolorizes by Gram's method. The presence of such a diplococcus in meningeal exudates is, however, sufficient for its identification in clinical work. In acute meningitis the diplococci can sometimes be found within neutrophilic leukocytes in ordinary blood smears (Fig. 305).

When the meningococci are not found in direct smears from a spinal fluid which contains many pus corpuscles we have had excellent results with Obe's enrichment method. Enough sterile 10 per cent dextrose solution is added to make about a 1 per cent solution, and

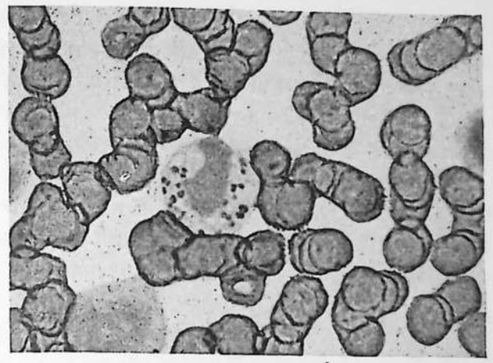


Fig. 305.—Meningococci in a leukocyte in a blood smear (photograph × 900).

the fluid is placed in the incubator for ten or twelve hours. The number of meningococci very greatly increases; they are taken up by the leukocytes and can be found in the sediment microscopically. Cultural methods are described on page 797.

Fig. 306.—Influenza bacilli in spinal fluid. Case of meningitis (photograph × 1000).

The meningococcus is present in the nasal discharge in most cases of this form of meningitis, and in very small numbers in the posterior nares and nasopharynx of some healthy persons who are dangerous as carriers. In this situation they can be identified only by cultures.

Various organisms have been found in other forms of meningitis—the pneumococcus most frequently, the influenza bacillus (Fig. 306) rarely. When the pneumococcus is present it is usually very abundant. In some cases no micro-organisms can be detected even by cultural methods.

(2) **Fungi.**—There have been an increasing number of cases of Torula meningitis reported. This organism resembles true yeast in that it reproduces by budding, but it differs from yeast in that it is incapable of fermenting sugars. In a routine examination of the spinal fluid, the organisms may be mistaken for lymphocytes. It is pathogenic for mice. (See page 631.)

(3) **Parasites.**—The larvae of Trichinella have been found in the spinal fluid at the same time that they are present in the blood (p. 575). Trypanosomes are present in the late stages of African sleeping sickness.

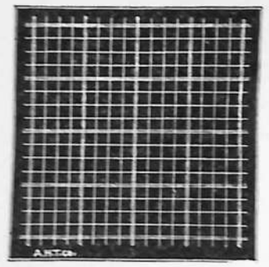


Fig. 307.—Fuchs-Rosenthal ruling of counting chamber for cells in spinal fluid.

(4) **Cytology.**—The fluid should be as fresh as possible, as the cells tend to degenerate. To avoid formation of a coagulum which might entangle the cells and interfere with the count it is well to secure the fluid in two tubes, one of which, used for the cytologic examination, contains a trace of powdered potassium oxalate. Early coagulation is, however, not common in the diseases in which the cell count is most important.

The routine examination should include both a total and a differential count.

The **total cell count** is best made with the Fuchs-Rosenthal counting chamber in a manner similar to the counting of leukocytes in blood. The ruled area (Fig. 307) in this chamber covers 16 sq. mm. and the depth below the cover glass is 0.2 mm. The capacity is thus a trifle more than 3 cu. mm. (sixteen fifths). Unna's polychrome methylene blue or a staining fluid consisting of crystal violet, 0.1 Gm., glacial acetic acid 1 c.c., water 50 c.c., 5 per cent phenol a few drops, is drawn into the leukocyte pipet to the mark 1, and the fresh spinal fluid, which has been well shaken, is drawn up to the mark 11. After mixing, a drop is placed in the counting chamber and covered. The number of cells in the entire ruled area is counted and this number divided by 3 to give the number of cells for each cubic millimeter of spinal fluid. The error incident to this calculation is practically balanced by the opposite error due to the dilution.

When the Fuchs-Rosenthal chamber is not at hand the ordinary hemacytometer counting chamber may be substituted. The number of cells in the ruled area is counted, the number for each cubic millimeter is calculated, and this is multiplied by $\frac{10}{9}$ to compensate for the dilution with staining fluid.

The **differential count** is made as described on page 265. A weak aqueous solution of methylene blue is probably preferable to Wright's stain for this purpose. Ordinarily, only two kinds of cells are seen: Lymphocytes and polymorphonuclear neutrophils.

The cells normally present are nearly all lymphocytes. They vary in number from 1 to 5 or 7 in each cubic millimeter; 10 is usually accepted as the maximum in health.

An increase in the cell count, together with predominance of lymphocytes (more than 70 per cent), strongly suggests tuberculous meningitis or syphilitic disease of the nervous system since it occurs in about 90 to 95 per cent of the cases. The number of cells present in these conditions varies greatly in different cases, but ordinarily lies between 25 and 100 in each cubic millimeter. Similar counts are frequent in lethargic encephalitis and anterior poliomyelitis, and may also sometimes be noted in cerebral hemorrhage, tumors, and the more chronic type of epidemic cerebrospinal meningitis.

In all forms of acute meningitis the total count is high, 100 to several thousand, and polymorphonuclear leukocytes prevail. A notable number of endothelial cells may also be present, especially in acute epidemic meningitis.

ANIMAL INOCULATION

Inoculation of animals is one of the most reliable means of verifying the presence of certain micro-organisms in fluids and other pathologic material, and is helpful in determining the species of bacteria which have been isolated in pure culture. Clinically, it is applied most frequently to demonstration of the tubercle bacillus when other means have failed or are uncertain.

Tuberculosis.—The guinea-pig is the most suitable animal for this purpose. When the suspected material is fluid and contains pus, it should be well centrifugalized, and 1 or 2 c.c. of the sediment injected, by means of a large hypodermic needle, into the peritoneal cavity or underneath the loose skin of the groin. If there is enough material, two guinea-pigs should be inoculated, one subcutaneously, and one intraperitoneally. Fluids from which no sediment can be obtained must be injected directly into the peritoneal cavity, since at least 10 c.c. are required, which is too great an amount to inject hypo-

dermically, and several animals should be inoculated, since some are likely to die from peritonitis caused by other organisms before the tubercle bacillus has had time to produce its characteristic lesions. For intraperitoneal injection the animal should be held with its head downward to cause the intestines to gravitate toward the diaphragm. The needle is then introduced between the umbilicus and the pelvis. Solid material should be placed in a pocket made by snipping the skin of the groin with scissors, and freeing it from the underlying tissues for a short distance around the opening.

The animals should be killed at the end of six or eight weeks, if they do not die before that time; and a careful search should be made for the characteristic pearl-gray or yellow tubercles scattered over the peritoneum and through the abdominal organs, particularly the spleen and liver, and for caseous inguinal and retroperitoneal lymph glands. The tubercles and portions of the caseous glands should be crushed between two slides, dried, and stained for tubercle bacilli. The bacilli are not usually abundant in the caseous material and may, therefore, be difficult to find.

If two guinea-pigs have been inoculated, one should receive an injection of 0.5 c.c. of undiluted old tuberculin four weeks after the inoculation. If tuberculosis is developing, the guinea-pig will very likely die within twenty-four hours because of this overwhelming dose of tuberculin. The other guinea-pig may then be killed for necropsy. This method may hasten the report if this is an important consideration in a given case.

Diphtheria Virulence Test.—At times it is necessary to determine the virulence of diphtheria bacilli that have been isolated from the throats of carriers. Such tests are usually carried out in the State Board of Health Laboratories as this is a public health problem. Standard methods are followed; briefly, they consist in using white guinea-pigs weighing about 250 to 400 gm. A pure culture of the organism to be tested is diluted by placing a loopful of the culture in 10 c.c. of 0.85 per cent salt solution. Two-tenths c.c. of the suspension may be injected intracutaneously into one of the guinea-pigs. A control animal which has been protected against the disease is also inoculated in the same manner with 500 units of diphtheria antitoxin given subcutaneously. Virulent diphtheria bacilli in the unprotected guinea-pig will produce redness and slight induration at the end of twenty-four hours at the site of injection and after forty-eight to seventy-two hours a marked necrotic area will develop. The toxin of diphtheria bacilli may also be tested by injecting 1 c.c. of toxin broth culture subcutaneously into a test animal. Observe the animal daily

and perform a necropsy as soon as it dies and look for characteristic signs of diphtheria toxicity. Diphtheria bacilli should be present in a stained preparation of the peritoneal fluid, and may be cultured.

Webster's Mouse Test for Rabies.—Webster and Dawson[1] have developed a quick biologic test for the diagnosis of rabies. Swiss mice are particularly susceptible to this disease. Emulsify a bit of Ammon's horn from the brain of the suspected animal in twenty times the amount of sterile water or broth. Inject 0.03 c.c. quantities through the skulls into the brains of eight Swiss mice, two to three weeks of age. Kill one mouse on the fifth day, another on the sixth day and a third mouse on the seventh day, and examine their brains for Negri bodies. Observe the remaining mice for twenty days for signs of rabies. If the suspected material really contains rabies virus, Swiss mice will usually show Negri bodies on the fifth and sixth days, become sick on the seventh to tenth days and die on the ninth to twelfth days.

Smallpox or Vaccinia.—The diagnosis of smallpox may be made by inoculating the cornea of rabbits with the suspected virus obtained from a pustule. This is known as "Paul's test" and has been well described by Scott and Simon.[2] The contents of a pustule may be dried on a microscope slide. Both eyes of the rabbit should be cocainized by using a few drops of a 5 per cent solution. Delicately scarify with a fine dissecting needle the corneas and inoculate the rabbit's eyes with an emulsion of the inspissated material. In forty-eight hours definite lesions may be detected in the cornea as bleb-like elevations which can best be seen with a magnifying lens. The animals may be killed and the eyeballs enucleated and placed in a bath composed of 2 parts of saturated solution of mercuric chloride and one part of 96 per cent alcohol. They should be removed in from two to four minutes to 70 per cent alcohol. Elevated, opaque blebs due to smallpox will be very apparent when viewed with a lens. The histologic examination of the cornea will confirm the diagnosis, although the *in vivo* examination of the cornea will usually give the desired information.

Rat Bite Fever.—This disease is now known to be due to two organisms, the *Spirillum minus* which has not been grown on culture media, and *Streptobacillus moniliformis* which may be grown on veal infusion broth containing 20 per cent of rabbit serum. *Spirillum minus* has been demonstrated only by injecting blood of the patient intraperitoneally

[1] Webster, L. T., and Dawson, J. R., Jr.: Early Diagnosis of Rabies by Mouse Inoculation. Measurement of Humoral Immunity to Rabies by Mouse Protection Test, Proc. Soc. Exper. Biol. & Med., *32*:570–573 (Jan.), 1935.

Webster, L. T.: Rabies, New York. The Macmillan Company, 1942, 168 pp.

[2] Scott, J. M., and Simon, C. E.: The Diagnosis of Smallpox by the Paul Method, Amer. Jour. Hygiene, *3*:401–415 (July), 1923.

into white rats. The organism is found after a week to ten days by dark field examination of the blood of infected rats.

Leptospirosis.—White mice, three weeks of age, are inoculated intraperitoneally or subcutaneously according to the method used by Larson (see page 513). White guinea pigs are also used for demonstrating the presence of this organism by animal inoculation. When infected, they become markedly jaundiced.

CHAPTER IX

MISCELLANEOUS EXAMINATIONS

THE NOSE, MOUTH, AND PHARYNX

MICRO-ORGANISMS are always present in the mouth in large numbers. Among these is *Leptotrichia buccalis* (Fig. 308), which is especially abundant in the crypts of the tonsils and the tartar of the teeth. The whitish patches of *pharyngomycosis leptothrica* are largely composed of these fungi. They are slender, segmented threads, which generally, but not always, stain violet with Lugol's solution, and are readily seen with a 4-mm. objective. Often the segments are separated, and then have the appearance of very large non-motile bacilli. At times they are observed in the sputum and stomach fluid. In the former they might be mistaken for elastic fibers; in the latter, for Boas-Oppler bacilli. In either case the reaction with iodine will distinguish them.

The prevalence of endamebae and spirochetes in the mouths of normal persons and of those suffering from pyorrhea alveolaris has already been mentioned (pp. 504 and 514).

Thrush is a disease of the mouth seen most often in children, and characterized by the presence of white patches upon the mucous membrane. It is caused by the thrush fungus, *Endomyces* (*Monilia*) *albicans*. When a bit from one of the patches is pressed out between a slide and cover and examined with a 4 mm. objective, the fungus is seen to consist of a network of simple or branching segmented filaments (Fig. 309). The segments measure about 4 by 20 μ. Globular or oval cells of somewhat greater diameter are attached to the filaments or lie free in the meshes between them. The meshes also contain leukocytes, epithelial cells, and granular débris. A related fungus, *Monilia psilosis*, is found in scrapings from the tongue lesions of sprue.

Acute pseudomembranous inflammations, which occur chiefly upon the tonsils and nasopharynx, are generally caused by the diphtheria bacillus, but may result from streptococcic infection. In many cases diphtheria bacilli can be demonstrated in smears made from the membrane and stained with Löffler's methylene blue or 2 per cent aqueous solution of methyl green or, much better, Albert's stain, given below. It is generally necessary, however, and always

safer, to make a culture from the throat swabs upon Löffler's blood serum, and to examine smears from the growth at the end of eight, twelve, twenty-four, and thirty-six hours. The bacilli are straight or

Fig. 308.—Gingival deposit (unstained): *a*, Squamous epithelial cells; *b*. leukocytes; *c*, bacteria; *d*, *Leptotrichia buccalis*. (Jakob.)

Fig. 309.—Thrush fungus (*Endomyces albicans*). (Kolle and Wassermann.)

curved rods, which vary markedly in size and outline and stain very irregularly. A characteristic form is a palely tinted rod with several

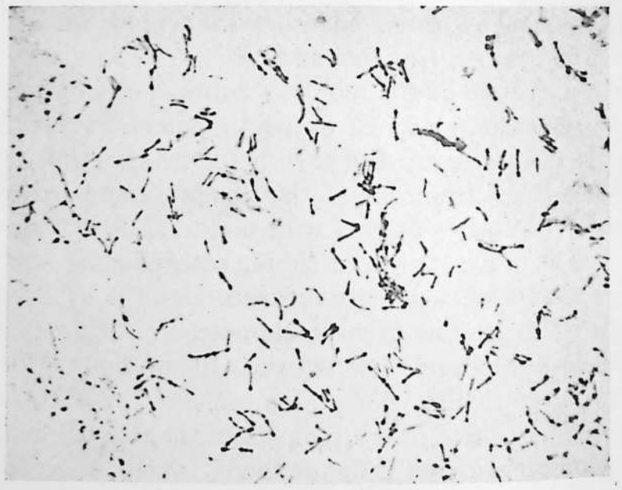


Fig. 310.—*Bacillus diphtheriae* stained with methyl green; culture from throat (photograph × 1000).

deeply stained granules (metachromatic granules), or with one such granule at each end (Fig. 310). Sometimes they are club shaped or barred. There is a strong tendency to parallelism—for several to lie

side by side. They are gram-positive. In clinical diphtheria the bacilli generally dominate every field, provided the swab was properly made. In "carriers" often only occasional small groups can be found in the film.

Sometimes bacilli are found which are morphologically typical, but avirulent. These may be recognized by isolating them and inoculating a guinea-pig subcutaneously or intraperitoneally with 1 c.c. of a forty-eight- to seventy-two-hour broth culture. If the strain be virulent, the animals should die within two days. As a control it is well to inject into a second guinea-pig the same amount of culture to which have been added 100 to 200 units of diphtheria antitoxin. This pig should not die.

Neisser's stain has long been the standard differential stain for the diphtheria bacillus. It colors the bodies of the bacilli brown and the metachromatic bodies blue.

1. Make films and fix as usual.
2. Apply the following solution, freshly filtered, for about one-half minute:

Methylene blue	0.1 Gm.
Alcohol (96 per cent)	2.0 c.c.
Glacial acetic acid	5.0 "
Distilled water	95.0 "

3. Rinse in water.
4. Apply a saturated aqueous solution of Bismarck brown one-half minute.
5. Rinse, dry, and mount.

Albert's Method.—We have found this more satisfactory than Neisser's.

1. Prepare films, dry, and fix in the flame.
2. Apply the following staining solution for one minute:

Toluidine blue	0.15 Gm.
Methyl green	0.2 "
Glacial acetic acid	1.0 c.c.
Alcohol (95 per cent)	2.0 "
Distilled water	100.0 "

Mix well, let stand twenty-four hours, and filter.

3. Rinse with water and blot dry.
4. Apply the following iodine solution for one minute:

Iodine	2.0 Gm.
Potassium iodide	3.0 "
Distilled water	300.0 c.c.

5. Rinse in water, dry, and examine.

Diphtheria bacilli are green, bars dark green, granules black and very prominent. Nearly all other organisms take a light green stain. The formula as published has proved very satisfactory; however, some workers prefer to substitute malachite green for methyl green.

Vincent's angina is a pseudomembranous and ulcerative inflammation of mouth and pharynx, which when acute may be mistaken for diphtheria, and when chronic is very apt to be mistaken for syphilis. Stained smears from the ulcers or membrane show large numbers of spirochetes and fusiform bacilli, giving a striking and characteristic picture (Fig. 311). Before making the smears the surface of the

Fig. 311.—*Borrelia vincenti* from case of ulcerative stomatitis stained with gentian violet (photograph × 1200).

lesion should be gently cleaned by swabbing, otherwise so many saprophytic bacteria may be present that the characteristic picture is obscured. The bacillus is spindle shaped, more or less pointed at the ends, and about 4 to 8 μ long. The spiral organism is a very slender, wavy thread, about 10 to 20 μ long, and stains feebly. Diluted formalin-gentian-violet makes a satisfactory stain. Giemsa's stain is also satisfactory. With methylene blue the palely staining spirillum may easily be overlooked. Their reaction to Gram's stain is variable. Further description is given on page 512.

Tuberculous ulcerations of mouth and pharynx can generally be diagnosed from curettings made after careful cleansing of the surface. The curettings are well rubbed between slide and cover, and

the smears thus made are dried, fixed, and stained for tubercle bacilli Since there is much danger of contamination from tuberculous sputum, the presence of tubercle bacilli is significant only in proportion to the thoroughness with which the ulcer was cleansed. The diagnosis is certain when the bacilli are found within groups of cells which have not been dissociated in making the smears.

In **leprosy** with intranasal ulcerations smears made from the nasal secretions or, better, from swabs of the ulcers themselves, constitute a useful aid to diagnosis. They often show the leprosy bacilli in great numbers, generally lying within large cells. They are stained as are tubercle bacilli, but with especial care not to decolorize, since these bacilli are not so strongly acid-fast as are tubercle bacilli. Leprosy bacilli can also be found, and are often abundant in material aspirated from any accessible nodular lesion, as is described in a later section for aspiration of *Treponema pallidum* from lymph nodes.

SALIVA

The secretion of the salivary glands amounts to about 1500 c.c. in twenty-four hours. It is a colorless, odorless, slightly viscid liquid with alkaline reaction. Microscopically it contains a few squamous epithelial cells; a few "salivary corpuscles," which are chiefly mononuclear blood corpuscles, generally much swollen and often filled with granules in active brownian motion; and many miscellaneous bacteria, the number depending largely on the condition of the teeth and gums. Most of the micro-organisms are saprophytic and many will not grow on ordinary culture media; but pneumococci, chiefly Types III and IV, streptococci, and other pathogenic organisms are not infrequently present in the saliva of healthy persons.

Physiologically speaking, the most important constituent of saliva is salivary amylase or ptyalin. Its presence may be recognized by its digestion of starch. To a few cubic centimeters of saliva in a test tube are added a few drops of a 1 per cent solution of starch, and the tube is placed in an incubator or water bath at about 37° C. for ten or fifteen minutes. At intervals a drop of the fluid is removed and mixed with a drop of Gram's iodine solution on a white porcelain plate. If ptyalin be present, the successive drops so treated will turn blue, purple, red, and finally yellow, owing to the gradual transformation of starch into the successive products of digestion, erythrodextrin and achroodextrin, and finally into maltose, which is capable of reducing Benedict's copper solution.

From the clinical point of view an important constituent of saliva is urea. As is well known, there is a tendency to uniform distribution

of urea among the body fluids, and Hench and Aldrich have shown that the combined urea and ammonia nitrogen of the saliva approximates the concentration of urea nitrogen in the blood, and thus furnishes a useful index of renal functional capacity. The sum of the urea and ammonia nitrogen is taken because urea tends to break down into ammonium carbonate through the action of bacteria after the saliva is secreted, and the ammonia of the saliva therefore really represents urea. Saliva is obtained for examination as described below. It is filtered through several layers of gauze, and diluted as described in the footnote on page 366. Urea is then estimated by the method for blood urea (p. 365) which gives urea and ammonia nitrogen together. Normally, saliva contains from 6 to 16 mg. of combined urea and ammonia nitrogen for each 100 c.c. corresponding to about 13 and 36.5 mg. urea; this increases with any increase of urea in the blood, although the rate of increase is somewhat less. The concentration of urea in the saliva is apparently very little influenced by salivation or the action of pilocarpine.

More recently Hench utilized the mercury-combining power of saliva as an approximate method of determining urea and hence as an index of renal insufficiency. The power to bind mercury is due to various nitrogenous substances in saliva, but chiefly to urea. The method is extremely simple and requires not more than five or ten minutes, hence it is available as an office procedure. It cannot take the place of blood-urea determinations. When blood chemical methods are not available, or when venipuncture is impossible or impracticable, it offers a simple means of determining whether nitrogen retention exists and approximately the degree; and it may be used as a preliminary procedure when resort to blood chemistry is contemplated.

Mercury-combining Power of Saliva.—Method of Hench and Aldrich.[1]—*Reagents Required.*[2]—(*a*) Mercuric chloride solution, an accurately prepared 5 per cent solution of chemically pure mercuric chloride in distilled water. (*b*) Sodium carbonate, saturated solution in distilled water.

Collection of Saliva.—The mouth is well rinsed with water. Chewing of a small piece of paraffin or holding a small marble in the mouth will favor the flow of saliva, but this is not necessary. The saliva is collected in two portions of about 8 c.c. each. The first of these carries off food particles and epithelial débris and is discarded. The second is used for the titration. It need not be filtered.

Method.—1. By means of a pipet transfer 5 c.c. of the saliva to a small flask or beaker.

2. Add 5 per cent solution of mercuric chloride from a buret or pipet a few drops at a time, with constant stirring, until a drop of the fluid, when added to a drop of saturated solution of sodium carbonate on a white porcelain plate, gives a definite reddish-brown color. The color should appear within about three seconds. If it develops more slowly, the end-point is near, but not yet reached, and a few additional drops of the bichloride must be added.

3. When the end-point is reached note the number of cubic centimeters of mercuric chloride solution which have been added, and multiply by 20 to find the number of cubic centimeters which would be required for 100 c.c. of saliva. Record this as the "mercury-combining index."

Hench has found the mercury-combining index in normal persons to lie between 30 and 50 for 100 c.c. of saliva. When there is retention of urea in the blood the index rises with the blood urea, although it lags a little behind. The probable blood urea may be roughly calculated as follows:

1.43 × salivary index − 34 = Probable blood urea in milligrams for each 100 c.c.

Example: Suppose the salivary index were 100. Then 1.43 × 100 − 34 = 109 mg. urea in 100 c.c. of blood.

The significance of retention of urea in the blood as an index of kidney insufficiency is discussed on page 171.

[1] Hench, P. S., and Aldrich, Martha: A Salivary Index to Renal Function, Jour. Am. Med. Assn., *81*:1997–2003 (Dec. 15), 1923.

[2] For the convenience of the practitioner the necessary reagents and a simple apparatus have been assembled in a small box and put on the market by Hynson, Westcott, and Dunning, Baltimore, Md., and also by the LaMotte Chemical Products Co., Baltimore, Md. The apparatus may also be used for the Hench and Aldrich method of direct blood-urea determination based on the mercury-combining power.

THE EYE

For studying the bacteriology of the eye both smears and cultures should be made. The former are much the more useful in many cases. In the normal conjunctiva *Staphylococcus albus*, *Corynebacterium xerosis*, and Type IV pneumococcus are the bacteria most commonly found.

Staphylococci (Figs. 312 and 313), **pneumococci** (Fig. 314) and **streptococci** are probably the most common of the bacteria to be found in nonspecific conjunctivitis and keratitis. Serpiginous ulcer of the cornea is generally associated with the pneumococcus (Fig. 295, p. 589).

The usual cause of acute infectious conjunctivitis ("pink-eye"), especially in cities, seems to be the **Koch-Weeks bacillus.** This is a minute, slender rod, which lies within and between the pus corpuscles (Fig. 315), and is negative to Gram's stain. In smears it cannot be distinguished from *H. influenzae* (Fig. 316) although its length is somewhat greater.

The **diplobacillus of Morax and Axenfeld** gives rise to an acute

Spread the material on clean glass microscope slides. Fix in methyl alcohol (page 251) and stain with Giemsa's stain (page 255). Decolorize with ethyl alcohol. The predominating forms are small "elementary bodies," intracellular and extracellular. Occasionally, large "initial bodies" are seen (Plate XV). The disease is also seen among adults and is known as "swimming pool conjunctivitis." There is a striking similarity between the morphologic appearance of the inclusion bodies found in the disease of the newborn and the appearance of **trachoma bodies** found in the chronic disease, trachoma, transmitted from person to person. Inclusion bodies will be considered briefly in the next section.

Inclusion Bodies

According to van Rooyen and Rhodes,[1] true virus inclusions give negative Feulgen and MacCallum tests, resist solution in hydrochloric acid, do not react to tests for mitochondria, the Golgi apparatus and the centrosome, do not give a positive oxidase test, and frequently stain vitally with cresyl blue.

The types of inclusion bodies are classified as the trachoma type, small basophilic inclusions and eosinophilic inclusions according to these authors. Cowdry[2] described nuclear inclusions, cytoplasmic inclusions, and both nuclear and cytoplasmic inclusions.

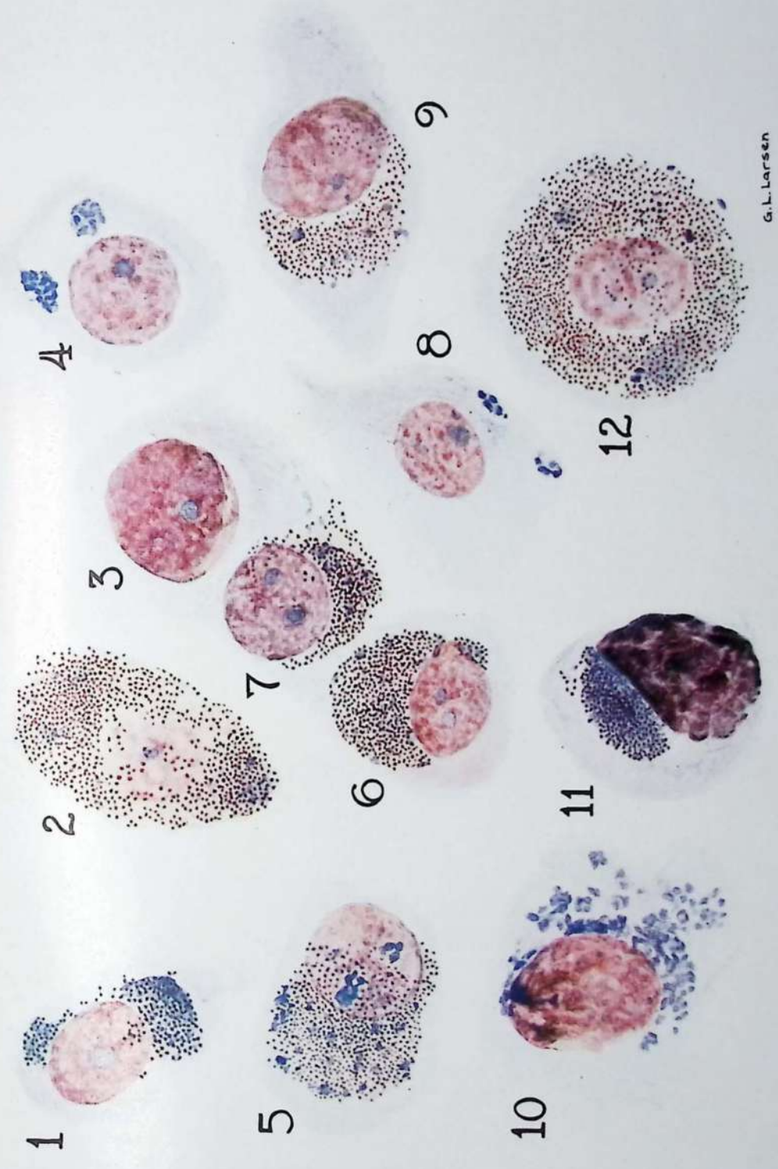
(*a*) **The Trachoma Type.**—The cytoplasmic inclusions of trachoma (0.200 microns), of inclusion blennorrhea (0.233 microns) and of psittacosis (0.275 microns) all stain readily with Giemsa's stain and all show the elementary bodies and the large plaques called "initial bodies" by Thygeson (see plate XV). These inclusion bodies are all so similar that they can be differentiated only by knowing the source of the material. The inclusion bodies that cause infectious blennorrhea may also be recovered from certain forms of cervicitis and urethritis. They also cause swimming pool conjunctivitis. The trachoma bodies are obtained from scrapings of the eyelids in cases of trachoma. The inclusion bodies of psittacosis can be recovered only by inoculating the sputum or blood of a patient into mice or pocket gophers. The matrix of inclusion bodies in trachoma and in inclusion blennorrhea can be demonstrated by staining with Lugol's solution. This matrix is probably of glycogen nature because it reacts with iodine.[3] The iodine

[1] van Rooyen, C. E., and Rhodes, A. J.: Virus Diseases of Man. London, Oxford University Press, 1940, 932 pp.

[2] Cowdry, E. V.: Identification of Inclusions in Virus Diseases, Amer. Jour. Clin. Path., *10*:133–146 (Feb.), 1940.

[3] Rice, C. E.: The Carbohydrate Matrix of the Epithelial-cell Inclusion in Trachoma Amer. Jour. Ophthal., *19*:1–8 (Jan.), 1936.

PLATE XV



Drawing of inclusion bodies from a case of severe inclusion blennorrhea in the acute stage. Cell 3 is normal. Cells 4, 8 and 10 contain young inclusions made up of initial bodies. In cell 5 the change from initial bodies to elementary bodies is almost complete. In cells 2, 6, 7, 11 and 12 the inclusions are mature, being made up almost entirely of elementary bodies. (Phillips Thygeson in Transactions of the American Ophthalmological Society.)

stain is distinctly valuable as a laboratory procedure in the diagnosis of trachoma.[1] (Figs. 318 and 319.)

(*b*) **Small basophilic Inclusions.**—Typical discrete blue granules (Giemsa's stain) lying in a more or less circumscribed area, as cytoplasmic inclusions, may be demonstrated by injecting into animals material from lymphogranuloma inguinale.

(*c*) **Eosinophilic Inclusions.**—Small acidophilic nuclear inclusions may be demonstrated in animals that have received injections of material obtained from herpes febrilis. The Guarnieri bodies of variola-vaccinia are acidophilic nuclear and cytoplasmic inclusions. The large molluscum bodies of molluscum contagiosum are filled with cyto-

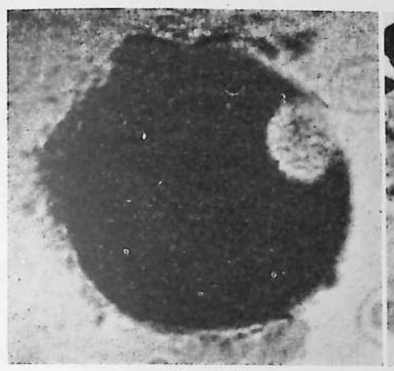


Fig. 318

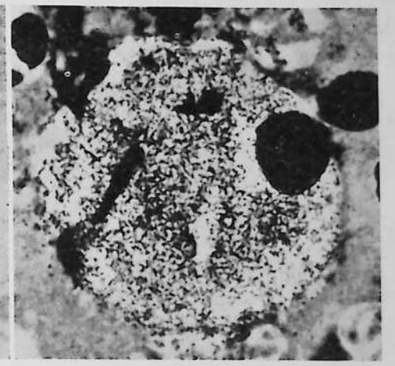


Fig. 319

Fig. 318.—Large inclusion body occupying the entire cytoplasm of an epithelial cell; stained with Lugol's solution (× 2000). (Thygeson, Am. J. Path., Vol. 14.)

Fig. 319.—The inclusion body shown in Fig. 318, restained by Giemsa's method after removal of the iodine with water. It is seen to be composed of myriads of elementary bodies. (Thygeson, Am. J. Path., Vol. 14.)

plasmic inclusions of small eosinophilic elementary bodies. The Negri bodies found in the brains of animals suffering from rabies are eosinophilic cytoplasmic inclusions. (See Plate XV.)

DIAGNOSIS OF RABIES

In view of the brilliant results attending prophylactic treatment by the Pasteur method, early diagnosis of rabies (hydrophobia) in animals which have bitten human beings is extremely important.

The most reliable means of diagnosis is the production of the disease in a Swiss mouse two to three weeks old by subdural or intracere-

[1] Thygeson, Phillips: The Matrix of the Epithelial Cell Inclusion Body of Trachoma, Amer. Jour. Path., *14*:455–462 (July), 1938.

bral injection of a little of the filtrate from an emulsion of the brain and medulla of the suspected animal. The diagnosis can, however, usually be quickly and easily made by microscopic demonstration of Negri bodies. Whether these bodies are protozoan in nature and the cause of the disease, as held by Negri, or whether they are products of the disease, it is certain that their presence is pathognomonic.

Negri bodies are sharply outlined, round, oval, or somewhat irregular structures which vary in size, the extremes being 0.25 and 30 μ. They consist of a hyalin-like cytoplasm, in which when properly stained one or more chromatin bodies can usually be seen. They are situated chiefly within the cytoplasm of the large cells of the central nervous system, the favorite location being the multipolar cells of the hippocampus major (Ammon's horn). In many cases they suggest red blood corpuscles lying within nerve cells.

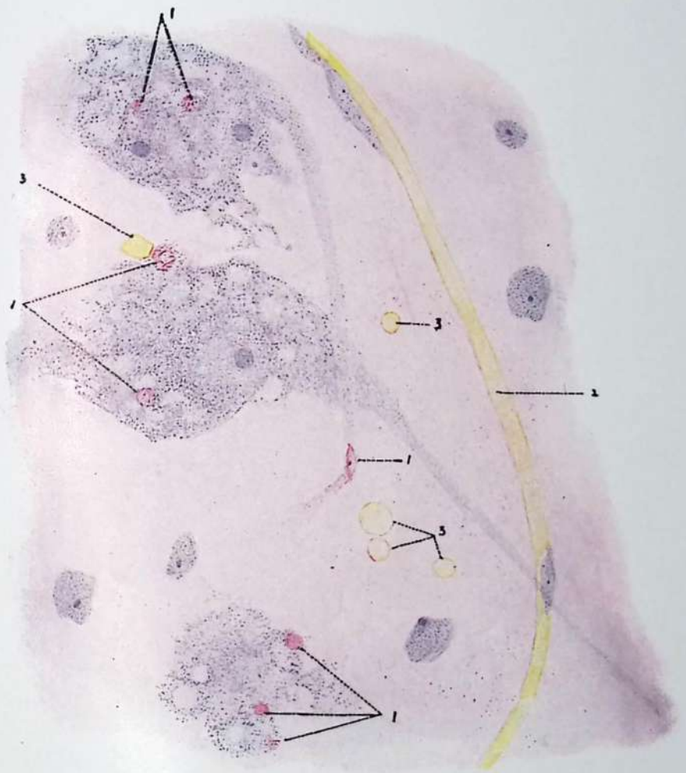
Probably the best clinical method of demonstrating Negri bodies is the impression method of Langdon Frothingham, which is carried out as described below.

1. Place the dog's brain[1] upon a board about 10 inches square, and divide into two halves by cutting along the median line with scissors.
2. From one of the halves cut away the cerebellum and open the lateral ventricle, exposing Ammon's horn.
3. Dissect out Ammon's horn as cleanly as possible.
4. Cut out a small disk at right angles to the long axis of Ammon's horn, so that it represents a cross section of the organ.
5. Place this disk upon the board near the edge, with one of the cut surfaces upward.
6. Press the surface of a thoroughly clean slide upon the disk and lift it suddenly. The disk (if its exposed surface has not been allowed to become too dry) will cling to the board, leaving only an impression upon the slide. Make several similar impressions upon different portions of the slide, using somewhat greater pressure each time. Impressions are also to be made from the cut surface of the cerebellum, since Negri bodies are sometimes present in the Purkinje cells when not found in Ammon's horn.
7. Before the impressions dry immerse in methyl alcohol for one-half to two minutes.
8. Cover with van Gieson's methylene-blue-fuchsin stain, warming gently for one-half to two minutes. This stain, as modified by Frothingham, is as follows. It remains effective for three or four days:

Tap water	20 c.c.
Saturated alcoholic solution basic fuchsin	1 drop
Saturated aqueous solution methylene blue	1 "

[1] For Dr. Frothingham's method of removing a dog's brain see American Journal of Public Hygiene for February, 1908.

PLATE XVI



Nerve cells containing Negri bodies.

Hippocampus impression preparation, dog. Van Gieson's stain; × 1000. 1, Negri bodies; 2, capillary; 3, free red blood corpuscles. (Courtesy of Langdon Frothingham.)

9. Wash in water and dry with filter paper. Examine with a low power to locate the large cells in which the bodies are apt to be found, and study these with an oil-immersion lens.

The Negri bodies are stained pale pink to purplish red, and frequently contain small blue dots (Plate XVI). The nerve cells are blue, and red blood corpuscles are colorless or yellowish copper colored.

When the work is finished the board with the dissected brain is sterilized in the steam sterilizer.

Demonstration of Negri bodies by this method is quicker and, possibly, more certain than by the study of sections. It has the decided advantage over the smear method that the histologic structure is retained. One or more of the impressions generally shows the entire cell arrangement almost as well as in sections, and it is very easy to locate favorable fields with a 16-mm. objective.

Seller's stain is satisfactory. The stain is prepared as follows:

Fuchsin (saturated solution of basic fuchsin in 95 per cent ethyl alcohol or absolute methyl alcohol fuchsin)	2–4 c.c.
Methylene blue (saturated solution in absolute methyl alcohol)	15 "
Methyl alcohol (absolute acetone-free)	25 "

Mix the methylene blue and alcohol in a Coplin jar and add 2 c.c. of fuchsin solution. A trial is made. The stain will probably be too blue. If so, add portions of 0.5 c.c. of fuchsin solution, and test the stain again until the proper balance is obtained. Macroscopically, the properly stained smear, when held up to the light, should appear violet in the thinner portions, shading into purplish blue in the thicker portions. The stain greatly improves after twenty-four hours and will keep indefinitely, but must be kept covered to prevent evaporation.

Smears are prepared in the usual way from the hippocampus major, and while still moist are plunged into the stain for two or three seconds and waved back and forth. Remove and rinse in running tap water, blot and examine.

Negri bodies are stained bright, cherry red; the nuclear material is stained black and blue and forms sharp contrast; nerve cells are stained purplish-blue; stroma is stained pink; nerve fibers are stained a faint pink or are not stained; bacteria, if present, are stained deep blue; red blood cells are stained a copper color; muscle fibers are stained deep pink or a copper color; the nuclei of leukocytes are stained deep blue with pink or pale blue border.

RICKETTSIAL DISEASES

According to Olitsky,[1] Rickettsia can be defined as minute, pleomorphic, gram-negative, bacilloid structures which cannot be stained satisfactorily by means of ordinary dyes and cannot be cultivated out-

[1] Olitsky, P. K.: Hans Zinsser and His Studies on Typhus Fever, Jour. Am. Med. Assn., *116*:907–912 (Mar. 8), 1941.

side the body except in the presence of living tissue cells. They are contained within the bodies of arthropods which in turn convey them to mammalian hosts, sometimes through an intermediary host, such as a rodent. In addition, infection by certain of them is associated with the development of a positive Weil-Felix reaction. (See page 625.) The pathogenic forms develop intracellularly. Typhus Rickettsia are found in the cytoplasm while Rickettsia are found in the cytoplasm and in the nucleus of cells from cases of Rocky Mountain spotted fever. Rocky Mountain spotted fever is transmitted chiefly by *Dermacentor andersoni* (fig. 278, page 580). The eastern form of the disease is transmitted by the common eastern dog tick, *Dermacentor variabilis*.

Typhus fever is transmitted through the body louse, *Pediculus humanus*. The European type of "rickettsia body" is named *Rickettsia prowazeki*. It is closely related to but differs serologically from similar bodies that are found in cases of typhus fever in this country. Another type of disease which is transmitted by the body louse is so-called "trench fever." The bodies that are found in this disease have been named *Rickettsia quintana*, or *Rickettsia pediculi*. It has also been demonstrated that the rickettsia which are found in the Mexican type of typhus fever (tabardillo) also may be found in fleas, bedbugs, and ticks; this suggests that these insects, as well as body lice, may be vectors.

Mooser[1] demonstrated that 90 per cent of male guinea-pigs could be infected with the "virus" of the Mexican type of typhus fever. He inoculated them intraperitoneally with 3 or 4 c.c. of blood which was diluted with an equal amount of sterile physiologic saline solution. The scrotum of a reacting guinea-pig becomes markedly swollen and reddened. If the scrapings from the tunica vaginalis are properly stained, minute bodies resembling diplobacilli can be found. Castaneda[2] developed the following method:

Castaneda's Stain for Rickettsia.—Prepare a buffer solution by dissolving 1 Gm. of monobasic potassium phosphate in 100 c.c. of distilled water, and 25 Gm. of dibasic sodium phosphate in 900 c.c. of distilled water. Mix the two solutions. The *p*H should be 7.5. Add 1 c.c. of formalin as a preservative. To 20 c.c. of the buffer solution, add 3

[1] Mooser, H.: Experiments Relating to the Pathology and the Etiology of Mexican Typhus (Tabardillo); Clinical Course and Pathologic Anatomy of Tabardillo in Guinea-pigs, Jour. Infect. Dis., *43*:241–260 (Sept.), 1928. Mooser, H., and Dummer, Clyde: On Relation of Organisms in Tunica Vaginalis of Animals Inoculated with Mexican Typhus to Rickettsia prowazeki and to Causative Agent of That Disease, Jour. Exper. Med., *51*:189–207 (Feb.), 1930.

[2] Castaneda, M. R.: A New Stain for Rickettsia Bodies, Jour. Infect. Dis., *47*:416–417, 1930.

drops of Loeffler's methylene blue, or probably better, 0.15 c.c. of a 1 per cent solution of methylene blue in alcohol. As a counterstain add 1 part of a 0.2 per cent aqueous solution of safranine "0" to 3 parts of 0.1 per cent solution of acetic acid.

To stain "rickettsia" or "Mooser bodies" scrape with an eye scalpel the inner surface of the tunica vaginalis from an infected guinea-pig. Make a thin layer on a slide. Stain the smear three minutes with the buffered methylene blue stain. Pour off the stain and, without washing, counterstain with the safranine stain for from one to four seconds. Wash with running water and dry with filter paper. The rickettsia bodies will be stained blue, and the protoplasm and nuclei will be stained red.

While this brief description of rickettsia has been included in this chapter on animal parasites, the morphology of the organisms would place them among the bacteria. On the other hand, the probability of a reservoir host and the transmission by an insect vector, suggests a protozoal nature.

Another very interesting but somewhat baffling diagnostic test for this group of diseases is known as the Weil-Felix reaction. A particular strain of *Proteus* bacillus, which is known as *Proteus X 19*, is agglutinated specifically with dilutions of 1 to 100 to 1 to 5000 of serum from patients who are suffering from typhus fever, trench fever, or Rocky Mountain spotted fever, but not with serum of patients who have typhoid fever, dysentery, or similar infections. The phenomenon has not been explained, as the organism has nothing to do with the disease etiologically. The technic for the test is discussed on page 662.

THE EAR

By far the most frequent exciting causes of acute otitis media are the pneumococcus and the streptococcus. The finding of other bacteria in the discharge generally indicates a secondary infection, except in cases complicating infectious diseases, such as typhoid fever, diphtheria, and influenza. Discharges which have continued for some time are practically always contaminated with the staphylococcus. The presence of the streptococcus should be a cause of uneasiness, since it much more frequently leads to mastoid disease and meningitis than does the pneumococcus. The staphylococcus, bacillus of Friedländer, colon bacillus, and *Bacillus pyocyaneus* may be met in chronic middle-ear disease.

In tuberculous disease the tubercle bacillus is present in the discharge, but its detection offers some difficulties. It is rarely easy to

find, and precautions must always be taken to exclude the smegma and other acid-fast bacilli (p. 51), which are especially liable to be present in the ear. Rather striking is the tendency of old squamous cells to retain the red stain, and fragments of such cells may mislead the unwary.

GOUTY TOPHI

Tophi (literally "porous stones") are small subcutaneous swellings. In cases of gout they are frequently found in an auricle, in the region of the helix, and often are connected with the cartilage. They may occur also in various other parts of the body, particularly in the olecranon bursa. A small incision into a tophus permits the extrusion

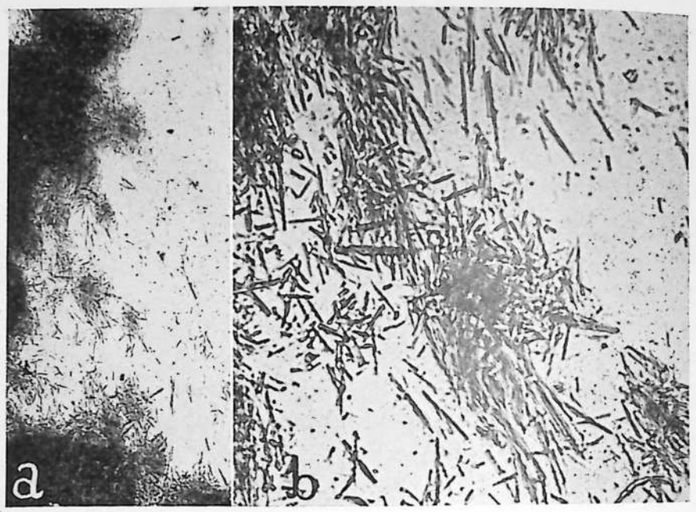


Fig. 320.—Sodium biurate crystals obtained from gouty tophus; *a*, × 100; *b*, × 400. (Photographs reproduced by courtesy of Dr. P. S. Hench.)

of a creamy or chalky mass of sodium acid urate. Typical, acicular, doubly refractive crystals of sodium biurate can be demonstrated easily with the microscope (Fig. 320).

DISEASES DUE TO FUNGI

Favus, tinea versicolor, and the various forms of ringworm are caused by members of the fungus group. To demonstrate them, a crust or a hair from the affected area is softened with a few drops of 10 per cent caustic soda solution, pressed out between a slide and cover, and examined with a 4-mm. objective. They consist of a more or less dense network of hyphae and numerous round or oval refractive spores.

Fungi grow best on media containing dextrose, and, as a rule, either Sabouraud's medium or tartaric acid medium (see p. 768), is used for the isolation of these organisms, as these media inhibit the growth of ordinary bacteria. As a rule, fungi grow slowly, best at 30° C. Cultures should not be discarded as negative until they have been incubated for several weeks. Mycotic diseases may be dermatomycoses, or they may be subcutaneous, or systemic.[1]

Tinea versicolor is caused by *Malassezia furfur* (see Fig. 321). Erythrasma, found in the inguinal folds, in the upper and internal surfaces of the thighs and of the axilla, is supposed to be caused by *Actinomyces minutissimus*. *Microsporon audouini* produces mild lesions of microsporosis on the scalp and nape of the neck, mostly in children. There is often nearly complete alopecia, covered with dry

Fig. 321

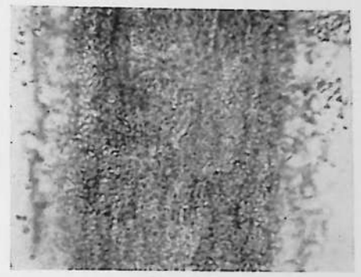


Fig. 322

Fig. 321.—*Malassezia* (*Microsporon*) *furfur;* appearance on direct examination of scales (× 277). (Lewis and Hopper, An Introduction to Medical Mycology, The Year Book Publishers, Inc.)

Fig. 322.—*Microsporon audouini*, infected hairs (× 292). (Lewis and Hopper, An Introduction to Medical Mycology, The Year Book Publishers, Inc.)

grayish scales with stumps of hairs. (Figs. 322 and 323.) There are also other forms of microsporosis which are contracted from the dog, cat and horse, and are caused by *Microsporon canis*, *Microsporon felineum* and *Microsporon equinum*. There are a number of fungi that cause ringworm; among the best known is *Trichophyton gypseum*. (See Figs. 324 and 325.) This organism may be found in lesions of the nails, hair on the scalp or face, trunk, arms and hands. A somewhat similar organism producing growth within the hair shaft is *Trichophyton tonsurans* (*T. crateriforme*). (See Figs. 326 and 327.) "Athlete's

[1] The list of diseases given has been taken from a chart "Mycotic Diseases" by Morris Moore, Mycologist at the Barnard Free Skin and Cancer Hospital. Many of the illustrations are from Lewis and Hopper: Introduction to Medical Mycology, Year Book Publication, 1939.

foot" is an epidermophytosis which may be caused by *Epidermophyton inguinale.* (See Figs. 328 and 329.) Favus, a crusting lesion of

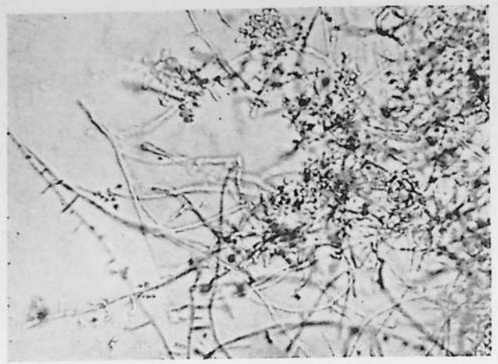


Fig. 323

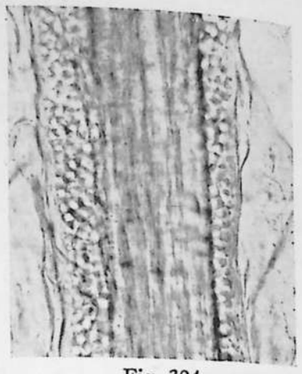


Fig. 324

Fig. 323.—*Microsporon audouini;* culture showing microconidia in clusters (× 180). (Lewis and Hopper, An Introduction to Medical Mycology, The Year Book Publishers, Inc.)

Fig. 324.—*Trichophyton gypseum;* infected hairs with the spores external to shaft (× 362). (Lewis and Hopper, An Introduction to Medical Mycology, The Year Book Publishers, Inc.)

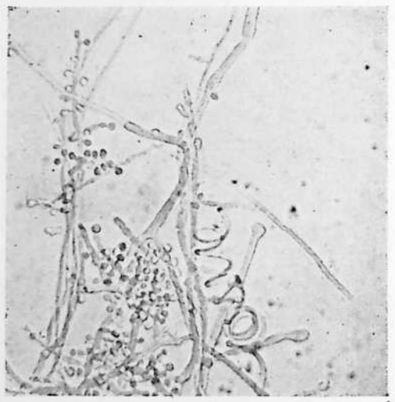


Fig. 325

Fig. 326

Fig. 325.—Culture of *Trichophyton gypseum* (× 348). (Lewis and Hopper, An Introduction to Medical Mycology, The Year Book Publishers, Inc.)

Fig. 326.—*Trichophyton tonsurans* (*Trichophyton crateriforme*). (Lewis and Hopper, An Introduction to Medical Mycology, The Year Book Publishers, Inc.)

the scalp, with typical yellow cups called scutulae, is one cause of alopecia. The fungus causing this dermatomycosis is *Achorion schoenleini.* (See Figs. 330, 331, and 332.)

Subcutaneous and systemic diseases that are caused by fungi are of importance. Many species of monilia have been described. These

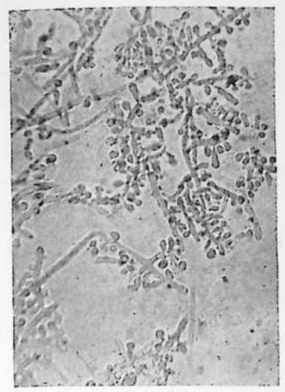


Fig. 327

Fig. 328

Fig. 327.—Culture of *Trichophyton crateriforme.* (Lewis and Hopper, An Introduction to Medical Mycology, The Year Book Publishers, Inc.)

Fig. 328.—*Epidermophyton inguinale* (wavy filaments in direct mount from scales) (× 200). (Lewis and Hopper, An Introduction to Medical Mycology, The Year Book Publishers, Inc.)

are simple, budding cells forming short filaments in old necrotic lesions. The typical species is *Monilis albicans.* (See Fig. 333.) *Sporo-*

Fig. 329

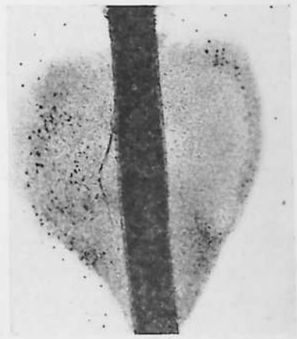


Fig. 330

Fig. 329.—*Epidermophyton inguinale,* culture (× 267). (Lewis and Hopper, An Introduction to Medical Mycology, The Year Book Publishers, Inc.)

Fig. 330.—*Achorion schoenleinii;* hair from scutulum (× 35). (Lewis and Hopper, An Introduction to Medical Mycology, The Year Book Publishers, Inc.)

trichum schencki causes a generalized cutaneous disease of the fingers, arms and body, known as sporotrichosis. It may also become systemic.

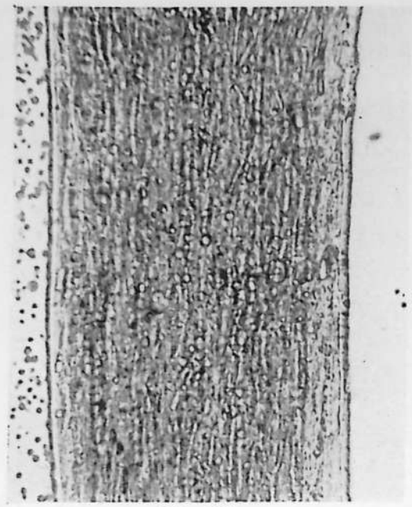


Fig. 331

Fig. 332

Fig. 331.—*Achorion schoenleinii;* filaments and spores in the hair shaft (× 206). (Lewis and Hopper, An Introduction to Medical Mycology, The Year Book Publishers, Inc.)

Fig. 332.—*Achorion schoenleinii;* culture (× 70). (Lewis and Hopper, An Introduction to Medical Mycology, The Year Book Publishers, Inc.)

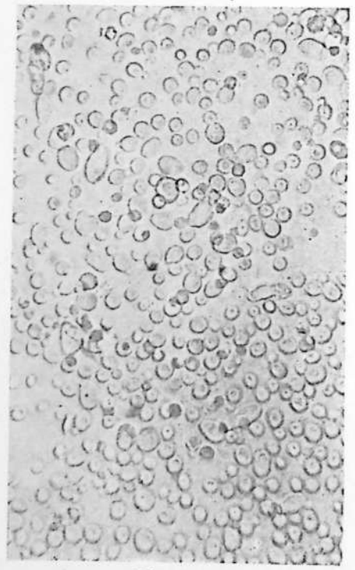


Fig. 333

Fig. 334

Fig. 333.—*Monilia albicans;* culture (× 390). (Lewis and Hopper, An Introduction to Medical Mycology, The Year Book Publishers, Inc.)

Fig. 334.—*Sporotrichum schencki;* culture showing clusters of spores on hyphae (× 1000). (Lewis and Hopper, An Introduction to Medical Mycology, The Year Book Publishers, Inc.)

(See Fig. 334.) *Blastomyces dermatitidis* is a yeast-like fungus causing Gilchrist's disease or blastomycosis. (See Figs. 335 and 336.) The organisms are recovered from skin lesions such as pustules, ulcers, vesicles and inflammation with raised borders and from systemic lesions which are miliary or large-sized nodules, abscesses and granulomas and other similar diseases.

Torulosis is caused by *Torula histolytica*. This organism may be found in the spinal fluid in cases of so-called torula meningitis. The organism resembles true yeast in that it reproduces by budding. It differs from true yeast in that it is incapable of fermenting sugars, except to the extent of slight acid formation in a few instances. In

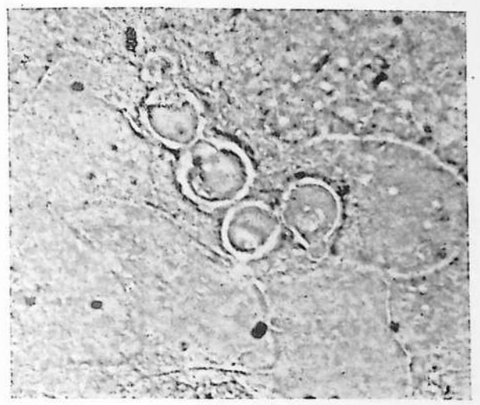


Fig. 335

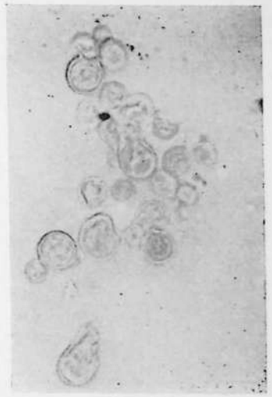


Fig. 336

Fig. 335.—*Blastomyces dermatitidis;* direct mount of pus showing double-contoured budding cells (× 716). (Lewis and Hopper, An Introduction to Medical Mycology, The Year Book Publishers, Inc.)

Fig. 336.—*Blastomyces dermatitidis:* culture showing double-contoured cells with budding (× 524). (Lewis and Hopper, An Introduction to Medical Mycology, The Year Book Publishers, Inc.)

routine examinations of the spinal fluid, the organisms are easily mistaken for lymphocytes by the unsuspecting observer. (See Fig. 337.)

Todd and Herrmann[1] have studied the life cycle of this organism and their work has been confirmed by Henrici.[2] Because of the fact that in old cultures there are two types of cells which conjugate, they propose instead of the usual name that this organism be called *Debaryomyces neoformans*.

[1] Todd, Ramona L., and Herrmann, W. W.: The Life Cycle of the Organism Causing Yeast Meningitis, Jour. Bact., *32*:89–104 (July), 1936.

[2] Henrici, A. T.: The Yeasts; Genetics, Cytology, Variation, Classification and Identification, Bacter. Reviews, *5*:97–179 (June), 1941.

Coccidioidal granuloma caused by *Coccidioides immitis* is an infectious granuloma which appears to be acquired by inhaling spores of the fungus, by cutaneous infections, through wounds, or rarely through the gastro-intestinal tract. Stiles and Davis[1] described the disease as chronic, progressive, highly fatal, affecting the lungs, skin, lymph nodes, bones, meninges, thoracic viscera and other tissues. Although the first cases were reported from California and the disease has been called the "California disease," its appearance both in man and animals in other localities showed that it is spreading or has not been sufficiently recognized. In regions in which man has acquired the infection, cattle, dogs, sheep and wild rodents may harbor the fungus. Granulomatous lesions containing caseous material should be examined for the double-contoured, spherical bodies of this organism.

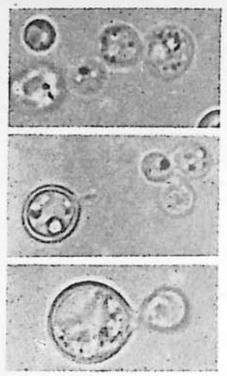


Fig. 337.—*Torula histolytica;* budding form of fungus found in spinal fluid. (Johns and Attaway, Am. J. of Clin. Path., Vol. III, The Williams and Wilkins Co.)

(See Fig. 338.) The organism may be grown on meat infusion agar after incubation for from forty-eight to seventy-two hours at 37° C. On culture media there will be mycelial growth producing a white, cotton-like colony. Only the spherical forms of the fungus are found in the tissues. An acute influenza-like disease called "valley fever" is caused by this organism. It may terminate with no sequelae and in this form is rarely fatal, or it may develop into the chronic form of coccidioidal disease.

Rhinosporidium seeberi causes polyps or pedunculated "strawberry" tumors of the nose, nasopharynx, conjunctiva, uvula, genital

[1] Stiles, G. W., and Davis, C. I.: Coccidioidal Granuloma, (Coccidioidomycosis); Its Incidence in Man and Animals and Its Diagnosis in Animals, Jour. Am. Med. Assn., *119:* 765–769 (July 4), 1942.

mucosa, lacrimal sac and skin. Elles[1] has made a very complete review of the literature and has described the lesions in the eye.

In 1905, Darling reported finding organisms which he called *Histoplasma capsulatum* in three cases of a disease which seemed similar to kala-azar in cases observed in the Canal Zone. It has since been proved that this disease is not leishmaniasis, but is due to a fungus. There has been an increasing number of cases reported each year so that histoplasmosis is either on the increase or is being diagnosed more frequently. Meleney[2] has made a review of the literature up to 1940.

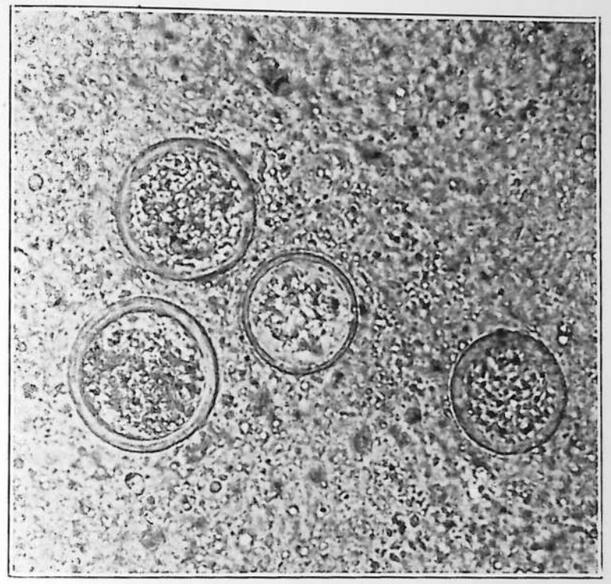


Fig. 338.—*Coccidioides immitis;* from purulent contents of lymph node (× 600). (Stiles and Davis, J. A. M. A., July 4, 1942.)

Henderson, Pinkerton and Moore[3] have reported on *Histoplasma capsulatum* as a cause of chronic ulcerative enteritis. They point out that gastro-intestinal lesions have been present in many of the cases that have been reported. In scrapings obtained from ulcers, the organisms

[1] Elles, Norma B.: *Rhinosporidium Seeberi* Infection in the Eye, Arch. Ophthal., *25:* 969–991 (June), 1941.

[2] Meleney, H. E.: Histoplasmosis (Reticulo-endothelial Cytomycosis): A Review, Amer. Jour. Trop. Med., *20:*603–615 (July), 1940.

[3] Henderson, R. G., Pinkerton, Henry, and Moore, L. T.: Histoplasma Capsulatum as a Cause of Chronic Ulcerative Enteritis, Jour. Am. Med. Assn., *118:*885–889 (Mar. 14) 1942.

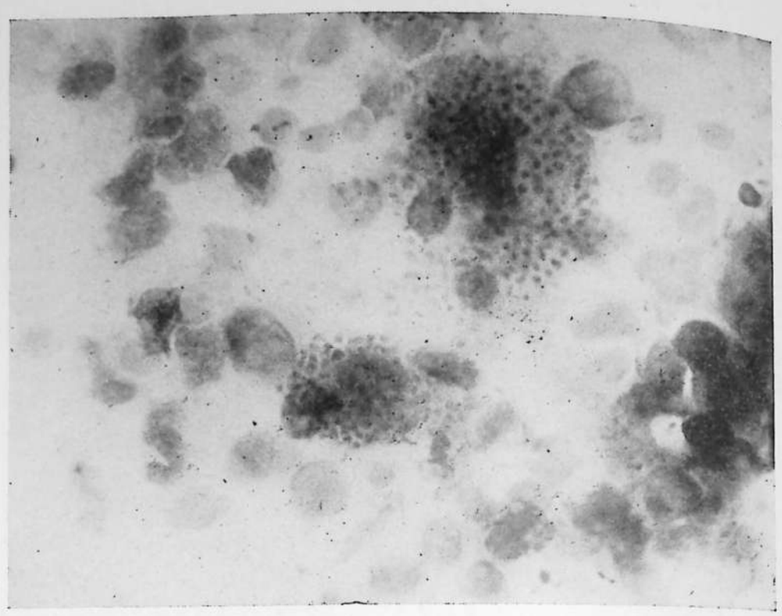


Fig. 339.—*Histoplasma capsulatum;* impression smear from rectal ulcer (Giemsa stain, × 850). (Brown, Havens and Magath.)

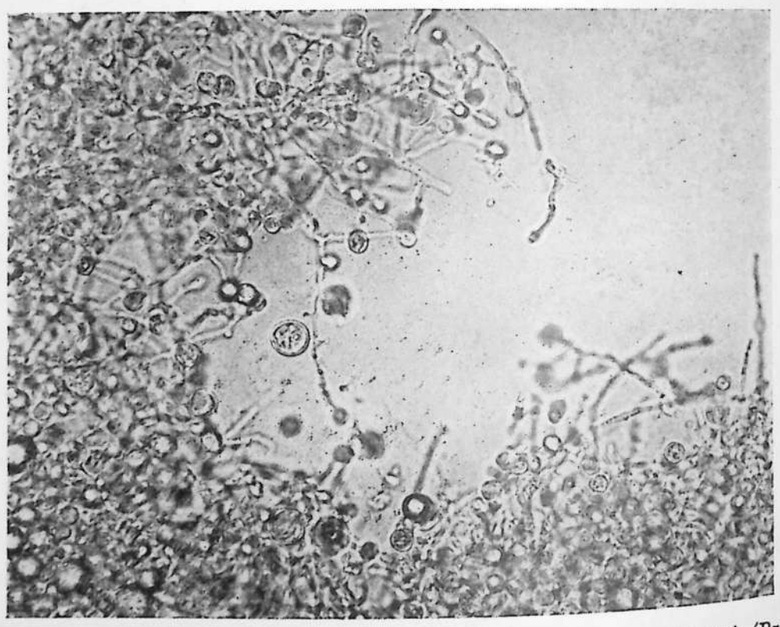


Fig. 340.—*Histoplasma capsulatum;* hanging drop preparation of culture (× 400). (Brown, Havens and Magath.)

may be found grossly packed together and resembling Leishman-Donovan bodies. The organisms stain readily with Giemsa's stain. (See Fig. 339.) They may also be stained with Wright's stain. The organism may be cultured on glucose agar, on which it grows slowly. (See Fig. 340.) Meleney stressed the susceptibility of suprarenal tissue to involvement with histoplasmosis. Van Pernis, Benson and Holinger[1] have suggested a specific, cutaneous skin test which may prove of value in clinical diagnosis.

Aspergillus fumigatus (see Fig. 341, also Fig. 82, p. 161). A saprophyte, often a contaminate on old culture media, may become established in the skin, nails, pulmonary system, ears, conjunctiva and

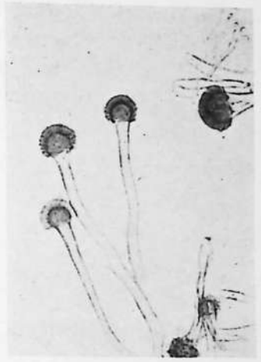


Fig. 341.—*Aspergillus fumigatus* (× 260). (Thom and Church, The Aspergilli, The Williams and Wilkins Co.)

cornea or in the sinuses and may produce an inflammatory granuloma in the internal organs. It may cause lesions resembling tuberculosis.

MILK

A large number of analyses of human and cows' milk are averaged by Holt as follows, Jersey milk being excluded because of its excessive fat:

	Human Milk Normal variations, per cent.		Cows' Milk Average, per cent.	
Fat	3.00 to	5.00	4.00 to	3.50
Sugar	6.00 to	7.00	7.00 to	4.30
Proteins	1.00 to	2.25	1.50 to	4.00
Salts	0.18 to	0.25	0.20 to	0.70
Water	89.82 to	85.50	87.30 to	87.50
	100.00	100.00	100.00	100.00

[1] Van Pernis, P. A., Benson, Miriam E. and Holinger, P. H.: Specific cutaneous reactions with histoplasmosis: preliminary report of another case. Jour. Am. Med. Assn. *117*:436-439 (Aug. 9) 1941.

The reaction of human milk is slightly alkaline; of cows', neutral or slightly acid. The specific gravity of each is about 1.028 to 1.032. Human milk is sterile when secreted, but derives a few bacteria from the lacteal ducts. Cows' milk, as usually sold, contains large numbers of bacteria, the best milk rarely containing fewer than 10,000 in each cubic centimeter. Microscopically, human milk is a fairly homogeneous emulsion of fat, and is practically destitute of cellular elements. Any notable number of leukocytes indicates infection of the mammary gland.

Chemical examination of milk is of great value in solving the problems of infant feeding. The sample examined should be the middle milk, or the entire quantity from one breast, or, as recommended by Talbot, 1 ounce obtained before nursing, 1 ounce

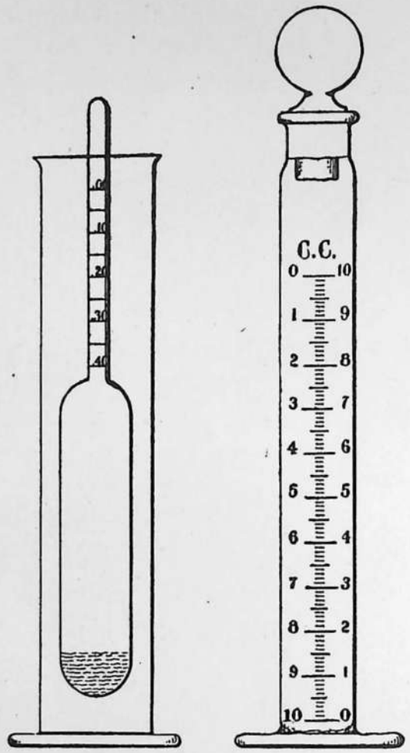


Fig. 342.—Holt's milk-testing apparatus.

Fig. 343.—Centrifuge tube for milk analysis.

after nursing, the two being then mixed. The fat and protein can be estimated roughly, but accurately enough for many clinical purposes by means of Holt's apparatus, which consists of a 10-c.c. cream gage and a small hydrometer (Fig. 342). The cream gage is filled to the 0 mark with milk, allowed to stand for twenty-four hours at room temperature, and the percentage of cream then read off. The percentage of fat is three fifths that of the cream. The protein is then approximated from a consideration of the specific gravity and the

percentage of fat. The salts and sugar very seldom vary sufficiently to affect the specific gravity, hence a high specific gravity must be due to either an increase of protein or decrease of fat, or both, and *vice versa*. With normal specific gravity the protein is high when the fat is high, and *vice versa*. The method is not accurate with cows' milk.

For more accurate work the following methods, applicable to either human or cows' milk, are simple and satisfactory:

Fat.—*Leffmann-Beam Method.*—This is essentially the widely used Babcock method, modified for the small quantities of milk obtainable from the human mammary gland. The apparatus consists of a special tube (Fig. 343) which fits a centrifuge cup, and a 5-c.c. pipet. Owing to its narrow stem the tube is difficult to fill and to clean. Exactly 5 c.c. of the milk are introduced into the tube by means of the pipet, and 1 c.c. of a mixture of equal parts of concentrated hydrochloric acid and amyl alcohol is added and well mixed. The tube is filled to the 0 mark with concentrated sulfuric acid, adding a few drops at a time and agitating constantly. This is revolved in the centrifuge at 1000 revolutions a minute for three minutes, or until the fat has separated. The percentage is then read off upon the stem, each small division representing 0.2 per cent of fat.

Proteins.—*T. R. Boggs' Modification of the Esbach Method.*—This is applied as for urinary albumin (p. 94), substituting Boggs' reagent for Esbach's. The reagent is prepared as follows:

1. Phosphotungstic acid	25 Gm.
Distilled water	125 c.c.
2. Concentrated hydrochloric acid	25 "
Distilled water	100 "

When the phosphotungstic acid is completely dissolved, mix the two solutions. This reagent is quite stable if kept in a dark glass bottle.

Before examination the milk should be diluted according to the probable amount of protein, and allowance made in the subsequent reading. For human milk the optimum dilution is 1:10; for cows' milk, 1:20. Dilution must be accurate.

Lactose.—The protein should first be removed by acidifying with acetic acid, boiling, and filtering. The copper methods may then be used as for glucose in the urine (p. 101), but it must be borne in mind that lactose reduces copper more slowly than glucose, and longer heating is, therefore, required; and that 10 c.c. of Fehling's solution (or 25 c.c. of Benedict's) are equivalent to 0.0676 Gm. lactose (as compared with 0.05 Gm. glucose).

Detection of Preservatives.—Formalin is the most common preservative added to cows' milk, but boric acid is also used.

To detect formalin add a few drops of dilute ferric chloride solution to a few cubic centimeters of the milk, and run the mixture gently upon the surface of some chemically pure sulfuric acid in a test tube. If formaldehyde be present, a bright red ring will appear at the line of contact of the fluids. This is not a specific test for formaldehyde, but nothing else likely to be added to the milk will give it. Jorissen's test, given for formaldehyde in urine, may also be used.

To detect boric acid Goske's method, as used by the Chicago Department of Health, is simple and satisfactory: Mix 2 c.c. of concentrated hydrochloric acid with 20 c.c. of the milk and place in a 50-c.c. beaker. In this suspend a long strip of turmeric paper (2 cm. wide), so that its end reaches to the bottom of the beaker. Allow to remain about half an hour. The liquid will rise by capillarity, and if boric acid be present a reddish-brown color will appear at the junction of the moist and dry portions of the paper. When this is touched with ammonia a bluish-green slate color develops. A rough idea of the amount of boric acid may be had by comparing the depth of color with that produced by boric acid solutions of known strength.

Bacteria.—At times, it is necessary to know the type of pathogenic organisms in a given sample of milk if it is suspected that the use of such milk has been the cause of disease. Epidemics of typhoid fever, or of a very virulent form of "streptococcic sore throat," have often been traced to a certain milk supply. Usually, however, a bacteriologic examination of milk consists of plating the milk in such a manner that an estimate can be made of the number of bacteria for each cubic centimeter. Excellent milk may have only a few hundred bacteria for each cubic centimeter. Milk with a count under 10,000 bacteria on delivery would be considered very good, and under 50,000 good. Poor milk, yet commercially usable, may have 500,000 bacteria for each cubic centimeter, while milk that should not be used may contain too many bacteria to be counted. The Standard Methods of Milk Analysis, as published by the American Public Health Association, should be used. Briefly the technic is as follows:

Technic for Milk Counts.—1. Prepare "water blanks," sterile bottles, preferably glass stoppered. Some of these should contain exactly 99 c.c. of water, and others only 9 c.c. Sterilize in the autoclave at 15 pounds' pressure for one hour.

2. Sterilize a number of 1-c.c. pipets in a hot-air oven. These are most easily handled if they are sterilized in a covered copper container in which they may be kept until ready for use.

3. Melt plain agar, slightly acid, and cool to 45° C.

4. Shake the sample of milk thoroughly, dilute as follows: (*a*) Place 1 c.c. of milk in a bottle containing 99 c.c. of sterile water; discard this pipet; (*b*) with a sterile pipet transfer 1 c.c. of the 1:100 dilution to a bottle containing 9 c.c. of sterile water; (*c*) with the same pipet transfer 1 c.c. of 1:100 dilution to a sterile Petri dish; (*d*) with a fresh sterile pipet make still another dilution by transferring 1 c.c. of 1:1000 dilution to a bottle containing 9 c.c. of sterile water; (*e*) with this same pipet transfer 1 c.c. of 1:1000 dilution to a sterile Petri dish; (*f*) finally use a fresh sterile pipet to transfer 1 c.c. of 1:10,000 dilution to a sterile Petri dish. All dilutions must be shaken twenty-five times before making any transfers.

5. Pour 10 c.c. of melted agar into each Petri dish. The milk dilution and the agar are thoroughly mixed by gently shaking.

6. Incubate cultures for forty-eight hours at 37.5° C.

7. Count the colonies. Only those plates are chosen in which there are not more than 300 and not less than 30 colonies in the entire Petri dish.

8. Calculate the number of organisms for each cubic centimeter from the count and the dilution; for example, there are 256 colonies in the plate; dilution 1:1000; number of organisms for each cubic centimeter is 256,000.

WATER

The approval of water supplies for domestic purposes and the examination of water in swimming pools and at beaches is a public health matter and is usually conducted in the State Board of Health or municipal laboratories; however, the private laboratory is sometimes called upon to pass judgment upon the potability of drinking water and also to make a routine examination of water that is known to be usable.

The color, turbidity and odor should be noted. Chemical examinations may be made for total solid nitrogen, as free ammonia and also nitrogen as albuminoid ammonia, nitrogen as nitrites or nitrates and organic nitrogen. The amount of oxygen and chlorides, the total hardness and the alkalinity may also be determined. All of these procedures are carried out according to the standard methods as laid down by the American Public Health Association.[1]

The bacteriologic examination of water also is carried out according to standard methods. Briefly, the examination is made for the number of bacteria per cubic centimeter. The sample of water in a sterile container should be shaken twenty-five times before plating, and if it is known to have a small bacterial count, 1 c.c. is plated in nutrient agar. If chlorine is present in the water, it is best to neutralize 100 c.c. with about 0.2 gm. of sodium thiosulfate. The plate should be incubated forty-eight hours before making the count. Use a $2\frac{1}{2}$ inch lens in order

[1] Standard Methods for the Examination of Water and Sewage, Ed. 8, American Public Health Association, New York, New York, Ed. 8, 1936, 309 pp.

to count all of the colonies present. Some form of plate counter is most satisfactory for this examination. (Fig. 344.) Tests should be made for the presence of members of the *E. coli* group. According to the standard methods, presumptive tests may be made with 10 c.c., 1 c.c. and 0.1 c.c. of the water sample placed in a lactose broth fermentation tube. If these tests are positive, confirmatory tests may be carried out to confirm the presence of gas-forming organisms. The complete test consists of plating and determining the presence of *Escherichia coli*. A very simple and customary method of testing for the presence of the *E. coli* group of organisms is the following: Place 10 c.c. of water in each of five fermentation tubes containing 10 c.c. of double strength

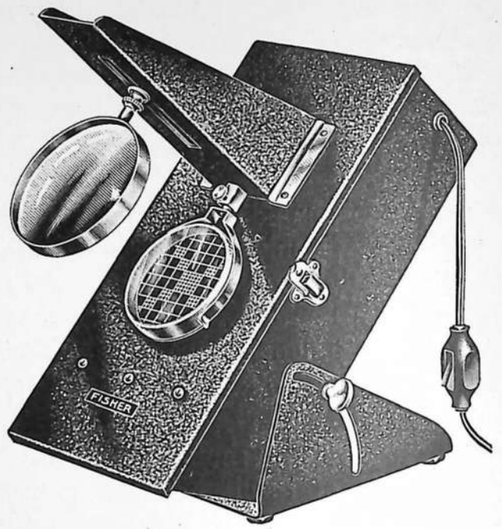


Fig. 344.—Queen bacteria colony counter.

lactose broth. If there is no gas formed in any of the tubes, *Escherichia coli* is not present in a 50 c.c. sample of the water, and it is probable that it is not present in 100 c.c. If one tube shows 10 per cent of gas, there is then at least one *Escherichia coli* present in 50 c.c. and the report may be made that at least two *Escherichiae coli* are present per 100 c.c. of the sample of water. This amount is considered permissible in drinking water. If more than one tube contains gas, it may be well to carry out the confirmatory or complete tests, as the water is contaminated. If all five tubes show gas, it would be evident that there are at least ten *Escherichiae coli* per 100 c.c. of the sample, and such water would be badly contaminated.

SYPHILITIC MATERIAL

In 1905 Schaudinn and Hoffmann described the occurrence of a very slender, spiral micro-organism in the lesions of syphilis. This they named *Spirochaeta pallida*, because of its low refractive power and the difficulty with which it takes up staining reagents. The name was later changed to *Treponema pallidum*. Its etiologic relation to syphilis is now universally recognized. It is found in primary, secondary, and tertiary lesions, but is not present in the last stage in sufficient number to be of value in diagnosis.

Treponema pallidum is an extremely slender, spiral, motile thread, with pointed ends. The organism varies considerably in length, the average being about

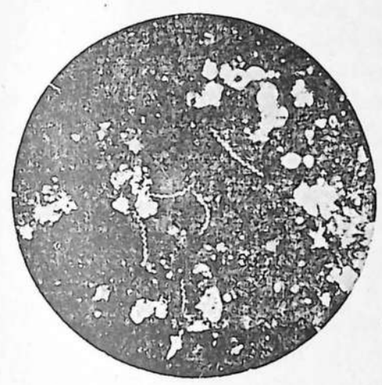


Fig. 345.—*Treponema pallidum* (× 1000) (Leitz 1/12 oil-immersion objective and Leitz dark-ground condenser). The parasite has the same appearance as in India-ink preparations.

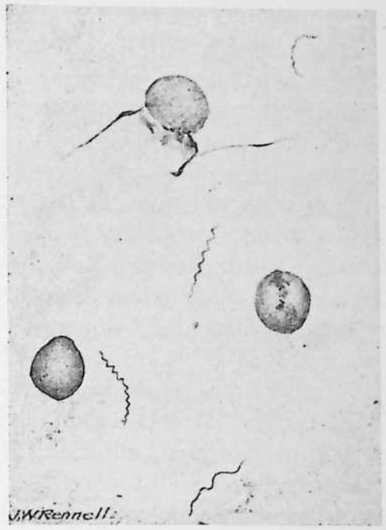


Fig. 346.—*Treponema pallidum, Borrelia refringens*, and three red blood corpuscles in a smear from a chancre (× 1200). From a preparation stained with Giemsa's stain, with alkali, for three hours. The treponemata were purplish red; refringens, bluish purple; red corpuscles; deep slate blue.

10 to 12 μ, or somewhat greater than the diameter of a red blood corpuscle; and it exhibits five to twelve, sometimes more, spiral curves, which are sharp and regular, and resemble the curves of a corkscrew (Figs. 345 and 346). As seen in fresh material by dark-field illumination it moves relatively slowly, forward or backward, rotating on its long axis and retaining its regular curves. It takes up stains so poorly and is so delicate that it is difficult to see even in well-stained preparations; a high magnification and careful focusing

are, therefore, required. Upon ulcerated surfaces it is often mingled with other spiral micro-organisms, which adds to the difficulty of its detection. The most notable of these is *Borrelia refringens*, described on page 514.

Treponema pallidum is most easily demonstrated in chancres and mucous patches, although the skin lesions—papules, pustules, roseolous areas—often contain large numbers. Tissue juice from the deeper portions of the lesions is the most favorable material for examination, because the organisms are commonly more abundant than upon ulcerated surfaces and are rarely accompanied by other micro-organisms. After cleansing, the surface is gently scraped with a curet or rubbed briskly with a swab of cotton or gauze. In a few moments serum will exude and very thin smears are then made from it. Exudation of serum may be hastened by gentle squeezing or by suction with a glass tube provided with a rubber nipple. In transferring the serum from the lesion to the slide or cover glass it is convenient to use a capillary pipet. The rubbing should not be so vigorous as to bring much blood, because the corpuscles may hide the treponemata; but a few red corpuscles are an advantage as an aid in locating favorable fields and as a check upon the quality of the staining. Best fields are those with the clearest background and with a few red corpuscles, which must be well stained, well preserved, and not shrunken. If the regional lymph nodes show enlargement the method of choice is aspiration. The skin is painted with iodine, and a stout hypodermic needle attached to a small syringe containing a few drops of sterile physiologic salt solution is inserted into the gland. Penetration of the capsule is shown by the movements of the gland when the needle is moved. The needle is rotated to break up some of the gland tissue, the salt is injected, the needle is again moved about slightly, and the fluid is withdrawn. Fluid thus obtained is often very rich in treponemata and is extremely unlikely to contain any other organism.

Methods for Treponema pallidum.—Undoubtedly the most satisfactory method for detection and microscopic study of any of the group of spirochetes is examination of fresh fluid by dark-field illumination (p. 5), but this is seldom available in the small laboratory. The practitioner will generally rely on staining methods. Of these the authors recommend Giemsa's method, and, especially, the Fontana-Tribondeau silver method.

Giemsa's stain (p. 255) is the most widely used and is one of the best (Fig. 346). It is best purchased ready prepared. Smears are fixed in absolute

alcohol for fifteen minutes. Ten drops of the stain are added to 10 c.c. of faintly alkaline distilled water (1 drop of a 1 per cent solution of potassium carbonate to 10 c.c. of the water), and the fixed smear is immersed on edge in this diluted stain for one to three hours or longer. It is then rinsed in distilled water, dried, and mounted. More intense staining may be obtained and the time shortened by conducting the process in the incubator. In well-stained specimens *Treponema pallidum* is reddish; most other micro-organisms, bluish. If desired, Giemsa's stain may be used as described for blood (p. 255), but the organisms do not then stand out quite so clearly.

It is a waste of time to search for treponemata in films in which the leukocytes and the red corpuscles are not well stained. The nuclei of the former should be dark purple; the latter should be deep copper red or salmon colored when the stain is used as for blood, and deep slate blue when alkali has been added.

Wright's blood stain, used in the manner already described (p. 253), except that the diluted stain is allowed to act upon the film for fifteen minutes, gives fair results. Medalia uses 1 per cent sodium carbonate instead of water for diluting the Wright stain on the slide, and stains, with very gentle steaming, for twenty minutes.

Silver Method.—The silver impregnation method has long been used for tissues. It is probably best applied to smears by the *Fontana-Tribondeau method:*

1. Thoroughly dry in the air.
2. Wash several times with a solution consisting of 1 c.c. glacial acetic acid, 2 c.c. of formalin, and 100 c.c. of distilled water. Rinse gently with alcohol and flame off the excess.
3. Cover with 5 per cent aqueous solution of tannic acid, heat until steam rises, and allow to cool for thirty seconds.
4. Rinse in water, cover with Fontana's silver solution, heat until steam rises, and allow to cool for thirty seconds.
5. Wash, dry, and mount.

Spirochetes are brown to black.

Fontana's silver solution must be freshly prepared as follows: To a 5 per cent solution of silver nitrate in distilled water add diluted ammonium hydroxide drop by drop from a capillary pipet until the heavy milky precipitate, which first forms, redissolves on shaking. To the clear solution add more silver nitrate drop by drop until a slight opalescence just appears on shaking.

India Ink Method.—A small drop of India ink of good grade (Günther and Wagner's "Chin-Chin liquid pearl" or Grübler's "nach Burri" recommended; "Bioloid Black" may also be used, and is more easily obtained) is mixed on a slide with 1 or 2 small drops of serum from the suspected lesion. The mixture is then spread over the slide and allowed to dry. After drying, it is examined with an oil-immersion lens. Micro-organisms, including *Treponema pallidum*, appear clear white on a brown or black background, much as they do with the dark-ground condenser (Fig. 345). If desired,

the mixture of ink and serum may be covered with a cover glass and examined in the moist state, the living organisms being thus demonstrated. Because of its extreme simplicity this method has been favorably received. It cannot, however, be absolutely relied upon, since, as has been pointed out, many India inks contain wavy vegetable fibrils which might easily mislead a beginner, and sometimes, indeed, even an experienced worker. Instead of India ink, collargol, diluted 1:20 with water and thoroughly shaken, has been recommended.

In staining spirochetes in tissue Miss De Galantha[1] has been very successful with the following technic:

1. Fix in formalin, 10 per cent. Tissues fixed for many years have given positive results.
2. Embed in paraffin by the usual technic and cut sections about 5 microns in thickness. Sections cut after freezing would serve as well, if cut thin enough.
3. Remove paraffin in usual manner.
4. Immerse in nitric acid, 20 per cent, for ten minutes.
5. Wash well in tap water and rinse in distilled water.
6. Immerse in silver nitrate, 3 per cent, heated to 50° C., for fifteen minutes.
7. Place slides in Petri dish, sections facing up, and pour over them gently the following solution, shaking dish until sections are light brown: 5 c.c. of silver nitrate, 3 per cent, to which are added 20 c.c. of gelatin, 3 per cent, at 40° C., and 1 c.c. of hydroquinone, 1 per cent. The hydroquinone must be added quickly and the resulting mixture must be used immediately and made fresh for each batch of slides.
8. Wash slides in distilled water at 50° C. to remove gelatin; then in tap water.
9. Immerse in 1 per cent formalin for two minutes.
10. Wash in distilled water.
11. Place in sodium hyposulfite, 2 per cent, for two minutes.
12. Dip in tap water.
13. Dehydrate, clear, and mount in Canada balsam.

SEMEN

Absence of spermatozoa is a more common cause of sterility than is generally recognized. In some cases they are present, but lose their motility immediately after ejaculation.

Semen should be kept warm until examined. When it must be transported any considerable distance a vacuum bottle may be used. In its absence the method suggested by Boston is convenient: The

[1] De Galantha, Elena: Modified Silver Stain for Treponema Pallidum, Am. Jour. Clin. Path., *2*:63 (Jan.), 1932.

fresh semen is placed in a small bottle, to the neck of which a string is attached. This is then suspended from a button on the trousers, so that the bottle rests against the skin of the inguinal region. It may be carried in this way for hours. When ready to examine, place a small quantity upon a warmed slide and apply a cover. The spermatozoa are readily seen with a 4-mm. objective (Fig. 77, p. 156). Normally, they are abundant and in active motion.

If an estimation of the number is desired, spermatozoa may be counted as are white corpuscles, using 0.5 per cent chlorazene as a diluent. Draw the semen to the 0.5 mark in a white blood corpuscle pipet and draw the chlorazene to the 11 mark. Shake for three minutes and place a drop on a hemocytometer counting chamber. Count the number of spermatozoa in 2 square millimeters; add 5 ciphers to give the count per cubic centimeter. This will normally be many millions.

Smears may be made as are blood smears. They should be dried by holding them face down high above a flame and then fixed by the heat of a flame. Allow the slides to stand for several hours to dry thoroughly before staining by the following technic: Place in 0.5 per cent chlorazene for three minutes and then wash in water. Dehydrate by placing in 95 per cent alcohol and allow the slides to dry. Stain with crystal violet (0.25 per cent) for two to three minutes, wash in water and decolorize with 95 per cent alcohol for twenty seconds. Again wash in water and stain with rose bengal (1 per cent) for ten seconds. Wash in water, dry in the air and mount in Canada balsam. Make a differential count of from 200 to 500 spermatozoa and record the percentage of normal and abnormal heads. A normal head is uniformly oval with good demarcation between the lightly stained forepart and the deeply stained posterior portion. Any deviation in size, shape or tinctorial characteristics of the head classifies the spermatozoon as abnormal. Any increase above 20 per cent of abnormal heads may be considered pathologic. For a discussion of the importance of sperm examinations and for references to the literature, the reader is referred to the studies of Moench.[1]

Detection of semen in stains upon clothing is often important. The finding of spermatozoa, after soaking the stain for an hour in normal salt solution or dilute alcohol and teasing in the same fluid, is absolute proof that the stain in question is semen, although it is not possible to distinguish human semen from that of the lower animals in this way. A little eosin added to the fluid will bring the

[1] Moench, G. L.: The Relation of Certain Seminal Findings to Fertility, with Special Reference to Sperm Concentration and the Significance of Testicular Epithelial Cells in Semen, Amer. Jour. Surg., *47*:586–596 (Mar.), 1940.

spermatozoa out more clearly, staining the heads red and the tails pink. The tails of most of the sperms will be broken off by the teasing.

Florence's Reaction.—The suspected material is softened with water, placed upon a slide with a few drops of the reagent, and examined at once with a medium power of the microscope. If the material be semen, there will be found dark brown crystals (Fig. 347) in the form of rhombic platelets resembling hemin crystals, or of needles, often grouped in clusters. These crystals can also be obtained from crushed insects, watery extracts of various internal organs, and certain other substances, so that they are not absolute proof of the presence of semen. Negative results, on the other hand, are practically

Fig. 347.—Seminal crystals (medium size) (× 750) from a stain on clothing. A single thread ⅛ inch long was used in the test, the stain being three years and four months old. (Peterson and Haines.)

conclusive. Very rarely does the reaction fail, even when the semen is many years old.

The reagent consists of iodine, 2.54 Gm.; potassium iodide, 1.65 Gm.; and distilled water, 30 c.c.

Hektoen[1] has elaborated a precipitin test, which is specific not only for semen but for the particular species.

PREGNANCY TESTS

The early diagnosis of pregnancy is sometimes of importance. Various laboratory tests for this condition have been suggested, but

[1] Hektoen, Ludwig: Specific Precipitin Test for Human Semen, Jour. Am. Med. Assn., *78*:704–705 (Mar. 11), 1922.

none proved practical until Aschheim[1] and Zondek described a method. Their test is based on the fact that early in pregnancy a great excess of the hormone from the anterior lobe of the pituitary is produced. This hormone is excreted in the urine, and can be demonstrated by the effect on the sex organs of laboratory animals when urine is injected.

Aschheim-Zondek Test for Pregnancy.—Take five immature female mice weighing from 5 to 7 Gm. Inject subcutaneously with catheterized morning urine as follows: 0.2 c.c. into mouse No. 1; 0.25 c.c. into mouse No. 2; 0.3 c.c. into mouse No. 3, and also into mouse No. 4, and 0.4 c.c. into mouse No. 5. A sixth mouse may sometimes be used, and into this animal inject 0.25 c.c. of urine. One hundred hours after the first injection kill the mice with illuminating gas and perform necropsy. Because of the time interval it is suggested that Monday, Tuesday, and Thursday may be the best days for starting the test. Inspect the ovaries with a hand lens. Normal immature ovaries are pinhead in size, and pale in appearance. If all of the animals are found to be normal, the test is negative. A positive reaction is evidenced by enlargement of the ovaries to two or three times normal size, and by minute yellowish protrusions of corpora lutea, or cyanotic protrusions which are due to hemorrhages into a follicle or a corpus luteum. There is also often swelling and hyperemia of the uterus.

The authors and several other investigators claim that this test is positive in 98 per cent of cases of early pregnancy. It has been reported also positive in cases of hydatiform mole and in chorionepithelioma.

Friedman Modification.—Reinhart and Scott,[2] and also Schneider,[3] following the work of Friedman,[4] have greatly simplified the test for the demonstration of excess of this hormone during pregnancy by using a single, nonpregnant rabbit weighing not less than 4 pounds. Inject intravenously 10 to 15 c.c. of freshly passed urine. Examine the animal in twenty-four hours for the effects of the hormone. Corpora haemorrhagica, or several corpora lutea, are the signs of a positive

[1] Aschheim, S.: The Early Diagnosis of Pregnancy, Chorion-epithelioma, and Hydatiform Mole by the Aschheim-Zondek Test, Am. Jour. Obst. and Gynec., *19*:335–342 (Mar.), 1930.

[2] Reinhart, H. L., and Scott, Ernest: The Hormone Test for Pregnancy, Am. Jour. Clin. Path., *1*:113–126 (Mar.), 1931. Reinhart, H. L.: The Results of Two Years' Experience with the Friedman Test, Am. Jour. Clin. Path., *3*:9–15 (Jan.), 1933.

[3] Schneider, P. F.: A Hormone Test of Early Pregnancy, Surg., Gynec., and Obst., *52*:56–60 (Jan.), 1931.

[4] Friedman, M. H.: Mechanism of Ovulation in the Rabbit. II. Ovulation Produced by the Injection of Urine from Pregnant Women, Am. Jour. Physiol., *90*:617–622 (Nov.), 1929.

reaction. This simple procedure should be more popular than the more troublesome original test.

TESTS USED IN THE STUDY OF ACIDOSIS

The never-ceasing processes of metabolism result in the continuous production within the body of a marked excess of acid radicles, chiefly from the combustion of carbon, phosphorus, and sulfur. Notwithstanding this, the hydrogen-ion concentration, or true reaction, of the blood and tissues remains slightly alkaline, close to *p*H 7.5.[1] This standard reaction is one of the physiologic constants of the body. Any appreciable deviation means illness, and any marked deviation is inconsistent with life.

Among the mechanisms by which the standard reaction is protected against variable amounts of acid arising in normal metabolism, and especially against the very large amounts produced in some pathologic conditions, the buffer action of the blood demands first consideration. When successive small amounts of acid are added to blood in a test tube the reaction does not change with each addition of acid as would the reaction of a simple alkaline solution; the blood, in fact, seems to absorb a very considerable amount of acid before even the slightest change in reaction takes place. This "buffer action," whereby the hydrogen-ion concentration is protected against the addition of acids, is due partly to the proteins of the blood, which can act either as weak acids or weak bases, but mainly to the relatively large supply of mono- and dibasic carbonates and phosphates of the fixed bases, of which sodium is the most abundant. Each addition of acid to the blood neutralizes a corresponding amount of the base, but a compensatory readjustment of the proportions of the remaining mono- and dibasic salts takes place, whereby the original concentration of free hydrogen ions is immediately restored. Only when the reserve supply of fixed bases, sometimes called the "alkali reserve," has been largely neutralized does the buffer action fail and allow a change of hydrogen-ion concentration to take place.

It is thus the alkali reserve of the blood and tissues which most directly protects the hydrogen-ion concentration of the body fluids. This reserve is subject to marked depletion in disease, but in health

[1] The reader who is not familiar with the significance and derivation of the symbol *p*H which is commonly used to indicate the hydrogen-ion concentration or true reaction of a liquid, may consult any recent work upon physical chemistry. It will suffice to state that *p*H 7 represents the reaction of distilled water or neutrality, and that the *p*H value rises with decreased hydrogen-ion concentration (alkalinity) and falls with increased hydrogen-ion concentration (acidity). Since the figure represents a logarithm, an increase or decrease of one unit represents a tenfold decrease or increase of acidity; thus, a solution whose *p*H is 5 is ten times as acid as one whose *p*H is 6.

is maintained at a fairly constant level, because most of the acid arising in metabolism is removed from the body by one or other of the following mechanisms:

1. A certain amount of acid is excreted by the kidneys, partly as free acid, but chiefly as acid salts, mostly acid phosphates. The power of the kidneys to secrete a frankly acid urine from a slightly alkaline blood is one of their striking and important functions.

2. A notable amount of acid is neutralized by ammonia, of which there is a constant supply derived from protein catabolism, and is excreted as ammonium salts in the urine. Such ammonia as is not thus intercepted by acids, constituting the major portion under normal conditions, is built up into urea. In certain pathologic conditions, notably in diabetes, this is an extremely important means of dealing with acids: A large part of the ammonia is diverted to this purpose and the formation of urea is correspondingly decreased.

3. Carbon dioxide, the most important acid end-product of metabolism, is eliminated chiefly in two ways: (*a*) The larger part is carried off by the lungs as follows: Carbon dioxide combines with the normal carbonates of fixed bases which constitute the chief alkali reserve of the blood plasma, and is carried from the tissues to the lungs in the venous blood as bicarbonate. Here the carbon dioxide passes over into the alveolar air, and the alkali again takes the form of the normal carbonate and returns to the tissues, where it again takes up carbon dioxide. (*b*) Another portion of the carbon dioxide is neutralized by ammonia and is then built up into urea and excreted by the kidneys.

The mechanisms mentioned above are adequate to meet the ordinary demands made by acids arising in normal metabolism. Only a small portion of the acid is allowed to attack the important fixed bases of the blood and tissue. This forms neutral salts which are excreted in the urine, but the lost base is soon replaced from the food, and the alkali reserve of the body thus remains undiminished. Only when the loss of base is too rapid for replacement from the food, as may occur in certain pathologic conditions, does the alkali reserve become very seriously depleted. *The clinical symptoms recognized as characteristic of acidosis then appear.* Finally, as a terminal event, the hydrogen-ion concentration of the blood may become appreciably increased.

Acidosis, then, is not the occurrence of an acid reaction in the blood or tissues; nor does it necessarily imply even the slightest reduction of the normal alkalinity as measured by hydrogen-ion concentration. It may be defined, in the words of Sellards, as "a diminution in the reserve supply of fixed bases in the blood and tissues of the body, the

physicochemical reaction of the blood remaining unchanged except in very extreme conditions." For practical purposes it may be regarded as a decrease in the titratable alkalinity of the blood.

Since the fixed bases of the blood constitute the chief means of transporting carbon dioxide from the tissues to the lungs, depletion of the supply of fixed base reduces the capacity of the blood to carry carbon dioxide. This leads to accumulation of carbon dioxide in the tissues and consequent blocking of the processes of oxidation, so that the individual suffers from asphyxia exactly as if he were deprived of air. The respiratory center is stimulated, leading to increased pulmonary ventilation, which, when marked, becomes hyperpnea or air hunger, a most characteristic clinical sign of acidosis.

Conditions in which acidosis might occur may, following Macleod, be outlined as follows:

I. Increase of acids in the body.
   1. Excessive formation of acids.
      (*a*) Excessive formation of beta-oxybutyric and diacetic acids from defective oxidation of fats in disturbances of carbohydrate metabolism or in carbohydrate starvation. This is a common and important form of acidosis, sometimes called "ketosis," and is best seen in diabetes mellitus.
      (*b*) Excessive decomposition of proteins, as in fevers.
      (*c*) Formation of acid in excessive intestinal fermentation.
   2. Accumulation of acids because of defective elimination.
      (*a*) Accumulation due to diminished ability of the kidneys to excrete acid, as in chronic interstitial nephritis.
      (*b*) Accumulation of carbon dioxide in asphyxial conditions.
   3. Administration of acids. Large therapeutic doses of hydrochloric acid may cause acidosis of sufficient degree to be detected by certain of the laboratory tests.

II. Decrease of base. There may be a primary loss of alkali reserve by abstraction of fixed base, as in very severe diarrheal conditions, pancreatic and biliary fistulae, and so forth. Lack of sufficient base in the food to restore the normal loss might conceivably be a factor in some cases.

Clinically, acidosis of sufficient degree to have any serious significance occurs in only a few conditions: Diabetes mellitus, in which the acidosis is due to excessive production of beta-oxybutyric and diacetic acids; acute nephritis and advanced chronic interstitial nephritis, in which there appears to be accumulation of acid from failure of the kidneys to excrete acids normally; Asiatic cholera, in which

the acidosis is due partly to abstraction of base, partly to coexistent nephritis; and certain diarrheal conditions of childhood, in which the cause of the acidosis is uncertain.

Milder grades of acidosis without serious clinical significance may occur in a variety of conditions, notably acute rheumatic fever, advanced cachexias, and severe anemias.

It must also be borne in mind that there may occur a condition that is the opposite of acidosis, namely, "alkalosis." In these patients the CO_2 combining power of the blood is high. The condition is particularly important when renal function is impaired, or in pyloric obstruction when tetany may occur. The administration of sodium bicarbonate is, of course, decidedly contraindicated in such a condition.

Tests for Acidosis.—The characteristic clinical sign of acidosis is hyperpnea or air hunger, but this is a comparatively late symptom. The state of acidosis can be diagnosed by laboratory means long before definite clinical symptoms develop. The various laboratory tests are based upon one or another of the facts regarding the cause and nature of acidosis which have been mentioned in the previous pages and may be classified as follows:

I. *Tests which measure the hydrogen-ion concentration of the blood.* None of these need be given here. While an increase of hydrogen-ion concentration is indeed very definite evidence of acidosis, yet, as has been shown in the previous pages, the buffer action of the carbonates, phosphates, and proteins of the blood is so effective that hydrogen-ion concentration changes only when the acidosis is very severe.

II. *Tests which measure the alkali reserve*—practically the buffer power of the blood and tissues—either directly or indirectly. Since acidosis is essentially a depletion of this reserve, these tests are theoretically the best and most generally applicable, provided the technical methods are satisfactory.

1. Titratable alkalinity of the blood.

2. The bicarbonate tolerance test of Sellards, given in detail on page 69. This consists in finding the amount of sodium bicarbonate which can be given by mouth or intravenously without causing the urine to become alkaline to litmus. The tolerance of a normal individual is about 3 to 5 Gm.; in acidosis it may reach 100 to 150 Gm. It is assumed that this amount is retained in the body in order to restore the depleted reserve. This is one of the most satisfactory clinical tests for acidosis, for it is applicable to all forms, is sufficiently sensitive to detect the slight grades which produce no clinical symptoms, furnishes a very definite measure of the degree of acidosis, and at the same time supplies the approved treatment.

3. The carbon dioxide carrying power of the blood. Carbon dioxide is carried to the lungs chiefly in combination with the fixed bases which are the principal buffer substances of the blood. When these are diminished the capacity of the blood to carry carbon dioxide is correspondingly diminished. The carbon dioxide combining power of the blood plasma is then a useful measure of the reserve of fixed bases in the blood, and may be determined by the method of van Slyke and Cullen, described on page 397. This is probably the most reliable test for acidosis and is applicable to all forms.

4. Carbon dioxide tension of alveolar air. The percentage of carbon dioxide in the expired air depends upon the power of the blood to transport carbon dioxide, that is, upon the supply of fixed bases, and, other things being equal, varies directly with it. Other factors which influence the carbon dioxide tension of expired air are increased pulmonary ventilation and changes in the lungs which interfere with the exchange of gases. The former is best illustrated by the effect of high altitudes, where, as Yandell Henderson has remarked, all inhabitants suffer from acidosis if this test be taken as the sole criterion. The simplest method for determining the percentage of carbon dioxide in alveolar air is that of Marriott.

Marriott's Method for Carbon-dioxide Tension of Alveolar Air.—This is based upon the change in reaction and corresponding change in color of a standard carbonate solution containing the indicator phenolsulfonephthalein when the alveolar air is passed through it, the degree of change depending upon the tension of carbon dioxide in the air regardless of the amount of air which passes through the solution. The color change is measured by comparison with a series of color standards of known hydrogen-ion concentration.

The complete outfit (Fig. 348) for this method may be purchased ready for use.[1] It consists of a set of 8 neutral-glass tubes filled with standardized color solutions and numbered 10, 15, 20, 25, 30, 35, 40, 45, respectively; a long test tube; a capillary tube, and a comparison box. These are contained in a small wooden box. The outfit also includes a flask of indicator solution, a rubber bag of about 1000 c.c. capacity, with mouthpiece and pinch-cock, and a rubber inflation bulb. It is accompanied by full directions for use.

The reading is in terms of millimeters of mercury. In normal adults this generally falls between 40 and 45 mm.; in mild acidosis, 30 to 35 mm.; in severe acidosis, below 20 mm.

III. *Tests which show excessive or abnormal formation of acids*

[1] This is manufactured by Hynson, Westcott & Dunning, Baltimore.

within the body. When such is demonstrated acidosis or, at least, the tendency to acidosis is assumed.

1. Detection of abnormal acids. This is practicable only in the case of beta-oxybutyric and diacetic acids, which, together with the derivative acetone, can be detected in the urine (p. 107).

2. Detection of excessive elimination of acids, which presupposes excessive production.

(*a*) By quantitative estimation of ammonium salts in urine (p. 84). This is of much value in diabetes where a large proportion of the abnormal acid is neutralized by ammonia and excreted as ammonium salts in the urine. For some reason, not yet clear, ammonium salts of the urine are not increased in the acidosis of nephritis. In

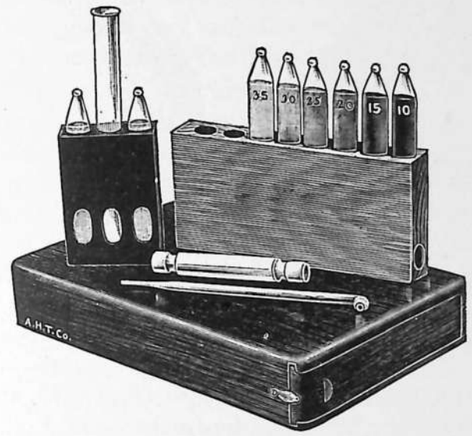


Fig. 348.—Marriott's alveolar air-testing outfit.

this connection the work of Nash and Benedict, indicating that formation of ammonia is a function of the normal kidney, is interesting. It must not be forgotten that other conditions, such as organic disease of the liver, which decrease the formation of urea, may increase ammonium salts in the urine.

(*b*) By titrating the total acidity of the urine (p. 68). This gives a rough index of acidosis, but has little practical value because the ability of the kidney to secrete an excess of acid is limited, especially in nephritis.

CAPILLARY MICROSCOPY

The principles of microscopic study of the capillaries were first laid down by Lombard in a study of the blood pressure in arterioles. If a drop of transparent oil, such as cedarwood oil, is placed on the

skin and examination made under a low-power microscope by a strong light, a remarkable view of the superficial terminal vessels may be obtained. Recently considerable attention was paid to this subject, which started as a physiologic study, as it has some clinical application in such conditions as polycythemia and Raynaud's disease. The studies are directed toward form, size, tonus, flow, and reaction to stimuli.

Several attempts have been made to utilize photography. The problems of sufficient light for quick exposures, prevention of movement, elimination of heat and actinic rays, and accurate focusing have all had to be met. Some of the best photographs have been obtained by a method described by Sheard (Figs. 349 and 350). The student is referred to the literature[1] for further details on this interesting subject which may prove to be of considerable value in studying certain rather rare diseases.

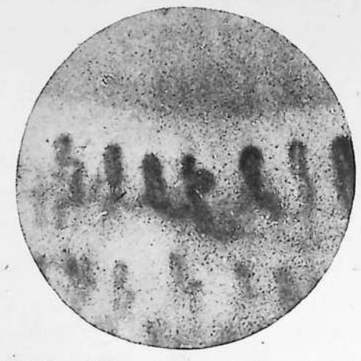


Fig. 349.—Normal capillaries (× 50). (Sheard.)

Fig. 350.—Capillaries in Raynaud's disease (× 50). (Sheard.)

[1] Sheard, Charles: Instantaneous Photomicrography of the Skin Capillaries in the Living Human Body, Science, *60*:409–410 (Oct. 31), 1924. Sheard, Charles, and Brown, G. E.: A Method for Instantaneous Photomicrography of the Skin Capillaries, Jour. Lab. and Clin. Med., *10*:925–929 (Aug.), 1925. Callander, C. L.: Photomicrographic Studies of Morphology of Surface Capillaries in Health and Disease. I. The Anatomy of Normal Surface Capillaries and a Photographic Method of Their Observation and Recording, Jour. Am. Med. Assn., *84*:352–356 (Jan. 31), 1925. Giffin, H. Z., and Brown, G. E.: Studies of the Vascular Changes in Cases of Polycythemia Vera, Am. Jour. Med Sc., *171*:157–168 (Feb.), 1926,

CHAPTER X

SERODIAGNOSTIC METHODS

PRELIMINARY CONSIDERATIONS

WHEN an individual suffers from an infectious disease there are formed by his tissues certain substances which possess the power to destroy or otherwise injuriously affect the disease-producing agent, or to neutralize its poisons. They reside in the blood plasma and lymph, and appear in the serum when the blood coagulates. They may appear in the blood very soon after the individual is taken sick, or they may be delayed until the disease is well advanced; and they may remain for a variable time after his recovery.

These substances, called "antibodies" or "immune bodies," are *specific*, that is, each is produced only as a result of the activity within the body of a particular disease-producing agent, or "antigen," and it acts antagonistically against this particular antigen alone. Therefore the presence of any of these immune bodies in an individual's blood may, with certain exceptions to be noted later, be regarded as pathognomonic of the corresponding disease, and the search for them may be resorted to for diagnostic purposes whenever they can be found more easily than can the disease-producing agent. Upon this foundation has grown up a long series of diagnostic procedures, some simple, some very complicated, which play an important and growing part in medicine under the name "serodiagnosis."

With the exception of the "flocculation" methods for the diagnosis of syphilis, the tests described in this chapter belong in this category, and it is therefore necessary to give such definitions of the immune bodies concerned as may enable the reader to undertake the tests with a reasonably intelligent conception of their mechanism. The mode of formation, structure, and action of the antibodies is customarily described in terms of Ehrlich's "side-chain theory of immunity" which divides them into three "orders."

Immune Bodies of the First Order.—These are antibodies with one combining portion only. They seize upon the antigen, which in this case is a toxin, while it is still free in the blood and lymph, in such a manner as to leave it no unsaturated affinities by which it may combine with the tissues. The toxin is thus rendered harmless. This order of immune bodies includes only the antitoxins, for example,

those of diphtheria and tetanus. They are extremely important in medicine, but are not utilized for diagnosis.

Immune Bodies of the Second Order.—These have a combining portion similar to that of the first order, and, in addition, a portion possessing a ferment-like action, by means of which the characteristic action of the body is affected. The ferment, or zymophore, portion is readily destroyed by heat, so that serum to be used for any of the purposes included in the group must not be heated. The group includes the agglutinins, responsible for the several applications of the Widal reaction; the precipitins, responsible for one of the biologic methods to be described later for the identification of blood stains, and the opsonins.

Immune Bodies of the Third Order.—These antibodies consist of two combining affinities only. One of these combines with the antigen, the other combines with a substance which is called complement because it "complements" or supplements or completes the specific action of the immune substance. Complement is normally present in the blood, but is unable to act upon the antigen without the mediation and aid of the immune body. The latter is, therefore, called the amboceptor. It is relatively thermostabile and keeps for a long time under suitable conditions. It is to be remembered that this is the specific immune substance whose presence or absence is indicative of the presence or absence of the corresponding disease. The native, normally present complement is relatively thermolabile, being destroyed in a few minutes by a temperature of 54° to 56° C., and keeps only a few days under the best conditions. It is nonspecific, and within certain limits the complement of one species may be substituted for that of another.

This group includes the bacteriolysins and hemolysins which are utilized in the various applications of the complement-fixation method to the diagnosis of syphilis (Wassermann reaction), gonorrhea, tuberculosis, echinococcus disease.

I. REACTIONS BASED UPON IMMUNE BODIES OF THE SECOND ORDER

A. The Agglutinins

Agglutination tests may be employed for the diagnosis of a variety of infections—typhoid, paratyphoid, bacillary dysentery, the plague, Asiatic cholera, epidemic meningitis, and others. In clinical work it is used chiefly for the diagnosis of typhoid and paratyphoid infections, and is then known as the Widal reaction. This method is also used in the diagnosis of tularemia and undulant fever.

1. The Widal Test.—(1) **Materials Required.**—1. A homogeneous suspension of bacteria of the species suspected of causing the disease, in this case typhoid or paratyphoid bacilli.

For the *macroscopic method* suspensions of killed organisms are used, and these may be purchased from the manufacturers of biologic preparations or may be prepared by the worker himself. In the latter case one uses a twenty-four-hour bouillon culture of a strain of the micro-organism which has been attenuated by at least several weeks' growth on culture media, with frequent transplantation. To each 10 c.c. of the bouillon culture is added 0.1 c.c. of a 10 per cent solution of the standard 40 per cent formaldehyde. The culture is then placed in the ice-box, and will be sterile in three or four days. It should be shaken vigorously several times a day. If any clumps remain, the

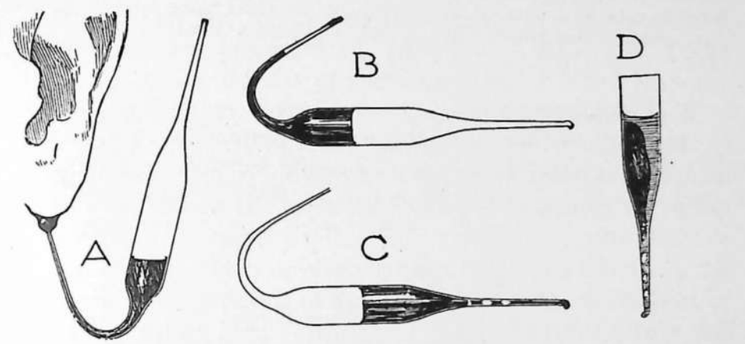


Fig. 351.—Method of obtaining blood in a Wright capsule: A, Filling the capsule; the long arm should be held more nearly horizontal than is here represented; B, the bulb has been warmed and the capillary end sealed in the flame; C, cooling of the capsule has drawn the blood to the sealed end; D, the serum has separated, and the top of the capsule has been broken off.

suspension should be filtered through a layer of sterile absorbent cotton. Such a suspension will keep for many months. Whenever any tendency to spontaneous agglutination becomes evident it must be discarded.

For the *microscopic method* living bouillon cultures of eighteen to twenty-four hours' growth are employed.

2. About 0.1 c.c. or the *patient's serum*. This is best obtained by venipuncture, withdrawing 1 or 2 c.c. of the patient's blood. It may also be obtained by pricking the cleansed finger or ear rather deeply, and collecting 10 or 15 drops of blood in one of the capsules shown in Figs. 351 to 353. More than one capsule should be at hand, so that a fresh one may be substituted should the first be plugged by fibrin before enough blood is obtained.

Sufficient blood may also be collected by allowing drops to dry on glass or unglazed paper (without heating), to be afterward macerated in water. In this case, however, dilutions can only be made approximately.

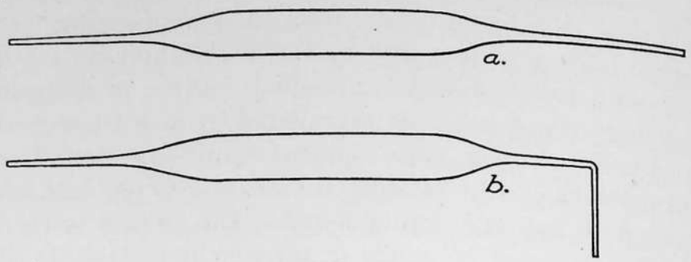


Fig. 352.—A satisfactory glass capsule for obtaining small quantities of blood, as for the Widal test. The straight tube (*a*) is more convenient to carry in the hand-bag than is Wright's capsule. It may be bent as shown in the lower figure by brief application of a match flame at the bedside. After the tube is filled the ends may be sealed with the match flame.

3. *Physiologic saline.*

4. *Small test tubes*, about 8 mm. in diameter, are required for the macroscopic test. These may be made from short sections of glass tubing by sealing one end in a flame.

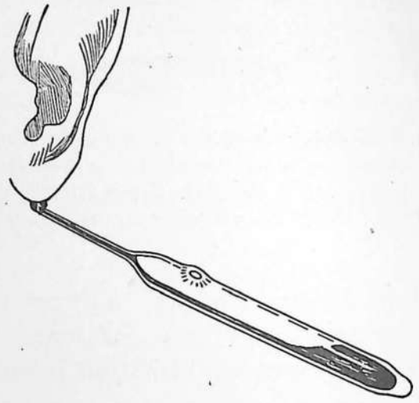


Fig. 353.—A very satisfactory blood capsule described by Lyon. A short section of glass tubing is heated and drawn out as in making a capillary pipet; the large end is sealed in the flame; and after it has cooled, the capillary tip is sealed. The side of the tube near the capillary end is then held in the edge of a flame until it softens and a "blow-hole" is formed through expansion of the contained air. The tip of the capillary portion is then broken off, and the capsule is filled as shown in the figure, the "blow-hole" allowing the blood to enter freely.

5. *Slides*, preferably hollow ground, *cover glasses*, and *vaselin*, are required for the microscopic test.

(2) **Methods.**—Two methods of performing the test, the macroscopic and the microscopic will be described. The former is probably preferable:

(*a*) **Macroscopic Method.**—Separate the clot and serum by centrifugation. Pipet off the serum, and place it in a clean test tube. Place seven small test tubes in a rack, and add to each one, *except the first*, 0.5 c.c. of physiologic saline by means of a serologic pipet. Then place, in the first tube *only*, 0.1 c.c. of the blood serum, and add 0.9 c.c. of physiologic solution of sodium chloride. Shake the first tube and transfer 0.5 c.c. of the diluted serum to the second tube. Shake the second tube, and with the pipet transfer 0.5 c.c. to the third tube. Shake this tube, and transfer 0.5 c.c. to the fourth tube, and so on, to the next to the last tube, from which 0.5 c.c. should be discarded. The last tube serves as a control and should contain only 0.5 c.c. of salt solution, without any serum. One thus arrives at a series of dilutions of the serum, as follows: 1:10, 1:20, 1:40, 1:80, 1:160, 1:320. Now add to each tube 0.5 c.c. of the suspension of killed bacteria. This doubles the dilution of the serum in each of the tubes, so that the final dilutions will be 1:20, 1:40, 1:80, 1:160, 1:320 and 1:640. At times it may be desirable to add one more tube containing a dilution of serum of 1:1280. Mix all the tubes thoroughly by shaking, and place the rack in a moderately warm place or in the incubator for eight to twelve hours. In those tubes in which the reaction is positive there will be found a sediment consisting of agglutinated bacteria at the bottom of the test tube, with a clear supernatant fluid. The control tube and the negative tubes will be cloudy and without sediment.

Bass and Watkins have described a modification of the macroscopic method (using very concentrated suspensions of the bacilli) by which the test can be applied at the bedside. Agglutination occurs within a few minutes. A similar technic is used by Huddleson for the rapid demonstration of agglutinins with a concentrated *Brucella abortus* antigen (p. 808).

(*b*) **Microscopic Method.**—Arrange a series of dilutions of the blood serum as above, or, if dried blood be used, macerate the dried clot with salt solution or tap water. In the latter case, unless the size of the original drop of blood be known, the color is the only guide as to the degree of dilution. A light amber color will roughly correspond to a dilution of 1:50. From such a dilution others can be prepared. On the center of each of several clean cover glasses place a loopful of each of the several dilutions, employing a platinum loop of about 2 mm. diameter. With the same loop add to each droplet of diluted serum a loop from a twelve- to twenty-four-hour-old bouillon culture of the organism in question, or of a suspension in salt solution

prepared from a young agar-slant culture. This doubles the dilution of serum in each case. One cover glass containing no serum should be prepared to serve as a control. Press over each cover glass a hollow-ground slide previously ringed with vaselin. Turn the slide over so as to bring the cover glass on top. Drying is prevented and the cover glass held in place by the vaselin.

When hollow-ground slides are not at hand, a drop each of the diluted serum and the bacterial suspension may be placed in the center of a ring of vaselin on an ordinary slide and a cover glass applied to this. Vaselin containing an antiseptic must not, of course, be used for this purpose.

Place the slides in a moderately warm place or in the incubator at 37° C. for two hours. Examine under the oil-immersion lens or, better,

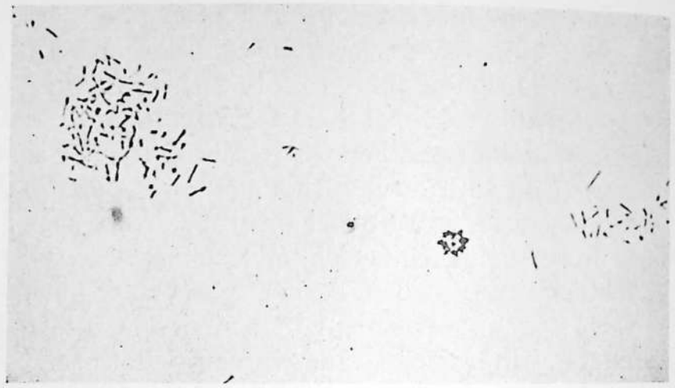


Fig. 354.—Showing clumping of typhoid bacilli in the Widal reaction. At one point a crenated red blood corpuscle is seen. (Wright and Brown.)

the high-power dry lens of the microscope, *using very subdued light.* In the negative slides and in the control the organisms will be found moving freely and not clumped, while in the positive slides the organisms are found motionless and gathered in tangled masses and balls, that is, they are agglutinated (Fig. 354). Pseudo-reactions, in which there are a few small clumps of organisms whose motion is not entirely lost, together with many freely moving organisms scattered throughout the field, should not mislead.

Jagic suggests that, after agglutination has taken place, a drop of the suspension be mixed with a drop of India ink and spread upon a slide. In this way a permanent record may be kept. To insure sterility the preparation may be fixed by heat.

(3) **Interpretation of Results.**—In recording the result of a Widal test it is necessary to state the highest dilution at which definite ag-

glutination occurred, and also the time required, counting from the first mixture of bacilli and serum. Experience has shown that not much significance attaches to reactions occurring in two hours with dilutions of serum less than 1:40 or 1:80. A number of other disease conditions may occasionally give rise to a positive reaction with the typhoid bacillus, notably infections with closely related organisms, such as the paratyphoid and colon bacilli. In such cases, if tests are made with several species, the species agglutinated in the highest serum dilution may be regarded as the cause of the infection.

Following antityphoid vaccination the Widal reaction becomes positive and remains so for at least a year, and in about 25 per cent of the cases for four or five years, or longer. A single Widal test is, therefore, of no value for diagnosis if the patient has received antityphoid vaccination within several years previously. However, a series of positive reactions of increasing strength from day to day may be accepted with confidence.

In typhoid fever the average time of first appearance of the reaction is the eighth or tenth day of recognizable illness, although, exceptionally, it may appear as early as the third or fourth day. In doubtful cases the test should be repeated at short intervals, and no disappointment should be felt if, as sometimes, though rarely, happens, the reaction does not appear until the third or fourth week. It is evident, therefore, that its value for early diagnosis is much less than that of the blood culture (p. 787). After the Widal reaction first appears it remains throughout the whole course of the disease and often persists for many years.

"H" and "O" Antigens.—For many years there has been an increasing interest in the difference in agglutination reactions with two different types of antigens of the typhoid-paratyphoid-coli group of organisms. "H" (*hauch*) strains represent actively motile forms and formalized "H" antigens react to antibodies formed against the *flagellar antigen*. On the other hand, a *somatic antigen*, made with "O" (*ohne hauch*) strains reacts to another agglutinin that has developed against this type of antigen. "H" and "O" antigens may be purchased from biological supply houses, or may be made by formalizing "H" and "O" strains of organisms. "H" antigen is destroyed by 50 per cent alcohol, and antigens so treated then become similar to "O" antigens. Therefore, an "O" typhoid antigen may be made by adding equal parts of absolute alcohol to a suspension of actively motile typhoid bacilli. In using these antigens according to the macroscopic method described on page 659 qualitative results are best detected by incubating for "H" agglutination at 55° C. for

from two to four hours, while "O" agglutination tests should be incubated for from four to twenty-four hours at 55° C. It is advisable at times also to place agglutination tests in the refrigerator over night after preliminary incubation.

2. Agglutination Method in Identification of Bacteria.—The agglutination test may also be used as a means of differentiating certain bacterial species, a suspension of the organisms being mixed with ascending dilutions of blood serum of an animal which has been immunized against the species in question. If agglutination occurs in high dilution, the organism is of the same species as that which was used for the immunization. Recently isolated cultures are, however, more resistant to agglutination than old attenuated ones. The technic is that given for the Widal test, the macroscopic method being generally the more satisfactory. The agglutination method is most frequently used to establish the identity of suspected typhoid and paratyphoid bacilli recovered from blood, urine, or feces, for the meningococcus when found in throat cultures, and for determining the types of the pneumococcus. Immune serum for these purposes can be secured from the biologic supply houses.

3. Weil-Felix Reaction.—Weil and Felix isolated proteus organisms from the urine of patients suffering from typhus fever. While these organisms are apparently nonpathogenic, one strain, particularly "X 19," used as an antigen is agglutinated by high dilutions of serums from cases of typhus fever, trench fever, and Rocky Mountain spotted fever. The antigen is made from the "O" strain (*ohne hauch*) of *Proteus X 19* by washing the culture off an agar slant and killing the growth with about 3 c.c. of 10 per cent formalin solution, or the killed culture may be obtained as an antigen from the commercial biologic laboratories. The agglutination test is set up in a manner similar to the macroscopic Widal test, which is described on page 659.

B. The Precipitins

These may be utilized for many purposes, notably for the biologic identification of unknown proteins, for diagnosis of echinococcus disease, and for determining the types of the pneumococcus.

1. Biologic Identification of Unknown Proteins.—This is applied chiefly to the differentiation of human and animal blood for medicolegal purposes.

(1) **Materials Required.**—The following equipment is needed:

1. *Blood serum* of an animal (rabbit) highly immunized against the protein to be determined. Immunize several rabbits by intravenous or intraperitoneal injections of a sterile solution of the desired

protein, for example, human blood, or, better, blood serum. The blood may be obtained from a vein. The doses may be given at five-day intervals, two doses of 8 c.c. each, two of 5 c.c., and two of 3 c.c. To prevent anaphylactic shock the rabbit may be desensitized by injecting 0.2 c.c. of the serum one-half hour before injection of each of the later doses. After the third or fourth dose draw 2 or 3 c.c. of blood from an ear vein, separate the serum, and determine its strength as follows:

Prepare dilutions of (in this case) human blood serum in the proportions 1:1000, 1:5000, 1:10,000, 1:20,000, and so forth, using physiologic salt solution as a diluent. Place 0.5 c.c. of the several dilutions in each of a series of very small test tubes. By means of a capillary pipet, place 0.1 c.c. of the rabbit's serum at the bottom of each of the tubes so as to form a sharp line of contact between the two fluids. A distinct white cloud should appear at the line of contact in the lowest dilution (1:1000) within a minute or two, rapidly deepening to a flocculent precipitate; the reaction develops more slowly in the higher dilutions, but no reaction is significant which is not definite within twenty minutes.

If the titration results as above described, anesthetize the rabbit *while it is in a fasting condition*, as otherwise the serum is likely to be opalescent. Bleed the animal from the heart, using large, sterile, all-glass syringes, and sterile needles, and observing aseptic precautions. Place the blood in sterile centrifuge tubes. After clotting has occurred, separate the clot, centrifugalize, remove the serum with a pipet and place in sterile bottles, and add a few drops of tricresol as a preservative. If the serum is opalescent it cannot be used; if cloudy it must be filtered clear through a sterile Berkefeld filter. Sometimes the cloudiness can be removed by simple centrifugation. The titration above described should be repeated and verified before the serum is used for making the test proper.

Precipitating sera for the proteins of horse, dog, sheep, beef, fowl, etc., may, of course, be prepared in the same way.

2. *A solution of the unknown substance in physiologic salt solution.* The stock dilution should be about 1:1000. If made from a dried clot this can only be approximate. The solution must be made perfectly clear—by filtration if necessary—and should meet the following conditions:

(*a*) It should be almost completely colorless by transmitted light.

(*b*) It should give only a slight cloudiness when heated with a little nitric acid.

(*c*) It should, nevertheless, foam freely on shaking.

(*d*) It should be neither strongly acid nor strongly alkaline to litmus. Very weak sodium hydroxide or hydrochloric acid may be used for neutralization.

(2) **Method.**—Arrange a series of 7 small test tubes and charge them as follows, placing the second fluid beneath the first by means of a capillary pipet as was described for the titration (p. 663).

Tube No. 1.—0.5 c.c. of the solution of the unknown protein, diluted 1:1000, plus 0.1 c.c. of the immune serum.
Tube No. 2.—0.5 c.c. of the unknown solution, diluted 1:1000, plus 0.1 c.c. of normal rabbit serum.
Tube No. 3.—0.5 c.c. of physiologic salt solution plus 0.1 c.c. of the immune serum.
Tube No. 4.—0.5 c.c. of 1:1000 dilution of known serum of the species suspected to be present in the unknown material plus 0.1 c.c. of the immune serum.
Tube No. 5.—0.5 c.c. of a 1:1000 dilution of serum of a species different from that suspected to be present in the unknown material plus 0.1 c.c. of the immune serum.
Tube No. 6.—0.5 c.c. of the unknown solution alone.
Tube No. 7.—If the solution of the unknown protein was made from a stain upon cloth, leather, or other material, this tube should be set up. It contains 0.5 c.c. of a salt solution extract of the material plus 0.1 c.c. of the immune serum.

When the first and the fourth tubes give a definitely positive reaction, as indicated by a distinct whitish ring at the zone of contact of the two fluids, and all the other tubes give a negative reaction, the presence of protein of the species tested for is established. When only a limited amount of material is available the test can be made in capillary tubes.

(3) **Interpretation of Results.**—The precipitin reaction is closely specific, and is fully established for medicolegal purposes. Here it is generally a question of the identity of blood stains on clothing or implements, and it must be remembered that the test does not prove the presence of *blood*, but only of a protein of the species indicated. Doubt can arise only between the proteins of very closely related species, as, for example, man and monkey, sheep and goat, etc., and this can practically always be removed by the use of adequate controls. The power of proteins to react with precipitin may be reduced or destroyed by alcohol, formaldehyde, strong acids and alkalis, great heat, and decomposition. Dried blood is much more resistant to these influences than is blood in the fluid state.

2. Other Precipitin Tests.—The precipitins are very useful in diagnosis of echinococcus disease. The test may be applied in the manner described above. The two fluids required are fluid from a hydatid cyst, which contains the precipitable substance, and blood serum of the patient, which contains the specific precipitin if he be

suffering from echinococcus disease. Both fluids are used undiluted. The test may also be applied by mixing equal parts of the fluids in a test tube. A positive reaction is indicated by the appearance of a flocculent precipitate within one-half hour. Control tests should be carried out with normal serum, and, when possible, with serum from a known case of hydatid disease.

For determining pneumococcus types in sputum, when the organism cannot readily be obtained in sufficiently pure culture to allow of agglutination tests, the precipitin test may be applied as described in the section upon Pneumococcus Typing (p. 793). Similar methods have been used for identifying diphtheria bacilli in swabs and cultures and typhoid bacilli in feces.[1]

C. The Opsonins

That phagocytosis plays an important part in the body's resistance to bacterial invasion has long been recognized. According to Metchnikoff, this property of leukocytes resides entirely within themselves, depending upon their own vital activity. The studies of Wright and Douglas, upon the contrary, indicate that the leukocytes are impotent in themselves, and can ingest bacteria only in the presence of certain substances which exist in the blood plasma. These substances have been named *opsonins*. They probably act by uniting with the bacteria, thus preparing them for ingestion by the leukocytes; but they do not cause death of the bacteria, nor produce any appreciable morphologic change. They appear to be more or less specific, a separate opsonin being necessary to phagocytosis of each species of bacteria. There are, moreover, opsonins for other formed elements—red blood corpuscles, for example. It has been shown that the quantity of opsonins in the blood can be greatly increased by injection of dead bacteria.

To measure the amount of any particular opsonin in the blood Wright has devised a method which involves many ingenious and delicate technical procedures. Much skill, such as is attained only after considerable training in laboratory technic, is requisite, and there are many sources of error. It is, therefore, beyond the province of this work to recount the method in detail. In a general way it consists in: (*a*) Preparing a mixture of equal parts of the patient's blood serum, a suspension of the specific microorganism, and a suspension of washed leukocytes; (*b*) preparing a similar mixture, using serum of a normal person; (*c*) incubating both mixtures for

[1] For diphtheria see Smith, G. H., and Kaufman, C. E.: The Precipitin Test in the Detection of Bacterium Diphtheriae, Jour. Lab. and Clin. Med., *7*:619–622 (July), 1922. For typhoid see Laird, J. L., Conover, J. R., and Butts, D. C. A.: The Autolysate-Precipitin Reaction in Typhoid Fever (a Preliminary Report), Am. Jour. Med. Sc., *165*:241–249 (Feb.), 1923.

a definite length of time; and (*d*) making smears from each, staining, and examining with an oil-immersion objective. The number of bacteria which have been taken up by a definite number of leukocytes is counted, and the average number of bacteria per leukocyte is calculated; this gives the "phagocytic index." The phagocytic index of the blood under investigation, divided by that of the normal blood, gives the *opsonic index* of the former, the opsonic index of the normal blood being taken as 1. Simon regarded the percentage of leukocytes which have ingested bacteria as a more accurate measurement of the amount of opsonins than the number of bacteria ingested, because the bacteria are apt to adhere and be taken in in clumps.

II. REACTIONS BASED UPON IMMUNE BODIES OF THE THIRD ORDER

The reactions of this group comprise the various applications of the Bordet-Gengou phenomenon of complement fixation. The principle may be applied to the diagnosis of any disease the antigen of which is known and obtainable in suitable form. The list includes syphilis, tuberculosis, gonorrhea, echinococcus and cysticercus disease, trichiniasis, typhoid fever, and pneumococcus, streptococcus, meningococcus, and staphylococcus infections. In several of these other and simpler methods of diagnosis are, however, available; and in some others the complement-fixation method is not sufficiently reliable to be of value in clinical work. The method as applied to the first three of the diseases above mentioned is given in the following pages. It is most useful in syphilis.

The mechanism of the complement-fixation reactions will be made clear by a review of the processes of bacteriolysis and hemolysis, upon which they are based.

Bacteriolysis.—In 1894 Pfeiffer, by his classical experiments with cholera spirilla placed in the peritoneal cavities of guinea-pigs which had recovered from cholera, definitely established the general principle that when an animal becomes actively immune to a disease, either experimentally or by contracting the disease and recovering in the natural way, its blood serum and tissue juices thereby acquire strong powers of killing and sometimes dissolving bacteria of the kind causing that particular disease, while they are relatively harmless to other kinds of bacteria. Such destruction of bacteria is known as bacteriolysis. It was later shown that this power is not due to a single substance in the immune animal's serum, but to two substances which act in combination, neither one being capable of causing bacteriolysis by itself. One of these exists only in the blood and tissue juices of the immune animal, and is relatively resistant to heat (thermostabile) and to drying. The other is present in the blood serum of nearly all warm-

blooded animals, whether immunized or not, and is very unstable and easily destroyed outside the body, especially by heating (thermolabile). Ehrlich explained the origin and mode of action of these two substances by his well-known side-chain theory, and named them "amboceptor" and "complement" respectively. To the bacterium, whose presence has induced the immunity and against which the newly acquired bacteriolytic activity is directed, the name "antigen" is given. According to Ehrlich the bacteriolytic power really resides in the complement, the specific amboceptor merely serving as an intermediate body or connecting link which binds the complement to a particular kind of bacterium, and thus enables the complement to act. Whenever this union of the three bodies takes place—whether within the body of an animal or in a test tube—bacteriolysis results. Should the appropriate amboceptor or bacteriolysin be absent, complement, even if abundantly present, cannot be bound to the bacteria, and hence does not attack them. If, upon the other hand, the complement is absent, union of the amboceptor and bacterin does, indeed, take place, but bacteriolysis does not occur. In such cases the bacteria are said to be sensitized, and subsequent addition of complement will quickly bring about bacteriolysis.

The process of bacteriolysis can be followed by careful microscopic study of the disintegrating bacteria, but is not visible to the unaided eye.

Hemolysis.—Many structures other than bacteria can act as antigens. Among these are various body cells, notably red blood corpuscles, whose destruction is known as *hemolysis*. The mechanism is entirely analogous to that just described for bacteriolysis: Injection of washed red blood corpuscles from another species of animal induces the formation of hemolytic amboceptor, or hemolysin, which is capable of binding complement to red corpuscles of that species and of thus bringing about their destruction.

The process of hemolysis when carried out in a test tube is easily followed with the unaided eye because of the liberation of hemoglobin from the damaged corpuscles. When the reagents are first mixed the corpuscles form an opaque reddish suspension. As hemolysis proceeds, their hemoglobin diffuses out through the fluid, which finally assumes a clear, transparent red color with no visible sediment. Should hemolysis fail to occur the intact corpuscles slowly settle to the bottom, forming a red sediment with a clear, colorless fluid above it.

In order to avoid confusion it must be said in passing that the name "hemolysis" is not limited to the biologic process just described; nor does it, as its etymology implies, necessarily indicate actual

solution of the red cells. In practice the term is applied to any injury whereby their hemoglobin is liberated and diffuses through the surrounding fluid, and this may be effected by a variety of agencies, such as hypotonicity of the fluid, certain bacterial toxins, and mechanical damage such as results from prolonged agitation. With these forms of hemolysis we are not concerned in serologic work, except in so far as human serum received for the test, especially when sent from a distance, or guinea-pig serum obtained for use as complement, may sometimes be colored red by hemoglobin derived from corpuscles damaged by mechanical, bacterial, or other agencies. Serum so colored is unsatisfactory for use.

Application of the Principles of Bacteriolysis and Hemolysis.—It is necessary to bear constantly in mind the three substances or "bodies" which are concerned in bacteriolysis and in hemolysis and the part which each plays. This may be outlined as follows:

BACTERIOLYTIC SYSTEM

Antigen (invading bacterium)	+	Bacteriolytic amboceptor (in serum of infected person)	+	Complement (in serum of any normal animal)	=	Bacteriolysis

HEMOLYTIC SYSTEM

Antigen (red blood corpuscles)	+	Hemolytic amboceptor (in serum of animal injected with red corpuscles)	+	Complement (same as in bacteriolytic system)	=	Hemolysis

The important fact in the above formulae is that, while antigen and amboceptor differ in the two systems, the complement is the same. Whatever the source of the complement, it will serve either for bacteriolysis or for hemolysis, and *this is the key to the complement-fixation tests*.

In the application of these principles it is possible so to adjust the test that any two members of a system being known, the third may be determined qualitatively and (roughly) quantitatively. In the clinical use of the test, however, one seeks the amboceptor, whose presence in the patient's serum establishes the diagnosis of the corresponding disease. To accomplish this one mixes in a test tube appropriate amounts of a culture or extract of the invading organism, blood serum from a suspected patient, and complement. One of two things will occur:

(*a*) If the patient suffers from the disease in question and his blood serum therefore contains the corresponding amboceptor, the complement will be fixed or bound to the antigen by this specific amboceptor, and no complement will be left in a free state.

(*b*) If the patient's serum does not contain the specific antibody to serve as a connecting link, the complement will remain unbound or free in the fiuid.

In either case there will be no visible change to show what has taken place, and it is necessary to add an indicator which will show whether the complement still remains free. This is found in the two specific elements of the hemolytic system—red blood corpuscles and hemolytic amboceptor. If free complement be present the hemolytic system is completed and the corpuscles will be hemolyzed. If, upon the other hand, all available complement had been bound to the antigen by the antibody, then hemolysis cannot occur.

III. SERODIAGNOSTIC TESTS FOR SYPHILIS

Many serologic tests for syphilis have been devised since the original Wassermann test was developed. This technic, known as a complement-fixation test, in theory depends upon antibodies of the third order. However, equally valuable tests, known as precipitation, or better, as flocculation, tests, do not employ complement, but merely an indicator of lipoidal emulsion similar to the so-called "antigen of the Wassermann test." Comparative evaluation studies conducted by the Health Organization of the League of Nations demonstrated the value of certain well-known American procedures. More recently, a special committee appointed by the Surgeon-General of the U. S. Public Health Service has demonstrated that, among many effective tests that have been developed in this country, there are at least five that may be considered standard and are recommended for use in all laboratories. The Kline test is a microflocculation test that requires a small amount of serum and antigen. The reaction is observed with the low-power objective of a microscope. The Kahn test, now known internationally, is a macroflocculation test that requires larger quantities of reagents than does the Kline test, and more time also is required for its performance. However, it is really much simpler than any complement-fixation test. The Hinton test, also a flocculation test, requires a specially prepared indicator, and over-night heating in an accurately controlled water bath. Eagle has devised a flocculation test which is best used in conjunction with his complement-fixation test. The Kolmer modification of the Wassermann test has been the standard complement-fixation test in this country for many years. All of these tests with their latest modifications were demonstrated at a conference of laboratory workers in

1938, and the technic as presented by each originator is herewith presented with the permission of each author. Cholesterol is added to all antigens to increase sensitivity by increasing the surface on

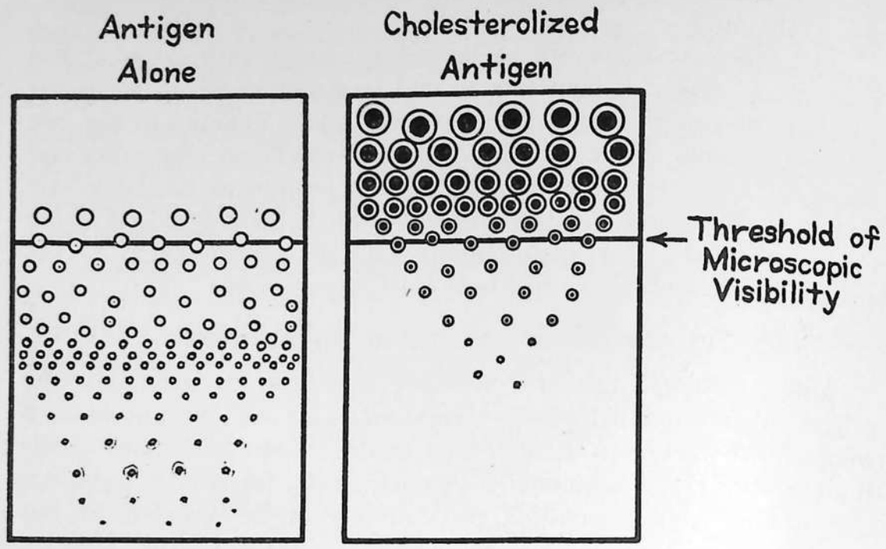


Fig. 355.—The effect of cholesterol upon the size, number, and composition of the particles in diluted antigen. (Eagle, "The Laboratory Diagnosis of Syphilis," courtesy of C. V. Mosby Co.)

which reagin may act. This is graphically represented by a diagram designed by Eagle (Fig. 355).

A. Kline Tests[1]

Materials Required.—*Sera.*—These are prepared as for the complement-fixation text, care being exercised that they contain no red blood cells, or foreign particles. (They are heated at 56° C. for thirty minutes.) In retesting sera within several days, reheating is unnecessary if the specimens have been kept at 8° to 12° C.

When blood is obtainable in small quantity only it is advisable to collect this in a narrow test tube (about 8 to 9 mm.) and to handle it in the same manner as a larger sample from the vein.

When blood is obtainable in very small quantity only it is advisable to collect this in a narrow glass tube with a capillary end. The end is then sealed, a narrow rod is passed through the open end to free the clot from the wall and after the tube is centrifuged at high speed, it is placed in a

[1] Kline, B. S.: Microscopic Slide Precipitation Tests for the Diagnosis and Exclusion of Syphilis, Official Publication, Am. Soc. Clin. Lab. Techn., vol. 1, No. 1, Nov., 1934. Revised, 1938.

water bath at 56° C. with water above the upper level of the serum. After heating, the tube is filed and broken just above the clot and the serum allowed to run into or is drawn into a 1-c.c. pipet, graduated in hundredths.

Glassware.—Microscope slides 2 x 3 inches as purchased are rubbed on both sides with Bon Ami paste (prepared by breaking up a cake of Bon Ami in a small quantity of hot water). As soon as the paste is dry (in about five minutes) it is completely removed from the slide with a soft muslin cloth. For convenience the slides covered with paste may be stuck to each other, allowed to dry, and cleaned at any time.

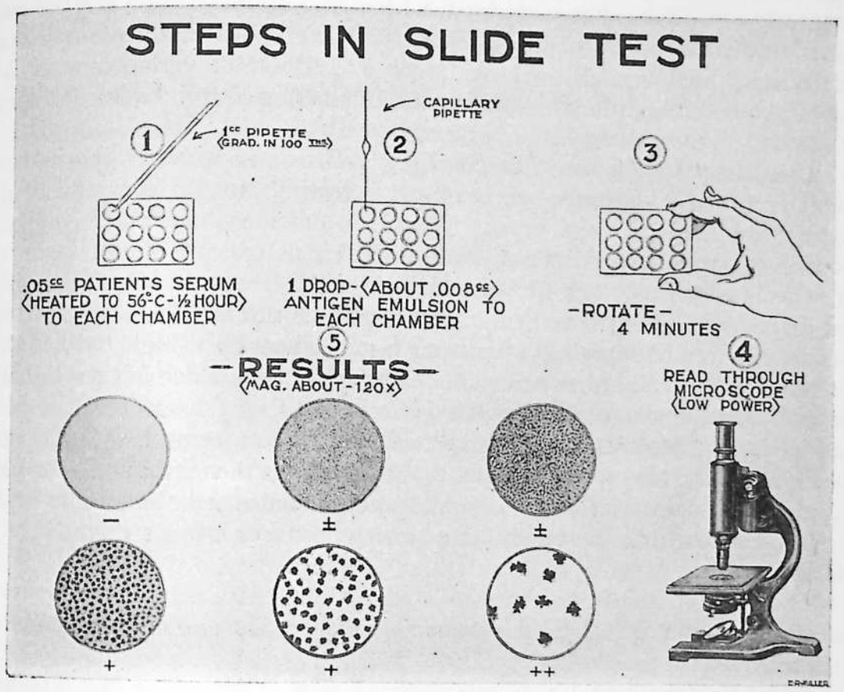


Fig. 356.—Steps in Kline heated serum slide precipitation tests for syphilis. (From B. S. Kline, Microscopic Slide Precipitation Tests for the Diagnosis and Exclusion of Syphilis, Williams and Wilkins Co., Publishers.)

Upon clean slides for the heated serum tests, 12 paraffin rings, each with an inside diameter of 14 mm., are mounted. (Inasmuch as the slide test results are influenced by the surface area of the chambers it is important that the paraffin rings be thin ones. With a little practice the required amount of paraffin can be ascertained. At first, it may be difficult to make complete rings. These incomplete rings may be completed by applying the loop a second time to the open areas.)

For the spinal fluid tests double ring slides are prepared as follows: Upon the clean slide a steel mold $3\frac{2}{16}$ x $2\frac{3}{16}$ x $\frac{1}{8}$ inches with two central

wells $1\frac{9}{16}$ inches in diameter is placed. A metal disk $1\frac{5}{16}$ inches in diameter and $\frac{3}{16}$ inch thick is then placed in the center of each well. The space between them is filled with hot wax (2 parts ordinary vaselin and 1 part parowax) from a 10-c.c. glass syringe. After the mixture cools a few minutes, each disk is elevated from the slide and separated from the wax wall by turning the central screw handle a few times to the right (holding mold down at edge). After the disk is freed, it is lifted out. The mold is removed by inserting a thin blade between it and the slide.

Pipets.—The pipets needed for delivering sera, and spinal fluid, and those for preparing the antigen emulsions are the ordinary finely graduated 0.2- to 10-c.c. pipets. The pipet for the 1 per cent acetic solution is a 0.2-c.c. pipet graduated in 0.001 c.c. The pipets for delivering the antigen emulsions are Wright pipets made from glass tubing 6 to 10 mm. in diameter with the tubes about 0.5 mm. in outside diameter, delivering a drop equal to about 0.008 c.c. (62 drops per 0.5 c.c.).

Instrument for Making Paraffin Rings.—This is essentially the instrument proposed by Green.[1] A piece of soft iron wire (No. 28) is wound twice tightly about a test tube (about 15 mm. in outside diameter) forming a double loop and leaving a double shaft about 1 inch in length. The two shafts are then twisted together to within $\frac{1}{4}$ inch of the free end. After removing the looped wire from the test tube, a piece of linen thread (No. 12) is started from the free end of the shaft after being fastened here by a single twist of the free ends. Three long turns are made reaching the loop which is then tightly wound with the thread. The winding is continued up the shaft to the free end where it is fastened between the two ends of the wire by twisting them. The loop is then bent at right angles to the shaft. It is then reshaped by working the loop against the bottom of the test tube mentioned above. The shaft is then inserted into the handle of a teasing needle or into a straight hemostatic forceps.

The paraffin rings are made by dipping the instrument into smoking paraffin (about 120° C.) draining quickly at one point and transferring the remainder to the glass slide.

Slide Holders (For 3 x 2 Inch Slides).—The slide holder is a wooden lid of a slide box ($3\frac{1}{2}$ x $6\frac{3}{4}$ x $\frac{1}{2}$ inches) containing an easily fitting thin wooden shelf having a small handle at each end.

Salt Solutions.—Sodium chloride (0.85 per cent) (C. P. or reagent, Merck) solution used in the tests is prepared with distilled water having a *p*H of about 6. (Such water gives a lilac color when 1 drop of chlorphenol red indicator [LaMotte] is added to 0.25 c.c. of it in a small chamber.) Distilled water having a *p*H of 5.2 or less gives a yellow color with this indicator and is not satisfactory.

One Per Cent Acetic Acid.—(C. P. Reagent.) It is advisable to use no less than 1 c.c. of acid (delivered from a 1 or 2 c.c. pipet) and accordingly 99 c.c. of distilled water.

[1] Green, G.: Paraffin Rings on Microscope Slides, Am. Jour. Pub. Health, *15*:651–652 (July), 1925.

Antigen.[1]—The purified antigen used in the microscopic slide precipitation tests for syphilis is prepared as follows:

Two hundred Gm. of dried beef heart powder (Difco) is placed in a 2-liter Erlenmeyer flask.

One liter of absolute ethyl alcohol (99+ per cent) (Rossville Commercial Alcohol Corp., Lawrenceville, Ind.) is added.

After the flask is stoppered with a cork covered with tin foil, it is shaken vigorously by hand at intervals for two hours. Better still two wide-mouth bottles (Difco bottles for 1 pound beef heart powder) each with 100 Gm. of beef heart powder and 500 c.c. of absolute ethyl alcohol (99+ per cent) are shaken vigorously in a machine for two hours. (This short extraction removes much of the desired antigenic substance in the powder.)

The extract is filtered into a liter cylinder through good grade filter paper of medium texture (Schleicher and Schull No. 597, 38.5 cm.).

During filtration the mixture is stirred with a wooden tongue depressor and toward the end pressed with the cork until the powder is quite dry.

The extract (about 775 c.c.) is placed in the refrigerator at 8° to 10° C. for twenty-four hours.

During this time a fairly heavy white precipitate settles out. This is filtered off and the filtrate in a large evaporating dish is concentrated on a water bath at 45° to 50° C. determined by a thermometer bulb within the extract. For convenience, the concentration of the extract may be started by leaving it in an air incubator at 50° C. until the following morning, and then completed by placing it on the water bath. During evaporation of the alcoholic extract an irregular festoon appears at the periphery. When the extract reaches the proper concentration the festoon disappears and the margin of the concentrated extract is sharp.

In a warm room the extract is now poured quickly into 500 c.c. of acetone, C. P. (Coleman and Bell), at 50° C. in a large evaporating dish. Stir acetone a few times with tongue depressor to distribute the extract.

The dish is then placed in an air incubator at 37° C. for fifteen minutes after which the acetone is decanted leaving a soft yellow brown wax adherent to the side of the dish. (Longer periods of precipitation and precipitation at lower temperatures permit of precipitation of adventitious substances as well and such antigens give more sensitive and less specific results.)

The dish is then placed on a water bath or in an air incubator at 50° C. until the little acetone remaining has evaporated (about thirty minutes).

The wax is then worked together and placed in a glass-stoppered bottle. Then 80 c.c. of absolute ethyl alcohol (99+ per cent) that has been kept in an air incubator at 50° to 56° C. for one-half hour or longer, is added and after a few minutes' shaking the bottle is placed in an air incubator at 50° C. and shaken gently after fifteen minutes and again after thirty minutes,

[1] Standard materials including antigen for the microscopic slide precipitation tests for syphilis may be obtained from the LaMotte Chemical Products Company, McCormick Building, Baltimore, Md.

when it is removed from the incubator and placed in the refrigerator at 8° to 10° C. for forty-five minutes.

The solution is then filtered at room temperature and the filtrate is evaporated down at 45° to 50° C. resulting in a soft brown wax (antigen wax). The wax is weighed and to each gram in a glass-stoppered bottle, 10 c.c. of absolute ethyl alcohol (99+ per cent) (at 50° to 56° C.) is added. After the bottle is shaken for a few minutes it is placed in an air incubator at 50° C. for thirty minutes, and then shaken a few minutes.

The slightly turbid solution is then placed at 8° to 10° C. for about an hour and then filtered at room temperature. The resultant clear filtrate is the antigen, and contains about 8.75 per cent of the alcohol-treated acetone-insoluble wax.

Kline suggested that owing to the fact that there are adventitious water-soluble substances in the powdered beef heart that may be carried through into the finished antigen, it may be advisable at times to purify the antigen further by a water purification process.[1]

The average yield of antigen wax from ½ pound of beef heart powder is 3.5 to 4 Gm. The discarded acetone-soluble lipoidal residue (impurities) ordinarily weighs at least three times this amount.

The antigen keeps best at room temperature hermetically sealed in glass containers. In small necked glass-stoppered bottles (opened from time to time) kept at room temperature it shows no appreciable change in specificity or sensitivity for at least six months. (As stated above it is important to follow the steps outlined in minute detail. Too long a precipitation in acetone, precipitation at too low a temperature and use of improper chemicals for instance result in waxes which contain some impurities that determine a sensitivity greater than standard.)

Preparation of Antigen Emulsions for Diagnostic and Exclusion Slide Tests of Heated Serum

Formula:

0.85 c.c. of distilled water (*p*H about 6).
1 c.c. 1 per cent cholesterol (C. P. Pfanstiehl) in absolute ethyl alcohol (99+ per cent).[2]

[1] Kline, B. S.: New Standard Slide Test Antigen (Water Purified), Amer. Jour. Clin. Path., *12*:48–61 (Jan.), 1942.

[2] The 1 per cent cholesterol solution for the emulsion is prepared in about forty-five minutes by placing the cholesterol flakes and absolute alcohol in a glass-stoppered bottle in an oven at 50° to 56° C. and shaking gently a few minutes at fifteen-minute intervals. The solution kept in the incubator at 37° C. is thoroughly satisfactory for use as long as two months.

The sensitivity of an emulsion is greatly influenced by the quantity of cholesterol present. The sensitivity is likewise influenced by the quality of the cholesterol used. The cholesterol (Pfanstiehl, C. P.) that has been found uniformly satisfactory in the slide tests is flaky, pearly and readily soluble to 1 per cent in absolute ethyl alcohol (99+ per cent). Powdery, white cholesterol incompletely soluble to 1 per cent in absolute alcohol (99+ per cent) has been found to give too sensitive results.

0.1 c.c. antigen.
2.45 c.c. 0.85 per cent sodium chloride (C.P. or reagent, Merck) solution (*p*H about 6).

The technic of preparing the emulsion according to the above formula is as follows: Into a 1-ounce bottle the required amount of distilled water (*p*H about 6) is pipetted.

The bottle is held at an angle, and the 1 per cent cholesterol in absolute ethyl alcohol (99+ per cent) is allowed to run along the side of the neck of the bottle.

The bottle is gently rotated from the neck for twenty seconds.

It is held at an angle again, and the proper amount of antigen is pipetted against the side of the neck of the bottle from a finely graduated pipet.

The bottle is promptly stoppered with a cork and shaken vigorously (the fluid thrown from bottle to cork and back) for one minute.

Lastly, the 0.85 per cent sodium chloride solution is allowed to run in quite rapidly, the bottle is stoppered again and shaken less vigorously than previously for one minute.

The emulsion, when examined under the microscope at a magnification of about 120 times, shows numerous very fine particles, but no clumps whatever.

For Diagnostic Test

Place 1 c.c. or more of the emulsion in a narrow test tube (12 mm. inside diameter) in a water bath at 35° C. (beaker or water in usual laboratory air incubator at about 37° C.) for fifteen minutes. The emulsion as soon as heated is ready for use.

For Exclusion Test[1]

Place 2 c.c. of the emulsion in a narrow test tube (12 mm. inside diameter) in a water bath at 56° C. for fifteen minutes. Then pour into a 3 x 1 inch tube and centrifuge for fifteen minutes (eighth setting rheostat, Centrifuge Size 1, Type SB). Decant the fluid and, with the tube inverted, dry the inside of the tube with a cloth almost to the level of the sediment. To the sediment add 1.5 c.c of 0.85 per cent sodium chloride solution.[2] Transfer to a narrow tube for use.

[1] Personal communication from Dr. Kline. In addition, for special purposes, such as the exclusion of syphilis in blood donors, in cases of suspicious sore, and for the control of adequate treatment (supplemented by clinical studies and interpretation) the following control exclusion test emulsion is recommended:

Control Antigen Emulsions for Microscopic Slide Precipitation Tests for Syphilis, Am. Jour. Clin. Path., *7*:490–497 (Nov.), 1937. 1. Pipet into a 1-ounce bottle 0.5 c.c. of 1 per cent standard cholesterol solution. 2. Pipetting with the left hand, allow 0.425 c.c. of distilled water to mix drop by drop with the cholesterol solution, rotating the bottle with the right hand during the mixture and for ten seconds thereafter on a flat surface. 3. Add 0.06 c.c. (not 0.05 c.c.) of standard antigen and after five to ten seconds *gently* ro-

These emulsions, kept at room temperature, are satisfactory for use for forty-eight hours after preparation.

Diagnostic and Exclusion Microscopic Slide Precipitation Tests for Syphilis with Heated Serum

1. Place three heated serum test slides, each with twelve small chambers, on a tray in a small holder.
2. Into each of the thirty-six rings, pipet 0.05 c.c. of the heated serum to be tested (eighteen sera in duplicate).
3. After all the sera are pipetted, 1 drop of the diagnostic test antigen emulsion (about 0.008 c.c.) is allowed to fall from a Wright pipet into one of the two portions of each serum. Into each of the other eighteen duplicate sera a similar drop of exclusion test antigen emulsion is allowed to fall from a Wright pipet.
4. The slides in the holder are rotated on a flat surface for four minutes.
5. The results are examined at once through the microscope at a magnification of about 120 times (low power 16-mm. objective, eyepiece 12) with the light cut down as for the study of urinary sediments and reported in terms of pluses according to the degree of clumping and the size of the clumps.

Any spilling from the chamber makes the reaction therein unsatisfactory, and the serum concerned should be retested.

If sufficient serum is available the exclusion test for syphilis may be done with 0.3 c.c. heated serum in a chamber similar to that employed for the spinal fluid test (33 mm. in diameter) and 1 drop (about 0.008 c.c.) of emulsion made by suspending the sediment of 8 c.c. of exclusion test emulsion (centrifuged fifteen minutes at eighth rheostat setting) in 1 c.c. of 0.85 per cent salt solution.

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tate the bottle in an upright position on a flat surface for five seconds. 4. Allow the mixture to stand for one minute and again *gently* rotate the bottle in an upright position on a flat surface for five seconds. 5. Add 1.25 c.c. of physiologic saline solution and again *gently* rotate the bottle in an upright position on a flat surface for five seconds. 6. *Gently* pour the emulsion into a narrow test tube and place in the water bath at 56° C., for fifteen minutes. 7. Transfer *gently* to a 3 by 1 inch tube. 8. Centrifuge for five minutes at the eighth rheostat setting, as for the standard exclusion slide test emulsion. 9. Decant the supernatant fluid and take up the sediment in about 1 c.c. of saline solution (it may require a little experimentation to determine the proper amount of saline solution to add to obtain the optimal number of particles in the emulsion). Vigorous shaking and rotation in the preparation and subsequent handling of the emulsion must be avoided. Emulsions properly prepared and properly handled have been found satisfactory for use for at least twenty-four hours after preparation.

[2] It may require a little experimentation with time and speed of centrifugation and quantity of saline to take up the sediment (1 c.c. to 1.5 c.c.) to make sure that the exclusion test emulsion contains the optimal number of particles.

Preparation of Antigen Emulsions for Diagnostic and Exclusion Slide Tests of Spinal Fluid

Formula:

0.85 c.c. distilled water (*p*H about 6).
1.25 c.c. of 1 per cent cholesterol (Pfanstiehl C. P.) in absolute ethyl alcohol (99+ per cent).
0.1 c.c. antigen.
2.2 c.c. of 0.85 per cent sodium chloride (C. P. or reagent, Merck) solution (*p*H about 6).
8.8 c.c. of the emulsion are made by using double the quantities given in the formula.

For Diagnostic test

Place 4 c.c. of the emulsion in a narrow test tube (12 mm. inside diameter) in a water bath at 35° C. for fifteen minutes. Then pour into a 3 x 1 inch tube. Centrifuge for fifteen minutes (eighth setting rheostat, 1, S.B.). Decant the fluid and, with the tube inverted, dry the inside of the tube with a cloth almost to the level of the sediment. To the sediment add 1 c.c. of 0.85 per cent sodium chloride solution (*p*H about 6).

Transfer to a narrow test tube for use.

For Exclusion Test

Place 4 c.c. of the emulsion in a narrow test tube (12 mm. inside diameter) in a water bath at 50° C. for fifteen minutes. Then pour into a 3 x 1 inch tube. Centrifuge for fifteen minutes (eighth setting rheostat, 1, S.B.). Decant the fluid and, with the tube inverted, dry the inside of the tube with a cloth almost to the level of the sediment. To the sediment add 1 c.c. of 0.85 per cent sodium chloride solution (*p*H about 6).

Transfer to a narrow test tube for use.[1]

These emulsions, kept at room temperature, are satisfactory for use for twenty-four hours.

Diagnostic and Exclusion Microscopic Slide Precipitation Tests for Syphilis with Spinal Fluid

Preliminary procedures with spinal fluids.

Spinal fluids, turbid with exudate, blood or bacteria, or containing injected substances including horse serum, are unsatisfactory for testing.[2] Spinal fluids with slight turbidity or few particles are centrifuged at high speed for ten minutes, and the clear fluid is withdrawn or decanted. The *p*H should be 8 or more.

[1] In making up the exclusion test emulsion, the centrifugation should be varied until the proper speed and length of time for obtaining the optimal amount of sediment (number of particles) is established.

[2] A satisfactory rough estimate of the protein content may be made by noting the turbidity when 1 c.c. of 95 per cent alcohol is added to 0.25 c.c. of spinal fluid.

Place the required number of test tubes, 6 x $\frac{5}{8}$ inches, each containing 5 c.c. of Benedict's solution (1909–1910), in a beaker (Pyrex). Add water halfway to the top. Heat. Keep the tubes in vigorously boiling water for five minutes.

Place the tubes in a rack. After making certain that no copper reduction has occurred in any of the tubes, add to each tube, properly numbered, 0.5 c.c. of spinal fluid. Shake each tube vigorously for ten seconds.

Replace the tubes in the beaker. Add water halfway to the top. Heat. Keep the tubes in vigorously boiling water for five minutes.

Replace the tubes in the rack, inspecting each immediately after removal from the beaker, for precipitate indicating presence of sugar.

Spinal fluids giving a negative reaction for sugar in the above test are unsatisfactory for testing for syphilis. These are fluids that have been acted upon by bacteria either inside or outside of the body. In the former case (bacterial meningitis) in which organisms and enzymes of the exudate have acted upon the sugar, the fluids may contain substances that give positive or unsatisfactory reactions in various tests for syphilis. Bacterial contamination subsequent to withdrawal of spinal fluids from cases of syphilis causes a steady loss both of sugar and of the specific reacting substance, if the fluids are kept at room temperature.

On the other hand, if spinal fluids containing sugar when withdrawn from the body are kept at low temperature (8° to 10° C.) they continue to give a positive reaction for sugar with the test described above for several weeks, and syphilitic spinal fluids under these conditions show no appreciable loss of specific reacting substance for at least a week.

The clear and cleared spinal fluids which give a positive reaction in the sugar test described above are then tested as follows:

Diagnostic and Exclusion Microscopic Slide Precipitation Tests for Syphilis with Spinal Fluid

Into each of twelve chambers (33 mm. in diameter) on six glass slides (in a holder tilted slightly by placing a small metal bar $\frac{1}{8}$ inch thick under one long slide) deliver 0.05 c.c. of 1 per cent glacial acetic acid solution from a 0.2-c.c. pipet graduated in thousandths. (It is of the utmost importance that the 1 per cent acetic acid be carefully prepared since this reagent in stronger concentration will precipitate the emulsion.)

Into each chamber allow 0.25 c.c. of the spinal fluid to be tested (six spinal fluids in duplicate) to fall from a 1-c.c. pipet graduated in hundredths. Hold the pipet directly above the acid and lastly touch the tip of the pipet to some dry portion of the chamber.

Rotate the slides in the holder on a flat surface with moderate vigor for one minute.

Into one half of the chambers allow 1 drop (about 0.008 c.c.) of diagnostic antigen emulsion to fall from a Wright pipet.

Into each of the duplicate spinal fluids 1 drop (about 0.008 c.c.) of exclusion antigen emulsion is allowed to fall from a Wright pipet.

Rotate the slides in the holder on a flat surface with moderate vigor for one minute to distribute the antigen and then for four minutes move the holder gently but rapidly (about three complete movements a second) back and forth a distance of $\frac{1}{4}$ to $\frac{1}{2}$ inch.

The results are examined at once through the microscope at a magnification of about 120 times (objective 16 mm., eyepiece 12) with the light cut down as for the study of urinary sediments and recorded in terms of pluses according to the degree of clumping and the size of the clumps. (For ease in reading the results, the slide is tilted on a piece of metal $\frac{1}{8}$ inch thick, $\frac{1}{2}$ inch wide, and 4 inches long, placed on the stage.)

B. Mazzini Flocculation Test[1]

I. EQUIPMENT

1. *Serologic pipets* of 5 c.c. capacity to measure the buffered saline solution; 1 c.c. capacity, graduated in hundredths to the tip, to measure serum and antigen; and 0.2 c.c. capacity, graduated in hundredths, to measure acetic acid.

2. *Bottles* of 30 c.c. capacity, round type, 3 cm. in diameter, for preparing one quantity of antigen suspension (3.4 c.c.), and 15 c.c. bottles, round type, approximately 2.3 cm. in diameter, for preparing half-quantity of antigen suspension (1.7 c.c.).

3. *Antigen Dropper.*—A 5 c.c. glass syringe or other convenient glass tube fitted with a 25-gauge needle.

4. *Serum Tube Rack.*—Any substantially made rack is satisfactory.

5. *Water Bath.*—A constant temperature 55° C. or 56° C. bath for heating sera.

6. *Serum Tubes.*—Any convenient size to fit the tube racks.

7. *Centrifuge* of suitable size to the volume of work done.

8. *Rotator.*—Rotation by hand is satisfactory provided it is carried out as recommended. Mechanical rotation, however, is superior since it provides a constant and uniform means of agitation.

9. *Microscope Equipped With a Low Power* (16 mm.) *Objective and a* 10X *Ocular.*

10. *Glass Slides.*—The slide of choice for the serum test is the microscopic slide 2 x 3 inches upon which wax rings have been mounted. For the spinal fluid test it is necessary to employ all glass concavity slides; the concavity should not be less than 2 mm. deep and 16 mm. in diameter.

[1] By permission of the U. S. Public Health Service.

11. *Slide Holder.*—Made of any convenient material to accommodate from one to three 2″ x 3″ slides.

12. *Mounting the Wax Rings on the Glass Slides.*—The Fisher Rapid Maker, which has a base of 12 metal circles arranged in three rows of four each, is a convenient instrument for mounting the rings. The wax used is the inexpensive Zubian Sealing Wax employed by "home canners" to seal jars of fruits and vegetables. It is manufactured by Dicks-Pontius Co., Dayton, Ohio, and obtainable at most retail grocery stores. A mixture of the wax and paraffin, in the proportion of 50 Gm. wax to 20 Gm. paraffin, has been found to give the most satisfactory results. The wax mixture is placed in a convenient vessel, such as a pyrex petri dish, and heated with a micro-burner from 105° C. to 110° C. and mixed thoroughly. The rings are made by dipping the instrument into the melted wax mixture and then placed on the clean slide for a few seconds. The instrument is then lifted and the process repeated on new slides. If a deeper chamber is desired, a second and third layer may be applied after the wax has been allowed to solidify.

13. *Care of the Slides After Use.*—The wax rings should not be removed from the slides after use. Immediately after reading the tests the slides are placed in a metal slide holder, such as that used in tissue staining, which is kept immersed in distilled water. By this procedure drying of sera on the slides is prevented. After the completion of the tests, the slides are rinsed thoroughly in running tap water and finally rinsed in distilled water. The slides are then transferred to a slide box and allowed to dry at room temperature under cover, or they may be dried by rubbing the chambers of the rings with a soft cloth free from lint. If proper care is given the slides, they may be used indefinitely.

II. REAGENTS

A. **Preparation of the Antigen Extract.**—1. 20 grams of dehydrated beef powder (Difco), 10 grams of powdered egg yolk[1] and 200 c.c. of ether for anesthesia are placed in a 500 c.c. wide mouth, glass-stoppered bottle. The mixture is agitated in a mechanical shaker for 5 minutes, or by hand for 15 minutes.

2. The mixture is filtered through a fat free 24 cm. paper of medium texture into a 500 c.c. flask. The ether extraction is repeated four additional times using 100 c.c. of ether each time. A new filter paper is used for each filtration and all the ethereal filtrates are collected in the 500 c.c. flask. The combined filtrates will be used in step 6.

[1] Powdered egg yolk may be purchased at any reliable wholesale dairy supply company, or from Bessire & Co., Inc., Indianapolis, Indiana.

3. After the last extraction is completed the moist powder is spread on a new piece of filter paper. The powder may be left at room temperature or placed in a 37° C. incubator to allow the ether adhering to the powder to evaporate.

4. The dried powder is then placed in a 500 c.c. glass-stoppered bottle, 80 c.c. of absolute ethyl alcohol is added and the mixture agitated in a mechanical shaker for 4 hours, or the mixture is left at room temperature for 3 days, being shaken for 5 minutes three times each day.

5. The mixture is filtered through a fat free 24 cm. paper of medium texture into a 100 c.c. wide-mouth, glass-stoppered bottle. The powder is now discarded.

6. The combined ethereal filtrates are now placed in a large evaporating dish (8.5 inches in diameter) and the ether evaporated by placing the dish in a water bath at 55° C. until no ether bubbles rise to the surface of the liquid.

7. 100 c.c. of acetone (Merck's Reagent) which has previously been warmed to 55° C. are poured rapidly into the concentrated ether extracts. The mixture is stirred thoroughly with a steel spatula and immediately decanted into two 50 c.c. centrifuge tubes, and centrifuged at 2000 R. P M. for 5 minutes. The acetone is poured off and discarded. 10 c.c. of fresh acetone are added to each tube. The acetone insoluble lipoids are stirred with a glass rod, then placing the palm of the hand over the mouth of the tube it is inverted a few times. The supernatant acetone is poured off and discarded. The acetone insoluble lipoids are collected with a spatula and added to the alcoholic extract obtained in step 5. The bottle is placed in a 55° C. water bath for 30 minutes, shaking gently at frequent intervals. The extract is then allowed to cool by placing the bottle in the refrigerator for 30 minutes.

8. The extract is filtered through a fat free 12.5 cm. paper of fine texture. The antigen is then ready for titration. Any precipitate appearing after the extract has stood should be removed by filtration. The antigen is kept tightly stoppered at room temperature and remains unchanged indefinitely.

B. **Preparation of the 1 Per Cent Cholesterinized Alcohol.**—A sufficient quantity to meet the individual need is prepared. For example: To prepare 50 c.c. of the solution, place 500 mg. of cholesterin C. P. (Pfanstiehl Ash Free) and 50 c.c. of absolute ethyl alcohol in a 100 c.c. glass-stoppered bottle. Heat in the water bath at 55° C. until the cholesterin has completely dissolved; agitate at frequent intervals. Filter the solution through fat free paper of fine texture.

C. **Preparation of the Buffered Saline Solution.**—A sufficient

quantity of this solution, having a *p*H of 6.3 to 6.4 and a salt concentration of 10 grams per liter, is prepared. An example based on a 250 c.c. volume follows:

Sodium chloride C. P.	2.025	Gm.
Secondary sodium phosphate (Na_2HPO_4).12H_2O	0.425	"
Primary potassium phosphate (KH_2PO_4)	0.050	"
Double distilled water	250.00	c.c.
N/1 hydrochloric acid	0.8	"
Formaldehyde (Merck's reagent)	0.25	"

The solution is filtered and the *p*H checked. For practical purposes the colorimetric method of determination is sufficiently accurate. The solution is kept in a glass-stoppered pyrex bottle and will remain unchanged indefinitely. If through carelessness the solution becomes contaminated with particles of dirt, cotton fibers, or other debris, it is necessary to filter the solution because any debris which may be transferred to the suspension will appear in the field of vision during the reading of the test and, unless differentiated from specific flocculate, may lead to false reading.

D. **Preparation of Serum.**—The patient's serum is separated from the clot by centrifugalization and heated for 30 minutes in the water bath at 55° C. to 56° C. For emergency pretransfusion test the serum may be heated at 60° C. for 10 minutes. Inspection of sera for visible precipitate after heating should be done as a matter of routine. Occasionally heated serum throws off a precipitate which, if not removed by re-centrifugalization, may interfere with serologic reactions in general. There is not, however, the possibility that the precipitate, if overlooked and left in the serum, will be mistaken for the true flocculate of a positive reaction when read microscopically, which does occur when specimens containing this pseudo-precipitate are read macroscopically. Another potential source of error to the inexperienced is the occasional presence of oil globules which have been transferred from lubricated syringes at the time the blood was drawn. The globules grouping themselves in clusters of varying sizes simulate weakly and even strongly positive reactions. Specimens containing an excessive amount of oil should be reported as unsatisfactory and a new sample requested. If it is necessary to retest a weakly positive reacting serum which has been heated and thereafter kept at room temperature or in the refrigerator for several hours, it is advisable to draw off a new portion of the serum from the clot. In strongly positive sera re-heating for 15 minutes does not cause sufficient destruction of reagin to make an appreciable change. Hemolyzed sera can be tested with accuracy provided they are not excessively viscous after the heating period.

III. TITRATION OF THE ANTIGEN

Titration of any antigen is necessary because it is impossible to obtain extracts of uniform antigenic value. Once the antigen has been titrated to the desired sensitivity, however, the routine technic for the preparation of the suspension is simple and rapidly done.

A. **Determination of the Optimum Lipoid-Cholesterin Ratio.**—1. Set up 5 clean and dry serologic test tubes in a rack. Label them 1 to 5.

2. Place 0.1 c.c. of the antigen extract directly into the bottom of each tube.

3. Add 0.9 c.c., 1.4 c.c., 1.9 c.c., 2.4 c.c., and 2.9 c.c. of 1 per cent cholesterinized alcohol to each of the five tubes respectively. Mix thoroughly the contents of each tube. Tube No. 1 contains a 1:10 ratio, tube No. 2 1:15, tube No. 3 1:20, tube No. 4 1:25, and tube No. 5 1:30.

4. Take five 30 c.c. bottles and label them 1:10, 1:15, 1:20, 1:25 and 1:30 respectively.

5. Pipet 3 c.c. of buffered saline solution into each of the five bottles.

6. With a 1 c.c. pipet graduated to the tip measure 0.4 c.c. (reading from the bottom of the pipet) of the 1:10 cholesterinized antigen; hold the bottle in the left hand and impart a rapid rotating motion to it as the antigen is being discharged directly and at once into the buffered saline from the pipette held in the right hand. Draw the suspension into the pipet and blow in and out two or three times.

7. Proceed in the same manner in preparing the 1:15, 1:20, 1:25, and 1:30 suspensions. The bottles are corked and allowed to stand at room temperature for 3 hours without further manipulation.

B. **Trial of the Suspensions.**—1. At the end of 3 hours suspension No. 1 is shaken gently from bottom to cork and back 10 times, then it is poured into a 5 c.c. syringe fitted with a 25-gauge needle.

2. Select 30 sera from cases known to be free from syphilitic infection.

3. Place 0.05 c.c. of serum from each of the 30 negative specimens in the corresponding one of the thirty chambers of three glass slides which have been placed on a slide holder.

4. Discharge one drop of the 1:10 suspension into each of the 30 sera.

5. Rotate the slides with a circular slightly "jerky" motion for 4 minutes at 120 rotations per minute. It is important that the number of rotations be that indicated, and that the proper motion be given to the slides to insure that the antigen particles become well dispersed

throughout the area of the rings. It is not necessary that the motion be of such nature as to cause the sera to "jump" the rings.

6. Examine the results under the low power (16 mm.) objective of the microscope with subdued light. Record the results. Every one of the sera should show numerous, very small, round or slightly elongated particles of lipoid-cholesterin complex. These particles uniformly dispersed throughout the field should not show the slightest clumping.

7. Place three other slides on the holder and using the same sera as previously used proceed to try out the 1:15 suspension. Follow this with the 1:20, the 1:25, and finally with the 1:30 suspension. Record the results. Usually within the range of 1:10 to 1:30 will be found one or more ratios in which the cholesterin is in excess of the lipoids, allowing the spontaneous clumping of the particles. Obviously these ratios cannot be employed in the test proper since false positive reactions will be obtained with such suspension.

C. **Determination of the Antigenic Quality of the Suspension.**—Having already determined the lipoid-cholesterin ratios which will not cause false positive reactions with negative sera, the next step in the standardization of the antigen is the evaluation of the antigenic properties of these suspensions. For this purpose select at least 10 partially positive sera, preferably those from long treated cases. The object is to employ sera containing as few reacting units as possible.

1. Place 0.05 c.c. of serum from each of the ten partially positive specimens into the corresponding chambers of a glass slide.

2. Discharge one drop of the 1:10 suspension into each serum. Rotate the slide for 4 minutes at 120 rotations per minute. Examine through the microscope. Record the results.

3. Proceed to try out the rest of the suspensions which were found to give clear-cut negative reactions with known negative sera by following the same procedure as for the 1:10 suspension. When these trials are conducted with sera containing relatively few reacting units, it will be observed that the lower the lipoid-cholesterin ratio the weaker the reaction, the flocculate increasing in size as the ratio increases. After recording all the results and having made a study of them, a final lipoid-cholesterin ratio is selected which is designated as the titer of the antigen. For *maximum sensitivity*, the suspension containing the highest lipoid-cholesterin ratio which *does not cause the least clumping in the presence of negative sera* is selected as the titer. If a less sensitive antigen suspension is desired a lower ratio is chosen. Naturally, a greater degree of safety is obtained by using a lower ratio but the sensitivity usually will be decreased.

When the titer of the antigen has been determined, a sufficient amount of cholesterinized antigen is prepared to meet the individual need for approximately one month. For example: If the ratio selected is that of 1:20, then by taking 0.5 c.c. of the antigen extract and adding 9.5 c.c. of 1 per cent cholesterinized alcohol, a supply for about one month is obtained, since 0.4 c.c. of the cholesterinized antigen, regardless of the ratio, is the fixed amount to use in 3 c.c. of buffered saline solution. This volume (3.4 c.c.) will be sufficient for about 300 tests. The cholesterinized antigen keeps unchanged for several months provided it is tightly stoppered. If evaporation of alcohol from the antigen is allowed to take place, it results in the precipitation of cholesterin, making the antigen unfit for use.

It would seem that the antigenic determination could be eliminated and the titer be based solely on the lipoid-cholesterin ratio. By this procedure less time and labor would be involved in the standardization of the antigen. However, experience has shown that this determination is very desirable if not essential. Sometimes it can be demonstrated that two different ratios give approximately the same degree of flocculation with weakly positive sera, and while there is little choice between the two, the logical dilution to employ is the one having the lower ratio, because the sensitivity is the same while the margin of safety (specificity) is increased. Obviously if the antigenic determination had not been carried out the dilution of choice as determined by the lipoid-cholesterin ratio titration would be the higher of the two. *It is possible to eliminate all titrations and to set an arbitrary mean which past experience has shown is the average lipoid-cholesterin ratio.* For instance, the most frequently encountered ratios which give clear-cut negative reactions with negative sera are the 1:10, 1:15, 1:20, and 1:25. Therefore, an arbitrary selection of the dilution containing the 1:15 or 1:20 ratios could be made provided strict adherence to details of technic is observed. Nevertheless, if the highest sensitivity consistent with safety is to be obtained it follows that both titrations are necessary. In general, the accuracy with which serologic reagents are standardized largely determines the quality of the work performed in any laboratory irrespective of the merits of the technic involved.

IV. THE TEST PROPER

Once the desired sensitivity has been determined the daily routine technic for the preparation of the suspension is very simple and quickly done. The reagents for the test proper consist of two solutions: buffered saline solution and cholesterinized antigen solution.

A. **Qualitative Test with Serum.**—1. Pipet 3 c.c. of buffered saline solution into a 30 c.c. bottle.

2. With a 1 c.c. pipet graduated to the tip measure 0.4 c.c. (reading from the bottom of the pipet) of the cholesterinized antigen; hold the bottle in the left hand and impart a rapid rotating motion to it as the antigen is being discharged directly and at once into the saline solution from the pipet held in the right hand. Draw the suspension into the pipet and blow in and out two or three times. The bottle is corked and allowed to stand at room temperature for 3 hours, at which time the suspension reaches its optimum sensitivity, or the bottle may be placed in the refrigerator at 6° C. to 8° C. for 15 minutes to accelerate the ripening of the antigen suspension and then it can be used immediately. However, at this stage of ripening it will occasionally fail to react with weakly positive serum. The suspension continues to be usable for 24 hours after which it decreases in sensitivity; therefore, dependable results require that it should be used within this period of time. If the number of tests to be performed is small one-half quantity of the reagents can be prepared.

3. At the end of 3 hours at room temperature, or 15 minutes in the refrigerator, the suspension is shaken gently from the bottom to cork and back 10 times and transferred to a 5 c.c. glass syringe fitted with a 25-gauge needle and is then ready for instant use. It has been ascertained that some antigens do not disperse completely when added to the buffered saline solution, hence it is advisable that the suspension be shaken gently, yet thoroughly, after the "ripening" period in order to obtain a "smooth" suspension.

4. Place one, two or three glass slides on a slide holder, depending on the number of specimens to be tested. Pipet 0.05 c.c. of each patient's serum, which has previously been heated for 30 minutes at 55° C. to 56° C., into the corresponding chamber of the glass slide. Discharge one drop of the ripened antigen suspension into each of the sera in the chambers. Known negative and positive sera as controls on the antigen should be included.

5. Rotate the slide holder for 4 minutes at 120 rotations per minute.

6. First examine every one of the rings macroscopically to make certain that no serum has "jumped" the ring and contaminated another. Then examine the results microscopically under the low power objective (16 mm.) with subdued light. Inspection of the periphery of the rings for clumps should be made a routine practice, for occasionally the flocculate is very compact and has a tendency to locate in the outer portion of the ring. Record the results as follows: No clumping, negative; very small clumps, 1+; small clumps, 2+; medium size clumps, 3+; large clumps, 4+.

An alternative method of reading may be used as follows: No clumping, negative; very small to small clumps, doubtful; medium to large clumps, positive.

It should be obvious that every specimen found to react positively, whether it be strongly positive, moderately positive or weakly positive, must be retested before being reported if mechanical errors are to be excluded.

In reading weakly positive reactions care should be taken to differentiate red blood cells, oil globules or debris—which may be contained in the serum, or in the slide, or in the antigen suspension—from the true flocculate of a positive reaction. The reading of any serologic test requires judgment and experience since no accurate standard can be prepared. Although no experience is needed to read strongly positive reactions, time and observation alone will lead to correct interpretation of weakly positive reactions.

Zone reactions are usually due to an off balance in the antigen-reagin ratio, although surface area and perhaps other factors play a role in causing these reactions. There are two general types of zone reactions as they apply to this test: in one the cause is an insufficient amount of antigen, and in the second it is due to an excess of reagin. In the majority of cases of type I zones, the phenomenon is readily recognized by the appearance of irregular aggregates. These clumps, varying in size from small to large floccules, are scattered in fields in which smaller clumps predominate. The correction of this deficiency is effected by adding a second drop of antigen suspension to the serum and re-rotating the slide for 4 minutes. If the addition of the second drop of antigen fails to increase materially the degree of flocculation, then the reaction is probably a type II zone. The second type of zone reaction is one seldom encountered, but more difficult to detect because of the almost complete inhibition of flocculation and, usually, there is also absence of irregular aggregation. Zone reactions of this type are corrected by preparing serial dilutions of the serum, such as 1:2, 1:4, 1:8, 1:16, etc., and proceeding as for routine testing. *Since zone reactions are frequently misinterpreted and at times not even recognized, it is recommended that the addition of a second drop of antigen be made a routine practice whenever weakly positive reactions are obtained.*

B. **Quantitative Test with Serum.**—The procedure for the quantitative test is exactly the same as for the routine test with the exception that each serum is tested in serial dilutions.

Set up 6 empty tubes, more if necessary, in a row in a rack. Place 0.5 c.c. of the heated serum in tube No. 1, add 0.5 c.c. of physiological saline solution, mix thoroughly, transfer 0.5 c.c. to tube No. 2, add

0.5 c.c. saline solution and continue the process until all the dilutions have been made.

The results may be reported in terms of actual quantity of serum tested, for example: 0.025 c.c., 4+; 0.0125 c.c., 4+; 0.0062 c.c., 4+; 0.0031 c.c., 2+; 0.0015 c.c., 1+; and 0.0007 c.c., negative; or they may be reported in terms of positiveness according to the highest dilution giving a positive reaction, such as positive up to a 1:16 dilution.

C. **Qualitative Test with Spinal Fluid.**—Spinal fluid should be tested as soon as possible after it is drawn. A grossly contaminated fluid is not dependable with this or any other test. It should not contain blood in appreciable quantity. The heating of spinal fluid is neither necessary nor desirable.

Rapid Test.—1. Centrifuge the fresh fluid at 2000 R.P.M. for 5 minutes, pour off the clear supernatant fluid into a clean tube.

2. With a 0.2 c.c. pipet graduated in hundredths place exactly 0.01 c.c. of 6 per cent acetic acid in one side of as many chambers of a glass slide as there are fluids to be tested. Accurate preparation of the acid solution and the amount delivered to each chamber is obviously necessary, for stronger concentrations or larger amounts will flocculate the suspension even in the absence of reagin.

3. With a 1 c.c. pipet deliver 0.1 c.c. of spinal fluid in the opposite side of the chamber from where the acid is located. Mix the acid and fluid evenly over the surface of the chamber with a wood applicator.

4. Rotate the slide holder with a circular motion for 1 minute. Thorough mixing of the acid and the fluid is essential.

5. Add one drop of the same antigen suspension, as is used for testing serum, to each chamber containing spinal fluid.

6. Rotate the slide holder for 10 minutes at 120 rotations per minute. The results are examined and recorded in the same manner as for the test with serum.

Concentration Test.—If a moderately positive (3+) or a strongly positive (4+) result is obtained with the acid test, no further testing is necessary; the weakly positive and negative reacting fluids are subjected to the concentration test. The concentrating apparatus consists of a hot air blower—the ordinary hair dryer is satisfactory and inexpensive—which has been clamped on a steel rod support.

Procedure:

1. Place about 50 c.c. of cold water in a petri dish.

2. Pipet 1.5 c.c. of spinal fluid into the bottom of a 50 c.c. beaker (4 cm. inside diameter).

3. Set the beaker in the center of the petri dish containing the water.

4. Place the blower at a distance of 2 cm. above the beaker and turn on the lever of the dryer to the "hot air" position.

5. Evaporate the fluid to a volume of 0.2 c.c. to 0.3 c.c. This is accomplished in 6 to 8 minutes.

6. Proceed to test the concentrated fluid in exactly the same manner as that used in the acid test.

D. **Quantitative Test with Spinal Fluid.**—Serial dilutions as those prepared for the quantitative test with serum are made and the acid test only is carried out.

C. Kahn Tests

I. APPARATUS

The use of standard apparatus in the performance of the various procedures is essential for correct results.

1. *Test tubes* for performing test (with serum and spinal fluid) are 7.5 cm. in length and 1 cm. in inside diameter.

2. *Vials* (with straight wall and flat bottom) for preparing antigen suspension are 5.5 cm. in length and 1.5 cm. in inside diameter.

3. *Pipets*, 10-c.c., graduated to 0.1 c.c.
   1-c.c., graduated to 0.01 c.c.
   0.5-c.c. (or 0.45 c.c.) graduated to 0.15 c.c.
   0.5-c.c., graduated to 0.025 c.c. (antigen suspension pipet)
   0.25-c.c., graduated to 0.0125 c.c. (antigen suspension pipet)
   0.2-c.c. graduated to 0.001 c.c.

4. *The test-tube rack* is made of suitable material (sheet copper, bakelite, and so forth); it is 3 inches wide, $11\frac{1}{2}$ inches long, and $2\frac{3}{4}$ inches high, and consists of three shelves, the upper and middle ones containing three rows of ten holes, each approximately $\frac{1}{2}$ inch in diameter. The center row of holes is offset $\frac{1}{2}$ inch.

5. The *standard shaking apparatus* has a speed of 275 to 285 oscillations per minute, with a stroke of $1\frac{1}{2}$ inches.

6. The *water bath* (56° C.), centrifuge and centrifuge tubes are of standard type.

7. *Washing of Glassware.*—Tubes and vials are rinsed twice in tap water and boiled for fifteen minutes in soft water containing a mild soap, such as "ivory" flakes. Then they are rinsed thoroughly in soft water and placed in cleansing solution for about twenty-four hours, after which they are rinsed three times in soft water and twice in distilled water. They are then inverted in wire baskets and dried thoroughly in an oven.

Pipets are rinsed by forcing soft and distilled water through them. They are placed in the cleansing solution[1] overnight, after which they are rinsed thoroughly, twice in soft water and twice in distilled water. The pipets are then placed in a slanting position in a wire basket and dried thoroughly in an oven. If the cleansing solution is not employed, the glassware will accumulate a layer of organic material.

The transfer of cleansing solution from one jar to another may be carried out by a siphon arrangement as follows: A rubber tube is attached to the suction arm of a water vacuum pump connected with a faucet. The far end of the rubber tube is lowered to the bottom of the jar which is to be emptied of cleansing solution. By starting a stream of water through the faucet, a vacuum is created within the rubber tubing and the cleansing solution is at once drawn up into the tubing. By detaching this tubing from the faucet and placing it in another jar, the acid will continue to flow into that jar. A thin, acid resistant rubber tubing is employed for this purpose; this enables one to see readily the cleansing solution rising within the tube.

Some laboratories report good results by washing tubes and vials with "calgonite." The tubes and vials are washed in hot distilled water containing 1 ounce of "calgonite" per 20 liters. This is followed by individual cleansing of each tube by a rotary brush and rinsing twice with soft and twice with distilled water.

II. REAGENTS

1. *Standard Kahn Antigen.*[2]—This antigen is employed in the performance of the standard (diagnostic) tests with serum and spinal fluid and in the quantitative tests with serum and spinal fluid; in special instances it also is employed in microtests. The antigen is

[1] *Preparation of cleansing solution (dichromic acid):*

Concentrated sulfuric acid	460 Gm. (252 c.c.)
Water	300 c.c.
Sodium dichromate	60 Gm.

Procedure.—(1) Mix water and $Na_2Cr_2O_7$ until dissolved. (2) Add H_2SO_4 *very* slowly, stirring constantly until the $Na_2Cr_2O_7$ is reprecipitated. Great caution must be taken in adding the sulfuric acid to the sodium dichromate solution. The solution becomes intensely hot and may boil over.

[2] Extract 25 Gm. of powdered beef heart with 100 c.c. of anesthesia ether for ten minutes with shaking. Filter. Repeat the extraction three times with 75 c.c. of ether; use fresh filter paper for the last filtration. Dry and weigh the residue. Extract this with 95 per cent ethyl alcohol (5 c.c. for each gram) for three days at room temperature. Filter and add cholesterol (6 mg. for each cubic centimeter) to the clear yellow alcoholic extract. This antigen kept at room temperature is used in the test.

standardized to the degree of specificity and sensitivity required for the standard Kahn tests.[1]

Standard antigen maintains the same titer for many years and remains uniform in specificity and sensitivity provided the following precautions are taken:

(*a*) Only chemically clean and dry glass vessels should be used for storing antigen and these should be properly stoppered, as evaporation of alcohol from the antigen will not only cause a change in the sensitivity of the antigen, but may cause the separation of cholesterol.

(*b*) Antigen should be kept at room temperature; not in the icebox or in the incubator. It should be stored in the dark, as in a cupboard. The antigen bottle in daily use might be kept in a mailing container to avoid undue exposure to light.

(*c*) Antigen should not come in contact with rubber or cork, because each substance contains alcohol-soluble elements which affect the specificity of the antigen. Such stoppers should be covered with a thin high grade tin foil.

2. *Serum.*—(*a*) It is essential that the serum employed in the test be entirely free from cells or particles of any kind, since these may give the impression of a precipitate in the completed test.

(*b*) It is important to adhere to sterile technic in obtaining blood from patients. The tube into which the blood is emptied should be chemically clean, dry and sterile. If the tube is agitated before a clot is formed, there will be a tendency toward hemolysis. After a clot is formed, this tendency is reduced to a minimum.

(*c*) It is well to break up the blood clot with wooden applicators before centrifugation. The inner wall of the tube is encircled with the applicators so as to remove completely the adhering blood clot. If the clot is contracted, it may be removed before centrifugation. The same applicator should *never* be used for more than one specimen of blood.

(*d*) Centrifugation at 2000 revolutions per minute for ten minutes is usually ample for the separation of the serum from the clot.

(*e*) The clear supernatant serum is either poured off into a clean tube or is drawn off with a bulb-capillary pipet designed to transfer

[1] The standardization requires three steps. (1) Titration of antigen to determine the minimal volume of salt solution to be added to a given volume of antigen. (2) The determination of sensitiveness of the antigen by testing its titer with syphilitic and nonsyphilitic serum by employing a standard antigen as a control. (3) The correction of antigen to standard requirements when the sensitiveness is not similar to that of standard antigen. Kahn, R. L.: The Kahn test, Baltimore, Maryland, Williams and Wilkins Co., 1928, chap. 4, pp. 83–110.

serum. If the same pipet is used for removing serum from different blood specimens, it should be rinsed at least six times in each of two containers of fresh salt solution and completely drained before using.

(*f*) The clear serum should be heated for thirty minutes in a water bath at a temperature of 56° C. before performing the desired test. The temperature of the water bath should *not* be 54°, 55°, or 57° C. The heating period should not be less than thirty minutes.

(*g*) Serums should be tested as soon as possible after being heated. For uniformity, it is well to begin the performance of the tests within two to five minutes after the serums have been removed from the 56° C. water bath. Serums that have been heated five to twenty-four hours previously should be reheated for ten minutes, when they are to be reexamined; if more than twenty-four hours have elapsed, they should be reheated for fifteen minutes.

(*h*) Serums showing some hemolysis or containing chyle or bile do not affect the correctness of the results of the Kahn test, but if they have undergone marked hemolysis or decomposition because of bacterial contamination, they are not fit for serologic tests.

3. *Physiologic Salt Solution.*—This solution consists of 0.9 per cent of sodium chloride in distilled water. The sodium chloride must be chemically pure and the solution must be filtered before using. Sterility is not essential, but if the solution is contaminated, it is not fit for use. It is well to prepare quantities of salt solution sufficient for two-week periods. This step will assure the use of relatively fresh salt solution.

III. STANDARD KAHN PROCEDURES

A. Standard (Diagnostic) Test with Serum

The standard test is a three-tube test, each tube containing a different proportion of serum and antigen suspension. Optimal precipitation is obtained when the concentration of antigen and that of the antibody (reagin) approximate each other. Hence, it is well to employ a relatively large, moderate and small quantity of antigen suspension with each serum, since the serum may contain a large, moderate or small amount of antibody. The use of three proportions of serum to antigen suspension makes it possible to obtain highly sensitive precipitation results with *standard antigen* which is moderately sensitive, but highly specific, antigen.

The following outline gives the general plan of the standard Kahn test with four different ranges in precipitation:

	Tube 1	*Tube 2*	*Tube 3*
Ratio of serum to antigen in suspension	3:1	6:1	12:1
Antigen suspension, c.c.	0.05	0.025	0.0125
Serum, c.c.	0.15	0.15	0.15

Types of Precipitation Reactions

Negative in the three proportions	–	–	–
Positive in the three proportions	++++	++++	++++
Positive only with the smaller amounts of antigen suspension	–	++	++++
Positive only with the larger amounts of antigen suspension	++++	++	–

1. *Preliminary Preparations for Test.*—Proper coordination and sequence of the various steps of the test, in relation to the number of specimens to be examined, are important for maintaining a high level of accuracy and efficiency. The racks should be set up, the tubes numbered and the pipets ready for measuring antigen suspension and serum. Antigen suspension is prepared as needed; hence, the amount prepared must conform to the number of tests to be made.

2. *Heating of Serum.*—The serum, which has been heated for half an hour at 56° C. is examined for the presence of particles. If particles are present, the serum is cleared by recentrifugation.

3. *Preparation of Standard Antigen Suspension.*—This suspension is prepared shortly before the serums are taken from the 56° C. water bath. Antigen is mixed with salt solution according to the required titer. Thus, if the titer is 1 c.c. antigen plus 1.2 c.c. physiologic salt solution, the antigen is mixed as follows: (*a*) 1.2 c.c. of salt solution is measured into a chemically clean and dry standard antigen suspension vial; (*b*) 1 c.c. of antigen is measured into a similar vial; (*c*) the salt solution is poured into the antigen, and as rapidly as possible (without waiting to drain the vial) the mixture is poured back and forth six times to insure thorough mixing; (*d*) the antigen suspension is allowed to stand for ten minutes before using. The suspension is not to be used after standing a total of thirty minutes from the time of mixing. An old antigen suspension is not to be mixed with a newly prepared suspension.

More than 1 c.c. of antigen may be mixed with a proportionately larger amount of salt solution. Thus, in case of an antigen of the titer previously mentioned, 2 c.c. of antigen may be mixed with 2.4 c.c. of salt solution and 2.5 c.c. of antigen may be mixed with 3 c.c. salt solution. Do not use amounts of antigen less than 1 c.c. nor more than 2.5 c.c. for the preparation of an antigen suspension.

4. *Measuring Antigen Suspension.*—The antigen suspension is shaken well, and 0.05 c.c., 0.025 c.c., and 0.0125 c.c. are measured for each serum, delivering the suspension to the bottom of the tubes. The standard rack capacity is thirty tubes; 0.05 c.c. is measured and placed in each of the tubes of the first row, 0.025 c.c. is placed in each of the tubes in the second row, and 0.0125 c.c. is placed in each of the tubes of the third row.

5. *Measuring Serum.*—The serum is added as soon as possible after the antigen suspension has been placed in the tubes, to avoid undue evaporation from the suspension. When examining large numbers of serums, it is well for one worker to measure the antigen suspension and for another to

measure the serums. It is necessary to limit the tests performed in each group to a number which will allow the addition of antigen suspension *and* serum to be completed within five minutes. *It is of the utmost importance to use a clean dry pipet for each serum.* Fifteen hundredths of a cubic centimeter of each serum is added to the 0.05 c.c., 0.025 c.c., and 0.0125 c.c. of antigen suspension, and the rack of tubes is shaken vigorously for ten seconds to insure thorough mixing of the ingredients. The serum-antigen mixtures are permitted to stand for three to five minutes at room temperature before they are shaken mechanically for three minutes (see 7).

6. *Controls.*—Set up for separate tests, one with a strongly positive serum, one with a weakly positive serum, one with a negative serum and a fourth in which salt solution (with antigen suspension) is employed instead of serum. The antigen suspension for these controls should be pipetted immediately after pipetting the antigen suspension for the tests, and the serum for these controls, immediately after the serums for the tests have been pipetted.

7. *Shaking.*—The tests and controls are then shaken in a standard shaking machine for three minutes. The machine oscillates 275 to 285 times per minute, with a stroke of $1\frac{1}{2}$ inches.

8. *Addition of Salt Solution.*—After the tests have been shaken, 1 c.c. of salt solution is added to each tube of the first row of the rack (containing 0.05 c.c. of antigen suspension) and 0.5 c.c. of salt solution is added to the remaining tubes. The rack is shaken sufficiently to mix the ingredients.

9. *Time of Reading Results.*—Results are read immediately after the addition of salt solution and a check reading is made fifteen minutes later, the racks remaining at room temperature during this interval. The final result is the average of the first and second readings.

B. Interpretation of Results of Standard Test

1. *Methods of Reading Results.*—Optimal reading conditions in each laboratory should be determined by trial. The following points will be found helpful: (*a*) When utilizing daylight for reading the tests it is well to have but one source of light coming from a single window immediately in front of the reader. It will be found satisfactory to shade the upper and lower portions of the window, narrowing the source of light to a section several feet in height. Light from any other windows near the reader should be dimmed by lowering the window shades. (*b*) When holding the rack in front of the exposed section of the window, the definitely positive and the negative reactions are readily differentiated without lifting the tubes from the rack. (*c*) In case of weak reactions each tube should be examined individually, lifting it several inches above the level of the eye and tilting it until the fluid is spread into a thin layer. The precipitate will then become readily visible.

Those preferring magnification will find the microscopic mirror helpful.

Place mirror on reading table with concave surface upward. Hold the tube in slanting position 2 to 3 inches above the mirror and examine the image in the mirror. Both daylight and artificial light may be employed. One may also utilize an ordinary hand lense for reading the tests. A twofold or threefold magnification will be found satisfactory. Some workers prefer the use of a slit-light arrangement, the source of light being an electric bulb enclosed in a box which is provided with a narrow slit.

As far as possible, workers should limit themselves to one method of reading. The occasional use of magnification by readers who usually do not resort to it will be likely to affect the uniformity of their reading scale. It should be emphasized that certain highly magnifying agglutinoscopes show particles in serum alone, and are thus unfit for use in this test. The magnification must be sufficiently low to assure opalescent and clear-cut negative reactions, with entire freedom from visible particles.

2. *Interpretation of Results.*—(*a*) Each of the three tubes of the test is read independently of the other tubes in the rack. In each tube, a definite precipitate suspended in a clear medium is read four plus or completely positive. Proportionately weaker reactions are read three, two, and one plus and plus-minus or doubtful, respectively.

(*b*) Strongly potent serums show complete or four plus precipitation in each of the three tubes, but owing to the different amounts of antigen suspension employed, the precipitates are unequal in bulk, being greater in the first tube and least in the last tube.

(*c*) Serums that are not strongly potent do not show complete precipitation in each of the three tubes. Such serums show most marked precipitation in the third tube because the small amount of antibody (reagin) reacts best with a small amount of antigen suspension. These serums generally show weak precipitation in the middle tube, which contains a moderate amount of antigen suspension, and no precipitation in the first tube, which contains a relatively large amount of the suspension.

(*d*) Another type of precipitation reaction is met with occasionally, namely, one in which precipitation is marked in the first tube, and weak or negative in the second or third tube. In this instance, the serum generally is so markedly rich in antibody that it requires a relative excess of antigen suspension to produce maximal precipitation. When a reaction of this type is met with, it is necessary to set up a supplementary test in which the amount of antigen suspension in relation to serum is increased beyond that employed in the standard test. A supplementary test is set up in which 2:1 and 1:1 proportions of serum to antigen suspension are used, thus:

	Tube 1	*Tube 2*
Antigen suspension, c.c.	0.02	0.02
Serum, c.c.	0.02	0.04
Shake tests three minutes		
Salt solution, c.c.	0.3	0.3

These two tubes, or at least tube 2, should show definite precipitation reactions if the serum is strongly positive.

As an additional check on serums giving precipitation reactions in the first tube of the standard test and negative reactions in the remaining two tubes, a second supplementary test is made by setting up a partial quantitative test. Thus, dilute the serum 1:5, 1:10, and 1:20 with salt solution and test each dilution with antigen suspension in a proportion of 15:1 in accordance with the following outline:

	Tube 1	*Tube 2*	*Tube 3*
Antigen suspension, c.c.	0.01	0.01	0.01
Diluted serum, c.c.	0.15 (1:5)	0.15 (1:10)	0.15 (1:20)
Shake tests three minutes			
Salt solution, c.c.	0.5	0.5	0.5

If one or more tubes should show a definite precipitation reaction the serum can be considered positive.

If these supplementary examinations do not show positive reactions, then the three-tube test which shows marked precipitation in the first tube and negative precipitation in the remaining two tubes must be considered a weak or doubtful reaction.

Rarely, one finds that the three-tube tests show a borderline precipitate in each of the three tubes, such as ±, ±, ±; +, +, +, or perhaps ++, ++, ++. After ascertaining that these borderline reactions are not due to a serum precipitate, the two supplementary tests which have been described are carried out. If these two tests show definite precipitation, the results are reported as positive. If the results of supplementary tests are also borderline or questionable, the chances are that one is dealing with nonspecific precipitation owing to conditions other than syphilis.

3. *Recording Results.*—A permanent record must be made of findings in all tubes of each test at the time of reading. All tests should be read independently by two workers. When two workers are not available, the original reading should be checked by the same worker after an interval of fifteen minutes.

4. *Reporting Results to Physicians.*—The final results are reported to physicians as either *positive, doubtful,* or *negative.* In view of the fact that Kahn reactions are read on a plus-sign basis, it is recommended that:

A total of six pluses to twelve pluses in the three tubes be reported as *positive.*

A total of two and one half pluses to five and one half pluses inclusive be reported as *doubtful.*

A total of two pluses or less be reported as *negative.*

The following tables illustrate the method of reporting the results, based on the reactions in the individual tubes. (A ± reaction is considered as one half in totaling.)

TABLE A

TYPES OF REACTIONS INTERPRETED AS POSITIVE (SIX PLUSES TO TWELVE PLUSES)

	Tube 1	*Tube 2*	*Tube 3*
1.	++++	++++	++++
2.	+++	++++	++++
3.	++	++++	++++
4.	+	++++	++++
5.	±	++++	++++
6.	−	++++	++++
7.	−	+++	++++
8.	−	++	++++
9.	++++	++++	+++
10.	++++	++++	++
11.	++++	++++	+
12.	++++	++++	±
13.	++++	++++	−

TABLE B

TYPES OF REACTIONS INTERPRETED AS POSITIVE PROVIDED SUPPLEMENTARY EXAMINATIONS 1 AND 2 (SEE TEXT) ARE POSITIVE

	Tube 1	*Tube 2*	*Tube 3*
1.	++++	++	−
2.	++++	+	−
3.	++++	−	−
4.	++	++	+
5.	+	+	+
6.	±	±	±

TABLE C

TYPES OF REACTIONS REPORTED AS DOUBTFUL (2½ TO 5½ PLUSES)

	Tube 1	*Tube 2*	*Tube 3*
1.	±	++	+++
2.	−	+	++
3.	−	±	++

TABLE D

TYPES OF REACTIONS REPORTED AS NEGATIVE (2 PLUSES OR LESS)

	Tube 1	*Tube 2*	*Tube 3*
1.	−	+	+
2.	−	±	+
3.	−	±	±
4.	−	−	±
5.	−	−	−

5. *Repeated Examinations.*—If the slightest doubt exists as to the correctness of results owing to some error in technic, the specimen should be reexamined, and if no serum is available, another specimen should be requested.

6. *General Consideration with Regard to Reading.*—Some workers occasionally become so "sensitized" in reading results that they begin to see precipitates in tubes in which no precipitates exist. Readers of tests should become thoroughly familiar with the general appearance not only of negative serum-antigen suspension mixtures, but of negative serum alone. Under conditions of sufficient magnification these will begin to show evidence of the presence of very fine particles generally shown by colloidal solutions. The expert reader should be in a position to distinguish at once a specific precipitate from dust particles and from fibers or particles that are due to magnification.

STANDARD (DIAGNOSTIC) TEST WITH SPINAL FLUID

In this test, the globulins of the spinal fluid are precipitated by means of ammonium sulfate and are redissolved in an amount of physiologic salt solution equivalent to one tenth of the original volume of spinal fluid. The concentrated globulin solution thus obtained is then tested with standard antigen suspension.

1. *Preparation of Concentrated Globulin Solution Reagents.*—The material and reagents needed for the preparation of concentrated globulin solution are: (1) spinal fluid, (2) physiologic salt solution, and (3) a saturated solution of ammonium sulfate of the highest purity.

Procedure.—(*a*) Spinal fluid is centrifuged to render it free from cells and foreign particles.

(*b*) One and five tenths c.c. of the clear fluid is added to a standard Kahn test tube (7.5 by 1 cm.).

(*c*) To the same tube is added 1.5 c.c. of a saturated solution of ammonium sulfate.

(*d*) The fluids are mixed by covering the mouth of the tube with the thumb (protected with rubber), and shaking tube back and forth vigorously. The mixture is placed in a 56° C. water bath for fifteen minutes to hasten the precipitation of the globulins.

(*e*) The mixture is centrifuged at high speed for fifteen minutes to throw down the precipitated globulins completely.

(*f*) The supernatant fluid is removed as *completely as possible* with a finely drawn capillary pipet. An optional method for removing the supernatant fluid is to pour off the fluid and invert the tube in a standard rack over clean filter paper, allowing the moisture that adheres to the tube to drain and be absorbed by the paper for a period of ten minutes.

(*g*) After thorough draining, 0.15 c.c. of salt solution is added to the precipitate which is redissolved readily by gentle shaking. In adding this salt solution, the point of the pipet is held close to the bottom of the tube to avoid washing down the ammonium sulfate adhering to the inner wall.

(*h*) This globulin solution is now ready to be tested with antigen suspension.

2. *Preparation of Antigen Suspension.*—Salt solution is mixed with antigen in the same manner as for the standard test with serum, according to the antigen titer required for spinal fluid. The antigen suspension is allowed to stand ten minutes and must be used in the test within the next twenty minutes.

3. *Measuring Antigen Suspension.*—With a 0.2-c.c. pipet graduated to 0.001 c.c., 0.01 c.c. of antigen suspension is placed in the bottom of a standard Kahn test tube.

4. *Measuring Concentrated Globulin Solution.*—With a 0.2-c.c. pipet 0.15 c.c. of concentrated globulin solution is placed in the tube containing the antigen suspension. Tests are shaken vigorously for ten seconds to mix ingredients.

5. *Controls.*—Positive and negative spinal fluid controls are included; each concentrated globulin solution also is observed to establish that it is free from foreign particles.

6. *Shaking.*—After mixing the concentrated fluid with antigen suspension, the test is shaken at the standard speed for *four* minutes.

7. *Addition of Salt Solution.*—Normal salt solution (0.5 c.c.) is added to the tube.

8. *Reading Results.*—Four plus, three plus, and two plus reactions are reported as positive; one plus reactions are reported as doubtful and plus-minus and negative reactions are reported as negative.

9. *Check Examinations.*—Each test on spinal fluid should be performed in duplicate. Hence, the amount of spinal fluid required for a test is a little more than 3 c.c.

IV. SPECIAL KAHN PROCEDURES

A. Presumptive Test with Serum

The presumptive test is an auxiliary method to the standard Kahn test. The main difference between the two methods is that the presumptive test is more sensitive than the standard test. This increased sensitiveness is due to the fact that, in the presumptive test, sensitized antigen is employed, which is more sensitive than standard antigen. The test was named "presumptive" because it may give false positives; hence, when it is employed for diagnosis, the reactions should be considered as presumptive in character. The presumptive test is of value: (*a*) as a technical check on the standard test, (*b*) in cases of syphilis in which a highly sensitive method is desired after treatment has been employed, (*c*) as an additional criterion in establishing the absence of syphilis.

Sensitized Kahn Antigen.—This antigen is employed not only in the performance of the presumptive tests with serum and spinal fluid but also in special instances, in the quantitative tests with serum and spinal fluid. Statement (*b*) on page 691, relative to standard antigen, applies also to sensitized antigen.

The following outline describes the performance of the presumptive test with serum.

1. *Preparation of Antigen Suspension.*—(*a*) Pipet 1 c.c. of sensitized antigen into an antigen suspension vial. (*b*) Pipet an amount of physiologic salt solution, indicated by the titer of the antigen, into a similar vial. (*c*) Pour the salt solution into the antigen vial and, as rapidly as possible, pour the mixture back and forth approximately six times. (*d*) Allow the antigen suspension to stand ten minutes at room temperature before using. Set up control tests as outlined under "Standard test with serum" paragraph 6, page 694.

2. *Measuring Antigen Suspension.*—Measure 0.025 c.c. of the thoroughly mixed antigen suspension into a standard tube (7.5 cm. in length, 1 cm. in diameter) with a pipet graduated to 0.025 c.c. amounts, delivering the suspension to the bottom of the tube.

3. *Measuring Serum.*—Add 0.15 c.c. of serum, after it has been heated for thirty minutes at 56° C., and mix the serum with the antigen suspension by shaking the rack vigorously by hand for about ten seconds.

4. *Shaking.*—Shake rack in the usual manner for three minutes in a shaking apparatus.

5. *Adding Salt Solution.*—Add 0.5 c.c. of physiologic salt solution to the tube and examine for presence of a precipitate.

6. *Interpretation of Results.*—Complete precipitation ++++ or +++ is interpreted as positive; a moderate precipitation reaction, such as ++, is interpreted as doubtful, while very weak reactions, such as +, ±, are classed with the negative reactions.

7. *Serum Control.*—Examine each serum for foreign particles which might give the appearance of a specific precipitate. Particularly in the case of each positive reaction, it is essential to determine that the serum used in the test is free from foreign particles. In using the presumptive test as a check on the standard Kahn test, the same serum control is, of course, sufficient for both methods.

B. Presumptive Test with Spinal Fluid

The presumptive test with spinal fluid is carried out exactly as the standard test, except that sensitized antigen is employed instead of standard antigen. With sensitized antigen, the spinal fluid test is more sensitive than with standard antigen and is believed to possess a relatively high degree of specificity.

In many laboratories, a spinal fluid test with sensitized antigen is employed routinely as a check on the spinal fluid test with standard antigen, although reports to physicians are based on the results given by the standard test.

C. Quantitative Test with Serum

The standard test is essentially qualitative in nature. When the reaction is positive no indication is obtained as to the relative potency of the serum. Two serums giving positive reactions may vary greatly in their potency. The extent of this variation may be readily determined by means of the quantitative test with serum. The test consists of two steps. Positive serums are first diluted, in series, with salt solution. Each dilution of serum is then tested with standard antigen suspension. The highest dilution giving a positive precipitation reaction is the end-point desired.

Some workers prefer the use of standard antigen in the quantitative test, others prefer the use of sensitized antigen. It is important to be consistent and to limit oneself to the use of either one or the other in reporting the results of the test to physicians. Sensitized antigen will give different quantitative results than will a standard antigen, hence, these antigens should not be confused in routine quantitative examinations.

1. *Dilution of Positive Serum with Salt Solution.*—A series of dilutions of serum with physiologic salt solution is prepared so that the ratio of the volume of diluted serum to the volume of serum before dilution ranges from 5 (1 part serum: 4 parts salt solution) to 60 (1 part serum: 59 parts salt solution). The following scheme is employed:

Dilution number.	Dilution ratio.
(1)	1 = 0.2 c.c. of undiluted serum.
(2)	5 = 0.2 c.c. of undiluted serum plus 0.8 c.c. of salt solution.
(3)	10 = 0.7 c.c. of (2) plus 0.7 c.c. of salt solution.
(4)	20 = 0.2 c.c. of (3) plus 0.2 c.c. of salt solution.
(5)	30 = 0.2 c.c. of (3) plus 0.4 c.c. of salt solution.
(6)	40 = 0.1 c.c. of (3) plus 0.3 c.c. of salt solution.
(7)	50 = 0.1 c.c. of (3) plus 0.4 c.c. of salt solution.
(8)	60 = 0.1 c.c. of (3) plus 0.5 c.c. of salt solution.

2. *Performance of Test.*—The dilutions of serum being available, the antigen suspension is prepared in the usual manner. After it has stood for ten minutes 0.01 c.c. is placed in each of eight standard test tubes; the suspension is deposited at the bottom of the tubes with a pipet. With an appropriate pipet, 0.15 c.c. of the seven dilutions of serum are added in order, beginning with the highest dilution (8), to the tubes containing the suspension of antigen. The racks are shaken for three minutes in the usual manner; 0.5 c.c. of salt solution is added to each tube and the results are read.

3. *Determination of Kahn Units.*—A definite precipitate (++++, +++ or ++) is recorded as *positive*, while a very weak reaction is con-

sidered *negative*. If a serum gives a ++++, +++ or ++ reaction only in an undiluted state and is negative in the dilution series, it is considered as containing Kahn units as indicated by the plus signs (4 units, 3 units or 2 units, respectively). The potency of any serum which is positive on dilution is determined according to the formula S = 4 D, where S is the serum potency in terms of Kahn units and D is the highest dilution ratio giving a positive (++++, +++ or ++) reaction. Thus, if a dilution of 1:5 is positive and a dilution of 1:10 or higher is negative, the serum contains 5 times 4 or 20 Kahn units. If a dilution of 1:10 is positive and a dilution of 1:20 or higher is negative, the serum contains 40 units.

4. *Reporting Results.*—Serums showing four or more units are reported "positive, 4 Kahn units" or "positive, 40 Kahn units," as the case may be.

5. *Highly Potent Serums.*—If a serum gives a positive precipitation reaction with a dilution ratio of 60, still higher dilutions of serum are examined with antigen suspension until a positive reaction is no longer obtained. Higher dilutions are readily prepared by resorting to tube 3 in the dilution series (1:10 dilution) of which an excess is prepared.

D. Quantitative Test with Spinal Fluid

This method consists in first establishing that a given spinal fluid is positive by the standard technic and then determining the potency of the spinal fluid by serial dilutions of the spinal fluid globulin with salt solutions, as in the case of the quantitative test with serum. Details of these dilutions and the interpretation of the results are given in the "Kahn Test—A Practical Guide." The tests are shaken for four minutes; 0.5 c.c. of salt solution is added and results are read.

E. Microtests and Other Procedures

If there is insufficient serum for the regular three-tube test, the examination and report are made as follows:

1. If 0.21 c.c. of serum is available, a three-tube test is performed, employing smaller quantities of serum and antigen suspension than in the standard test, but maintaining the same 3:1, 6:1, and 12:1 proportions of serum-antigen suspension. Thus, 0.01 c.c. of standard antigen is measured into each of three tubes, and 0.03 c.c., 0.06 c.c., and 0.12 c.c. of serum are added, respectively, after which the steps of the diagnostic test are followed, except that only 2 to 3 drops of salt solution are added to the completed test before the reading is made.

2. If less than 0.21 c.c. of serum is available, a one-tube test (microtest) is made by employing ten parts of serum to one part of antigen suspension. Thus, if 0.1 c.c. of serum is available it is employed with 0.01 c.c. of antigen suspension. If 0.05 c.c. of serum is available, it is employed with 0.005 c.c. of antigen suspension. These reactions are reported as microtests.

3. Dependable serologic results may be obtained with fluid from chancres and other syphilitic lesions. After cleansing the area with physiologic salt solution, and under moderate pressure, fluid from chancres or other lesions is collected by means of a fine capillary pipet. The material is deposited at the bottom of a small agglutination tube and centrifuged to throw down cellular material. The clear supernatant fluid is mixed with standard antigen suspension in the proportion of 10:1. If 0.03 c.c. of fluid is available, it is mixed with 0.003 c.c. of antigen suspension. The tests are shaken for three minutes and 2 drops of salt solution are added before reading the results. A definite precipitate is read as *positive*, while freedom from a precipitate is read as *negative*. If it is desired to make a Kahn test with aqueous humor, it is necessary to employ the same technic as for the spinal fluid test. The aqueous humor is treated with an equal amount of saturated solution of ammonium sulfate and a test is made with the globulin concentrate.

D. Hinton Test (Third Modification[1])

This test requires precision in execution, because consistently accurate results cannot be obtained if minor variations in technic are allowed.

Equipment.—1. Test tube racks. To simplify numbering and pipetting serums, these racks should be constructed to hold ten or twenty tubes in a row.

2. Serum tubes 100 mm. long, with an approximately uniform inside diameter of 10 mm.

3. A water bath for inactivating serums.

4. A Wassermann bath or a bacteriologic warm air incubator. The former is preferred, because by its use the test is somewhat more sensitive. The water in the bath should be changed frequently to prevent a deposit from sticking to the outside of the tubes.

5. A centrifuge with a speed of more than 2000 revolutions per minute.

6. A maximal and minimal thermometer.

7. Graduated 100- and 250-c.c. cylinders to measure the reagents.

8. Dropping pipets with rubber bulbs of about 5-c.c. capacity, for drawing off serums.

9. Serologic pipets of 1-c.c. capacity, graduated in 0.1 c.c. to the tip, to measure serums, and 5- or 10-c.c. serologic pipets to measure reagents.

[1] Hinton, W. A.: Hinton Test for Syphilis; Third Modification, Jour. Lab. and Clin. Med., *18*:198–205 (Nov.), 1932.

10. Thick-walled Erlenmeyer flasks (Fig. 304) of 125- or 250-c.c. capacity, with an inverted V-shaped ridge blown into the bottom, for mixing glycerinated indicator. This ridge produces two semicir-

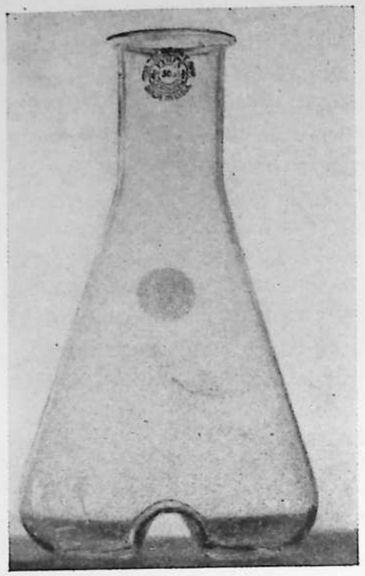


Fig. 357.—V-bottom flask for preparation of Hinton antigen.

cular compartments, each of which holds 3 to 5 c.c. in flasks with a capacity of 125 or 250 c.c.

PREPARATION OF STOCK SOLUTIONS

Stock Indicator.—Extract dried, ground, beef-heart muscle (Bacto-Beef Heart, Dehydrated, Difco Laboratories) by putting 100 Gm.[1] of the powder and 400 c.c. of ether (anesthesia) in a wide-mouthed, glass-stoppered bottle and shaking thoroughly by hand for ten minutes. Allow the bottle to stand five to ten minutes so that the solid material may settle out. Then pour as much of the ether as possible through filter paper into an Erlenmeyer flask, without pouring out any large quantity of the solid material. Scrape the solid material from the filter paper into the bottle for further extractions. Do not allow the main portion of extracted tissue to dry between extractions. Discard the filtrate in the Erlenmeyer flask. Make five separate extractions in all, using 400 c.c. of fresh ether and a new filter paper for each. After the final extraction, let the tissue dry on the filter paper. Obtain

[1] The extraction of larger or smaller amounts of the powder in one operation has appeared to yield an inferior reagent.

the net weight of this dried residue of ether-insoluble constituents. Place this dried residue in a glass-stoppered bottle with 95 per cent ethyl alcohol, using 5 c.c. of alcohol to each gram of residue. Extract for three days at room temperature (17° to 20° C.), shaking the contents of the bottle vigorously by hand for five minutes three times each day. Remove the tissue by filtering into a graduated cylinder; measure the alcoholic extract, and transfer to a glass-stoppered bottle. Add cholesterol (using 0.4 Gm. to each 100 c.c.), and warm at 37° C. in an incubator or water bath, occasionally shaking, until the cholesterol has dissolved. A solution thus prepared is called "stock indicator" (antigen).

Warning is given that the best results can be assured only if a stock indicator as prepared according to the given directions has proved to be accurate in comparative tests with an indicator that is wholly satisfactory, as determined by both clinical and serologic means. Materials designated for this test by the Difco Laboratories of Detroit, Michigan, have met this critical requirement.

The stock indicator should not be stored in a refrigerator, for chilling will precipitate the cholesterol; if, by inadvertent chilling, the cholesterol should precipitate, it must be redissolved (by heating in a water bath at 37° C.) Stock indicator kept in colorless, glass-stoppered bottles at room temperature for more than two years has given as good results as that freshly prepared.

Five Per Cent Salt Solution.—Prepare a 5 per cent solution of sodium chloride (C. P.) in sterile distilled water, and add 1 Gm. of salicylic acid (C. P.) to each 4500 c.c.

The salicylic acid helps to preserve the potency of glycerinated indicator (which will be described later).

Fifty Per Cent Solution of Glycerol.—Prepare by mixing equal volumes of Baker and Adamson's Glycerin (reagent)[1] and sterile distilled water.

The 5 per cent salt solution and the 50 per cent glycerol solution keep indefinitely.

Preparation of Glycerinated Indicator.—Directions for mixing this should be followed strictly. Thirty c.c. is the smallest, and 150 c.c. is the largest, amount that can be satisfactorily prepared at one time. If larger quantities are required, two or more batches should

[1] This is an especially pure redistilled glycerin.

be pooled. *If kept in a refrigerator at a temperature of about 8° C., it will remain unimpaired in strength one month and sometimes longer.* This stability of the glycerinated indicator is extremely advantageous for emergency work and for small laboratories.

Prepare the Glycerinated Indicator as Follows: Pipet 1 part of the cholesterinized heart extract into one compartment of the Erlenmeyer flask (with the inverted V-shaped ridge), and 0.8 part of the 5 per cent salt solution into the other.

Use great care, when pipetting the salt solution into the flask, to avoid admixture of the two solutions. A 125-c.c. flask is suitable for the preparation of 30 to 60 c.c. of glycerinated indicator, and a 250-c.c. flask for preparation of 90 to 150 c.c.

Mix by shaking the flask very rapidly from side to side for one minute. Let the mixture stand exactly five minutes. Without further delay add 13.2 parts of the 5 per cent salt solution and shake thoroughly. Finally, add 15 parts of the 50 per cent glycerol solution and shake until the suspension is homogeneous.

PERFORMING THE TESTS

1. Centrifuge the blood, if necessary, to aid in separating the serum from its clot; with a long dropping pipet remove the serum (free from blood cells) and deliver into an appropriately labelled serum tube.

To avoid contamination of one serum by another, after each serum has been drawn off, thoroughly rinse the dropping pipet at least three times with sterilized physiologic salt solution. To minimize bacterial contamination, after every twenty serums have been drawn off, empty the washing bottle (about 200-c.c. capacity) and fill it with fresh salt solution.

2. Heat the serums in the inactivating bath at 55° C. for thirty minutes. Be sure that the level of the water is above the level of every serum and that the temperature is kept at 55° or 56° C. throughout inactivation. Errors may result if this varies even 1° or 2° C. Serums should be inactivated on the day of testing. If it is necessary to retest a specimen, use serum freshly separated from the clot.

3. Select all serums that show (*a*) hemolysis, manifested by redness greater than is produced when 0.1 c.c. of blood is dissolved in 3 c.c. of distilled water; (*b*) bacterial contamination, shown by cloudiness; or (*c*) marked opacity from other causes. Place in a separate rack and test, as soon as possible, according to the rapid method (p. 709). This will avoid further deterioration, which by decreasing the sensitiveness of the test and increasing the capacity of the medium makes the test difficult to read.

4. Set up the racks with one properly numbered serum tube for each remaining specimen. Tubes should be clear and clean.[1]

5. With a 1-c.c. pipet, measure 0.5 c.c. of each heated (inactivated) serum into the tube that has been labelled for it. (Use a separate pipet for each serum.)

For routine purposes one tube is sufficient for each test. If, however, the result of this test is negative and there is reason to suspect syphilis it is desirable to retest the specimen by using 0.1 c.c. in one tube and 0.5 c.c. in the other. We have found that in approximately 1 of every 200 cases of syphilis the second tube, containing the 0.1 c.c. of serum, gives a positive reaction, while the first, which contains the 0.5 c.c. of serum, gives a negative reaction.

If less than ten tests are to be made at one time, use positive and negative controls.

6. Compare the appearance of each pipetted serum with that in the tube from which it was taken. This is to make sure that there has been no error in pipetting or in labelling the tubes.

7. Not more than thirty minutes before incubation, add 0.5 c.c. of glycerinated indicator to each serum with a clean, 10-c.c. pipet.

8. Pipet 0.5 c.c. of the same indicator and 0.5 c.c. of the 5 per cent salt solution into an empty serum tube. This serves as a control, the use of which will be indicated later (p. 708).

9. Incline the rack to an angle of about 45 degrees and then shake by thrusting it quickly upward and forward, then downward and backward with sufficient speed to cause the fluid to travel halfway up the tubes and make small bubbles. At least three minutes of shaking are required for accurate results. If there are enough tests, a shaking machine is desirable.

10. Place the rack in the Wassermann bath or incubator at 37° C. and let it remain for sixteen hours (conveniently from 5 P. M. to 9 A. M.), or in the warm air incubator for eighteen hours. Longer incubation makes the tests increasingly hard to read. Do not agitate the tubes before reading.

When the racks containing the tubes are removed from the bath or incubator, record the readings shown by the bath or incubator thermometer as well as those of the maximal and minimal ther-

[1] To clean the tubes, rinse them thoroughly, as soon after use as possible, with tap water, and then fill each tube with a warm solution of 5 Gm. of sodium hydroxide in 1000 c.c. of tap water; allow them to stay in this solution for about two hours and wash thoroughly with hot water to remove the alkali. This process usually removes (without the aid of a test-tube brush) any deposit which may have remained from previous use.

mometer. The temperature should not fall below 34° C. nor rise above 39° C.

Reading the Tests.—The tests should be read within an hour after the incubation has ended. To read them, sit in front of a window, but do not face the sunlight. (The light must be good, and for this reason suitable artificial light must be provided on dark days or at night.) In order to determine whether or not clearing of the fluid has occurred and a ring or band of white flakes or white coarse granules is present at the meniscus, lift each tube carefully from the rack, hold it at the level of the eye, tilt it at an angle of about 45 degrees, and view it in the direction of a darkened background on either side of a window or of a suitably placed light. While still viewing it at the same angle, slowly roll the tube between the fingers; this will make a faint ring visible. Finally, gently shake the tube and look for flocculation, which is evidenced by agglutination masses or by faint granularity.

The reactions are read and reported as positive (without indicating the intensity of the reaction), negative, doubtful, and unsatisfactory.

Such reports do not confuse physicians by implying that the intensity of the reaction is related to the clinical condition of the patient.[1]

Positive Tests (*Recorded* +).—At or a little above the level of the meniscus there is a ring or band, approximately 0.2 to 1.5 mm. wide, of white coarse granules or flakes of lipoids, slightly to moderately, but not strongly, adherent to the wall of the tube.[2] By gently shaking the tube, the ring or band is loosened and the particles scattered so that they are visible as agglutinated masses in a clear fluid, or as somewhat coarse granules in a cloudy field.

Negative Tests (*Recorded* —).—Usually there is at most only slight clearing, but no ring, band or floccules. However, with some indicators, the negative reactions may show a "scummy" nongranular deposit of lipoids on the wall of the tube, extending from the meniscus downward for from 1 to 3 mm.

Doubtful Tests (*Recorded* ±).—Centrifuge from ten minutes at high speed (about 2000 revolutions a minute) (1) those tubes which on gentle shaking showed only slight granularity beyond that observed in the control tube (containing 0.5 c.c. of indicator and 0.5 c.c. of the 5 per cent salt solution) and (2) those tubes which showed only a slightly flaky or slightly granular ring. If, as a result of the

[1] Hinton, W. A.: Syphilis and Its Treatment, New York, The Macmillan Company, 1936, p. 28.

[2] Hemolyzed serums that are also bacterially contaminated frequently produce a whitish ring, which is strongly adherent to the tube.

centrifuging, there is definite clearing, and on top a thin layer of lipoids which shaking breaks up into fine flakes or coarse granules, the result of the test is recorded as ± and is reported as "doubtful"; if these changes have not taken place, it is reported as "negative."

RAPID METHOD

This procedure may be used whenever there is urgent need for a quick report: for example, in testing donors for transfusions, and in the diagnosis of suspected primary or secondary syphilis. Although this method has not been officially evaluated it is, according to the experience of Hinton, from 98 to 99 per cent as accurate as the regular method.

Technic of Rapid Method.—Treat the specimens selected under No. 3 (p. 706) as indicated under directions 4, 5, and 6 (p. 707). Then add 0.5 c.c. of the glycerinated indicator to each tube and shake according to direction 9 (p. 707); place in Wassermann bath at 37° C. for twenty minutes or in a warm air incubator at 37° C. for one hour; next, centrifuge at about 2000 revolutions a minute for ten minutes, read and record as follows:

Positive (*recorded* +), if there are plainly visible flakes at the top of the fluid, and a well-marked flocculate precipitate is seen on shaking.

Unsatisfactory, if the specimen is hemolyzed or bacterially contaminated, unless the reaction is strongly positive. This interpretation is necessary, because even the moderately hemolyzed or bacterially contaminated serum of a known syphilitic patient usually gives a negative reaction.

Negative (*recorded* —), if centrifuging has caused no change, provided the specimen shows no decided hemolysis (see under 3 on p. 706) or no marked bacterial contamination.

Tubes that show only very fine floccules or none at all after the centrifuging should be shaken well and placed in the water bath for sixteen hours at 37° C. or in the incubator for eighteen hours. after which the reading and interpretation are made as if the tests had been conducted in the routine manner.

DAVIES-HINTON MICROFLOCCULATION TEST

In order to facilitate carrying out this test, specimens of blood should be collected in special rubber-capped glass collection tubes which are then placed in labelled glass tubes that are approximately 100 mm. long and 15 mm. in diameter, such tubes being ordinarily

called "Wassermann tubes." The collection tubes are made of glass tubing and are 80 mm. long by 2.5 to 3 mm. in inside diameter. Over each end fits a flexible rubber cap such as is used to stopper small bottles of vaccine or serum. The capacity of this tube is about 0.3 c.c.[1]

Laboratory Procedure.—Number the Wassermann tubes containing the specimens and place them in a suitable rack. Take each specimen in its collection tube from the rack, remove the rubber cap from one end, and loosen the clot from the wall of the tube with the stylet of an 18-gauge needle or with some other stiff wire. Replace the rubber cap on the collection tube and return it to the numbered Wassermann tube; centrifuge at high speed for ten minutes. Examine each specimen so handled to see if the serum is clear and well separated from its clot; if the serum is not clear, recentrifuge for another ten minutes, and if this still does not clear it, the specimen is unsuitable for the test. Place the rack containing the clear specimens (clot downward) in a suitable serologic water bath for thirty minutes at 56° C., having first filled the Wassermann tube with water from the water bath. Next, remove the rack from the water bath, pour the water off the tubes by inclining the rack, remove the cap from the serum end of each collection tube and notch it just above the junction of clot and serum with a file such as is used to open an ampule of arsphenamine. Hold the collection tube horizontally, break it and discard the part of the tube that contains the clot. Handle the part with the clear serum as follows:

1. Drain into one glass tube (similar to the collection tube previously described) enough serum so that the length of the column of serum is about 2.5 cm., and drain into a second similar glass tube enough serum so that the column is 0.5 to 1 cm. long.

2. With a capillary pipet, add to the first tube glycerinated Hinton indicator equal to the amount of serum in the tube, as estimated by the combined length of the column of serum and indicator.

The capillary pipet consists of a piece of glass tubing about 10 or 12 cm. long and 1 cm. in diameter; one end is tapered to a capillary tip about 1 mm. in diameter. The other end is capped with a rubber bulb. Provided no serum is drawn into the capillary pipet, it need not be rinsed. Care should be taken not to allow air to separate the serum and Hinton indicator, which would prevent mixing.

3. To the second tube, with the same capillary pipet, add diluted Hinton indicator equal to about five times the amount of the serum, as estimated by the combined length of the column of serum and indicator.

4. Mix the serum and the Hinton indicator in each tube by tilting the liquid toward alternate ends of the tube ten or fifteen times.

5. Cap both ends of each tube, return the tubes to the numbered Wassermann tubes in the rack and immerse in a 37° C. water bath for sixteen hours, having first filled the Wassermann tubes with water from the water bath.

6. Remove the rack from the water bath and centrifuge the tubes for five minutes at approximately 2000 revolutions per minute.

7. Read the results with the low-power objective of a microscope. The light should be cut down by lowering the condenser so that aggregates at the meniscus can be seen readily. Moreover, the stage of the microscope should be tilted at an angle of about 30 degrees and the tube placed under the lens so that the meniscus is uppermost. Readings are designated as follows:

Positive, if there are definite, discrete, compact clumps at the meniscus. Gentle thumping of the tube may help float the clumps into view.

Doubtful, if there are a few small clumps at the meniscus. In such cases, the clumps should be broken up by thumping the tube with a finger and the tube should be centrifuged for three minutes. The test is reported "doubtful" if small clumps are again visible at the meniscus, and "positive," if large, compact clumps are present.

Negative, if there are no clumps at all. Amorphous, cloudy material at the meniscus should be disregarded.

The technic that has been described requires about 0.1 c.c. of serum; smaller amounts of serum (0.05 c.c. or more) may be handled in the same way, in capillary tubes that are 10 or 11 cm. long and 1.25 to 1.5 mm. in inside diameter, except that the tubes are sealed with a small gas flame instead of rubber caps. Inactivation at 56° C. may be reduced to twenty minutes, and incubation at 37° C. may be reduced to thirty minutes without apparent loss of accuracy.

[1] Davies, J. A. V.: A Microflocculation Test for Syphilis, Jour. Lab. and Clin. Med., *22*:959–966 (June), 1937.

DAVIES-HINTON FLOCCULATION TEST OF CEREBROSPINAL FLUID[1]

Although this test has not been officially evaluated, it has in the experience of the originators proved to be far easier to perform and more efficient than the Wassermann and the flocculation tests with which it has been compared. At present it is being used by the Massachusetts Department of Public Health.

[1] Davies, J. A. V.: A Modification of the Hinton Test Applied to Spinal Fluid, Am. Jour. Clin. Path., *7*:240–245 (May), 1937.

Specimens of spinal fluid for this test should not be cloudy because of bacterial contamination, which is likely to give falsely negative reactions, nor admixed with more than a trace of blood, because in rare instances an excess of blood can cause a falsely positive reaction. These facts must be borne in mind when interpreting results, for even if the specimen has been cleared by centrifuging, these statements still hold.

The test requires the following materials:

1. *Glycerinated Hinton indicator*, which may be made in sufficient quantity to last a month, provided it is stored in the refrigerator at a temperature of 8° to 10° C.

2. *A 20 per cent solution of acacia*, which is prepared by diluting 2 parts of 30 per cent acacia,[1] with 1 part of physiologic salt solution. This should be kept in a refrigerator and discarded when it becomes cloudy.

3. *Hinton-negative human serum*, which may be obtained by pooling serums that remain after performing the routine serologic tests. Select for this pooling only Hinton-negative serums which are clear (without hemolysis) and which have been inactivated at 55° C. for thirty minutes. The rather remote possibility of a zonal effect may be ruled out by testing the pooled serum in the following amounts:

Tube 1—0.5 c.c. of serum and 0.5 c.c. of Hinton indicator.
Tube 2—0.1 c.c. of serum and 0.5 c.c. of Hinton indicator.
Tube 3—0.1 c.c. of serum and 1 c.c. of Hinton indicator.
Tube 4—0.1 c.c. of serum and 2 c.c. of Hinton indicator.

If all of these tubes show a negative reaction the pooled serum is suitable for use and should be passed through a Berkefeld "N" filter and collected under sterile conditions in rubber-stoppered bottles, so that each contains not more than three days' supply. These should be kept in a refrigerator at 8° to 10° C. Pooled serum should not be kept longer than three weeks and should be thrown away sooner if it becomes cloudy. Old serum is likely to give falsely negative reactions.

Laboratories that test only a few spinal fluids at a time may find it more convenient to prepare only enough serum for the day by selecting one or two of the clearer Hinton-negative serums of that day and retesting by the "rapid" method in the four amounts indicated previously. The rapid method consists in shaking the four tubes containing the serum and Hinton indicator for three minutes, then placing them in a water bath at 37° C. for thirty minutes, centrifuging them for ten minutes at high speed (about 2000 revolutions per minute) and then reading the results. If the reaction in all of the tubes is negative, the serum is suitable for testing spinal fluid.

[1] The solution of acacia (containing 4.5 per cent of sodium chloride) may be purchased from the Eli Lilly Co. in 100-c.c. ampules.

Procedure.—In a suitable rack, set up two tubes (one behind the other), measuring 10 by 100 mm., for each spinal fluid, and two tubes for controls. Label each tube.

Pipet 0.6 c.c. of the first spinal fluid into the first tube of the first row and the same amount into the corresponding tube of the second row, and continue in this way with each specimen of spinal fluid. Pipet 0.6 c.c. of physiologic salt solution into each control tube.

Mix the 20 per cent solution of acacia with the previously tested clear Hinton-negative human serum in equal parts; for example, if ten spinal fluids are to be examined, 1.5 c.c. of the serum and 1.5 c.c. of the 20 per cent solution of acacia, mixed together, will be enough to make up for these specimens. Pipet 0.2 c.c. of this into each tube of the first row (include control) and then pipet 0.2 c.c. of Hinton indicator into these same tubes. If several spinal fluids are to be examined at one time, the freshly mixed acacia-serum mixture may be added to an equal amount of Hinton indicator just prior to use, and 0.4 c.c. of this mixture pipetted into each tube of the first row.

Into each tube of the second row, including the control, pipet 0.2 c.c. of the acacia-serum mixture and 0.6 c.c. of Hinton indicator. If so desired, 1 part of the acacia-serum mixture may be added to 3 parts of Hinton indicator just prior to use, and 0.8 c.c. of this mixture measured into each tube of the second row.

Thoroughly and vigorously, either by hand or with a shaking machine, shake the rack containing the tubes, so that the contents are completely homogeneous.

Incubate in a water bath at 37° C. for sixteen hours, taking care that the water level is slightly above that of the contents of the tubes. Do not allow water to drop into the tubes.

Centrifuge at approximately 2000 revolutions per minute; then read the tests in front of a window or other suitable artificial light. Hold the tube at the top with one hand and tap near the bottom with a finger of the other hand. By such a procedure, any floccules at the meniscus are dispersed downward and are easily visible.

Report the results as follows:

Positive, if in *either* tube there is *flocculation* that is definitely visible. Tubes in which the reaction is positive should not be centrifuged a second time, because this may change the reaction to negative in cases in which it is truly positive.

Doubtful, if either of the tubes shows questionable flocculation which centrifuging for an additional five minutes does not amplify.

Negative, if the original ground-glass appearance persists in *both* tubes. Tubes in which the reaction is negative should be centrifuged a second time for five minutes, the test read again and the reaction reported as negative if the ground-glass appearance persists; otherwise, it should be reported as positive or doubtful, depending upon the visibility of the floccules.

Unsatisfactory, if the tubes containing spinal fluid that was originally turbid from bacterial contamination show no clearly visible particles, but cloudiness that is distinctly greater than that in the control tube; or if the reaction is positive in the presence of contamination with more than a trace of blood.

E. Eagle Tests

I. Qualitative Serum Flocculation Test

1. *Preparation of Serum.*—If the blood has been obtained by venipuncture, the clot is centrifuged and 0.1 c.c. of the clear supernatant serum is transferred to a numbered tube. If it is found convenient to work with larger quantities, 0.2 c.c. of the serum may be used, with a correspondingly larger amount of antigen. It is essential that the serum be free of red blood cells or suspended particles which might simulate lipoidal aggregates. The use of strongly hemolyzed serum does not affect the results save in so far as it makes it difficult to recognize aggregates with the naked eye. However, the results can usually be read with no difficulty under the microscope.

If the blood is obtained from a finger-tip puncture, it is allowed to flow into a capillary tube 110 mm. long and approximately 3 to 3.5 mm. in diameter.[1] Such tubes hold approximately 0.3 to 0.45 c.c. of blood when two thirds full, an amount which is adequate for the performance of the test. The free end of the tube is sealed in a flame, and the blood is allowed to coagulate. When the clot has begun to retract, the tube is centrifuged for three or four minutes at 2000 revolutions per minute, the glass is filed and broken above the clot, and approximately 0.1 c.c. of the clear serum is blown into the numbered glass tube used in performing the test. Thick-walled tubes without a lip, measuring either 10 by 75 mm. or 9 by 90 mm. will prove satisfactory.

2. *Preparation of Antigen.*—Fifty Gm. of dried powdered beef heart (Difco) are extracted for fifteen minutes at 30° to 37° C. with 250 c.c. of anesthesia ether, with frequent shaking. The mixture is

[1] These capillary tubes can be obtained from Friedrich and Dimmitt, Millville, New Jersey.

filtered with suction, the ether extract is discarded and the powder is similarly extracted with a second portion of fresh ether (250 c.c.). This is done four times. All the ether extracts are discarded. The beef heart powder is then washed on the filter with 100 c.c. of fresh ether, thoroughly dried, and extracted with 250 c.c. of absolute ethyl alcohol for three to five days at 20° to 37° C. The flask should be periodically shaken during this interval. At the end of this time, the alcoholic mixture is filtered, and the moist powder is washed with small portions of fresh absolute alcohol until the combined alcoholic extract and washings measure 250 c.c.

This basic extract is fortified with 0.6 per cent each of cholesterol and corn germ sterol (6 mg. of each sterol per cubic centimeter antigen). The required amount of sterol is added to a measured volume of antigen and is dissolved by boiling.[1] One c.c. of this completed antigen suffices for 500 tests.

Each lot of antigen should be checked by performing some tests with known negative and positive sera. The antigen remains serviceable for years if stored at room temperature in tightly stoppered containers.

3. *Preparation of Antigen Dilution.*—One volume of the fortified antigen is diluted with two volumes of 0.85 per cent ("physiologic") solution of sodium chloride. The measured amount of salt solution is blown into the antigen with a pipet. The tip of the pipet should have a sufficiently large bore to make the admixture of salt and antigen rapid and complete. The milky suspension which forms is the stock antigen dilution, which should be aged for at least twenty-four hours in the icebox before being used. Stored in the icebox, this primary dilution remains serviceable for at least five days.

For actual use in the test, a measured amount of this stock dilution (for example, 0.2 c.c.) is further diluted with eight volumes (for example, 1.6 c.c.) of a 4 per cent solution of sodium chloride. This second dilution should be made just before the antigen is added to the serum.

4. *Performance of Routine Serum Test.*—To 0.1 c.c. of inactivated serum is added 0.05 c.c. of freshly diluted antigen (the stock dilution freshly diluted with eight volumes of a 4 per cent solution of sodium chloride). If it is more convenient to use larger quantities, 0.1 c.c. of the antigen dilution is added to 0.2 c.c. of serum. The serum-antigen mixture is vigorously shaken for five minutes. If a shaking machine is used, it should be adjusted to approximately 180 to-and-

[1] The antigen can be obtained from the Digestive Ferments Company, Detroit, Michigan.

fro movements per minute, with a 4-cm. stroke. The tubes are then placed in a 37° C. water bath for thirty minutes.[1] At the end of this time, they are centrifuged at 1500 to 2000 revolutions per minute for ten minutes. Prolonged centrifugation at excessive speeds is to be avoided, as it causes partial aggregation of the antigen crystals and false doubtful reactions on microscopic examination.

The results are read at once, by either macroscopic or microscopic observation, or both.

5. *Emergency Serum Test.*—In case of emergency, as for a blood transfusion, the entire test can be completed within twenty to twenty-five minutes. The serum is inactivated for three minutes at 60° C.; antigen is added as in the routine test and the tube is shaken for five minutes in a shaking machine at 180 complete to-and-fro movements per minute, with a 4-cm. stroke. The incubation is omitted. The tube is then centrifuged for eight minutes at 1500 to 2000 revolutions per minute, and the results are read as in the routine test.

6. *Reading of Results.*—In general, aggregates are easier to see under the microscope than with the naked eye. It follows that very weakly positive sera may appear doubtful or even negative when viewed macroscopically, and yet the serum-antigen mixture may show unmistakable aggregation by microscopic examination. Wherever feasible, it is therefore recommended that the tests be read both macroscopically, in the tube, and microscopically, by pouring a little of the serum-antigen mixture onto a glass slide. If the number of tests makes this prohibitively laborious, it is suggested that the microscopic examination be used wherever there is the slightest doubt as to the macroscopic reading. Almost all otherwise doubtful reactions are thus resolved into positive or negative.

(*a*) *Macroscopic Reading.*—If the result is positive, one sees a few coarse aggregates floating in a comparatively clear fluid. In a few positive sera, the fluid is water clear and the aggregates have sedimented to form a coherent flake at the bottom of the tube. If the aggregation is questionable, the result is doubtful. If the result is negative, the tube at rest seems opalescent and homogeneous; when it is gently shaken, one sees a swirl of the refractile crystals of the antigen. Occasionally, these discrete crystals are floated to the top of the serum on centrifugation; these immediately redisperse on mild agitation and are readily distinguished from the coherent aggregates of a positive reaction. Vigorous shaking of the contents of the tube is to be avoided, as sedimented dirt particles or red blood cells are thus resuspended.

[1] May be omitted if the tubes have been shaken in a mechanical shaker for five minutes.

(*b*) *Microscopic Reading.*—The contents of the tube are poured onto a glass slide. It is convenient to use hollow ground slides, with depressions 1.5 cm. in diameter and 1.5 to 2 mm. deep. If these are not available, circles 2 cm. in diameter are inscribed on ordinary slides with a glass-marking pencil. The slide is gently rotated on a flat surface for five to ten seconds and the results read at once. It is essential that not more than two minutes elapse between the time the serum-antigen mixture is poured on the slide and its examination under the microscope. The magnification should be about 100 times

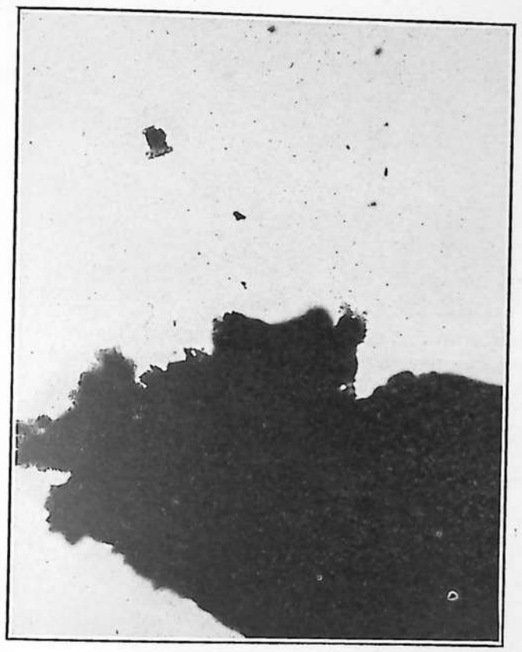


Fig. 358.—Macroscopic reading, positive—a single large clump of antigen floating in clear liquid; microscopic reading, positive; report to physician, positive. (Eagle and Brand, "Amer. Jour. of Syphilis, Gonorrhea, and Venereal Diseases," January, 1938.)

(10 × ocular, 16-mm. objective). The light should be adjusted so that a control negative serum appears homogeneous (Fig. 363) and should be kept in that position while the actual tests are read.

If a positive result, one sees irregular aggregates of varying degrees of coarseness floating in a clear fluid (Figs. 358, 359, 360, 361). If the aggregation is questionable, the result is doubtful (Fig. 362). In a negative reaction, one finds a uniform distribution of minute and discrete antigen crystals over the entire field (Fig. 363).

7. *Reporting the Results.*—In reporting the results to the physician, if the macroscopic reading is positive or doubtful, and the micro-

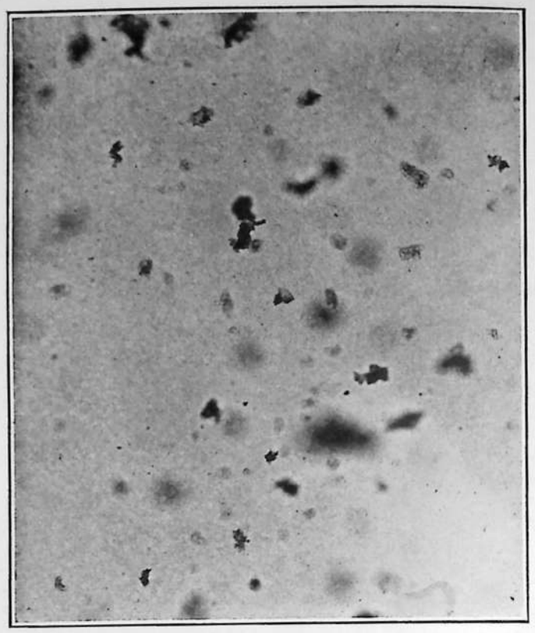


Fig. 359.—Macroscopic reading, positive—numerous clumps floating in clear liquid; microscopic reading, positive; report to physician, positive. (Eagle and Brand, "Amer. Jour. of Syphilis, Gonorrhea, and Venereal Diseases," January, 1938.)

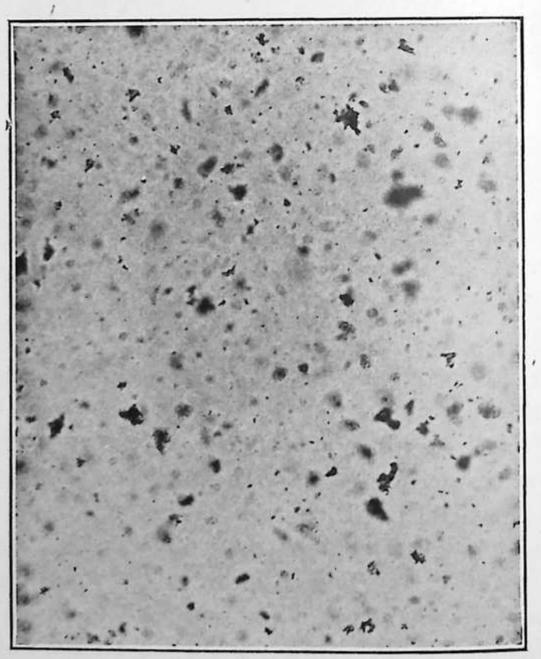


Fig. 360.—Macroscopic reading, doubtful—indefinite small aggregates; microscopic reading, positive; report to physician, positive. (Eagle and Brand, "Amer. Jour. of Syphilis, Gonorrhea, and Venereal Diseases," January, 1938.)

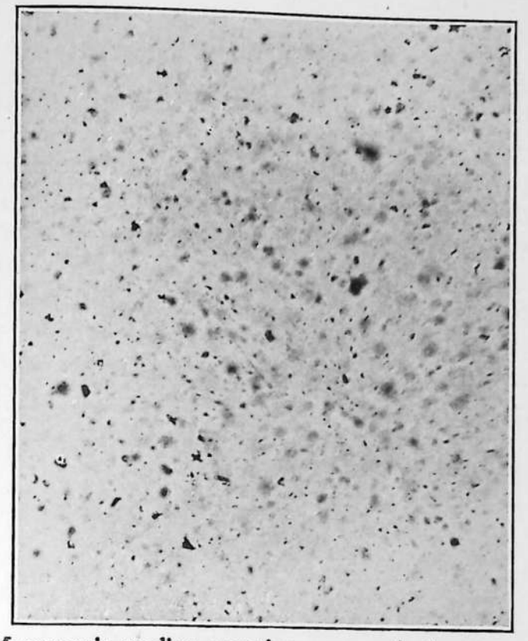


Fig. 361.—Macroscopic reading, negative—apparently homogeneous; microscopic reading, positive; report to physician, (1) positive, please repeat, (2) positive, confirmed by duplicate test; please repeat. (Eagle and Brand, "Amer. Jour. of Syphilis, Gonorrhea, and Venereal Diseases," January, 1938.)

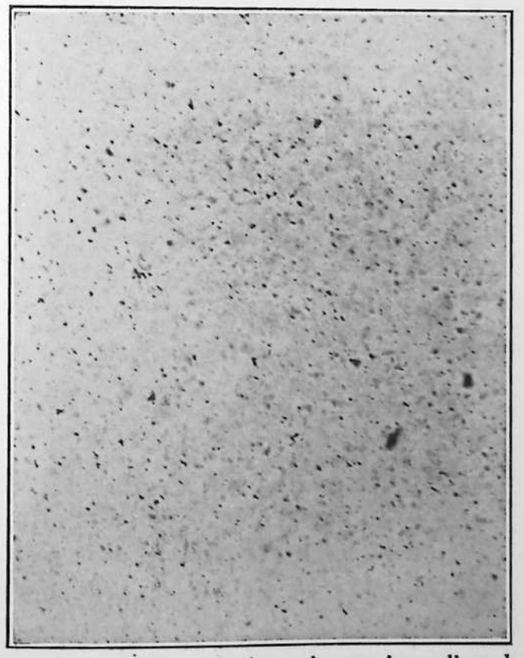


Fig. 362.—Macroscopic reading, negative; microscopic reading, doubtful; report to physician, (1) doubtful, please repeat, (2) doubtful, confirmed by duplicate test; please repeat. (Eagle and Brand, "Amer. Jour. of Syphilis, Gonorrhea, and Venereal Diseases," January, 1938.)

scopic reading is positive, the result is reported as positive (Figs. 358, 359, 360).

If the macroscopic reading is negative, but unmistakable aggregates are seen under the microscope, the result is positive (Fig. 361). If there is sufficient serum for another test, such discrepancies should be checked before being reported. If the result has been so confirmed by a second test, the report should include a statement to that effect (positive; confirmed by duplicate test).

If the microscopic examination shows indefinite aggregation (Fig. 362) the macroscopic reading is almost invariably negative, and the

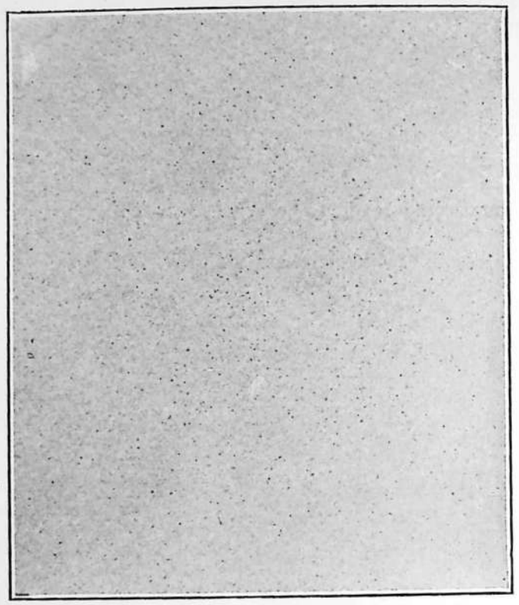


Fig. 363.—Macroscopic reading, negative; microscopic reading, negative; report to physician, negative. (Eagle and Brand, "Amer. Jour. of Syphilis, Gonorrhea, and Venereal Diseases," January, 1938.)

result of the test is reported as "doubtful—please repeat." If there is sufficient serum for another test, such results should be checked before being reported.[1] The report should include a statement to that effect ("doubtful; confirmed by duplicate test—please repeat").

II. QUANTITATIVE SERUM FLOCCULATION TEST

If it is necessary or desirable to ascertain the degree of positivity of a known positive serum, this can be done by doing the routine test on serial dilutions of serum.

[1] Since two hours suffice for the entire test, it can be repeated on the same day if the serum is available.

Whole inactivated serum, c.c.	0.2	0.1	0.05						
Serum, diluted 1: 8 with 0.85 per cent solution of sodium chloride, c.c.	..	..	...	0.2	0.1	0.05			
Serum, diluted 1:64 with 0.85 per cent solution of sodium chloride, c.c.	..	..	...	...	...	...	0.2	0.1	0.05 etc.
0.85 per cent solution of sodium chloride, c.c.	0	0.1	0.15	0	0.1	0.15	0	0.1	0.15
Final dilution of serum	1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Result of test	+	+	+	+	+	+	±	0	0

The serum is positive up to a 1:48 dilution, a reagin titer of 48.[1]

III. EAGLE WASSERMANN TECHNIC

(Inactivated serum; pooled guinea-pig complement; sheep red blood cells; rabbit amboceptor; strongly fortified beef heart antigen. All the reagents are used in a volume of 0.4 c.c., making the total volume of the test 2 c.c. If the conservation of materials is advisable, or if the technician is sufficiently expert, all the indicated quantities can be halved.)

1. *Preparation of Antigen.*—If a uniform preparation of dried powdered beef heart is not available, the technician may prepare his own. The heart tissue must be quite fresh, and the fat, pericardium and blood vessels should be removed. The muscle is ground in an ordinary meat grinder, and then dried by the addition of 150 c.c. of acetone to each 100 Gm. of tissue. After about an hour at room temperature, with frequent shaking, the acetone is removed by filtration, the tissue is shaken with a second portion of acetone (150 c.c.) for a few minutes and the mixture again is filtered. The acetone filtrates are discarded. The tissue is spread in a thin layer on a clean surface, and thoroughly dried in a 37° C. incubator for twenty-four hours, when it is turned and dried again for twenty-four hours. The dry sheet is now pulverized as finely as possible in a mortar, or, preferably, in a pulverizing machine.

(*a*) *Preparation of the Tissue Extract.*—Fifty Gm. of the dried powdered beef heart, preferably a pooled preparation such as one

[1] Very rarely, zone reactions are observed, in which strongly positive sera give a negative result in the routine test with whole serum, but are strongly positive if tested in a 1:10 or 1:20 dilution.

available commercially (for example, Difco), is extracted with 250 c.c. of pure anesthesia ether (5 c.c. per gram of powder) for fifteen minutes at 30° to 37° C., with frequent shaking. The mixture is filtered with suction, and the extraction is repeated with fresh ether until four extractions have been performed. All the ether extracts are discarded. After the fourth filtration, the beef heart powder is washed on the filter with 100 c.c. of fresh ether, dried and then extracted for five days with 250 c.c. of absolute alcohol (5 c.c. per gram of powder). The alcoholic extract is filtered, and the moist powder is washed on the filter with small portions of fresh alcohol until the volume of the combined alcoholic filtrate and washings is equal to 250 c.c. (5 c.c. per gram of powder). The clear, straw-yellow extract is the basic antigen, and contains approximately 1.2 to 1.5 per cent of solids. It is now fortified with cholesterol, which is added to a concentration of 0.6 per cent (6 mg. per cubic centimeter of extract).[1] The cholesterol is dissolved by boiling. This completed antigen, tightly stoppered, retains its reactivity almost indefinitely (more than eight years) at room temperature. For use in the test, it is diluted each day by slowly pouring from 80 to 200 volumes of 0.85 per cent salt solution into one volume of the antigen. The exact quantity of salt solution to be used is determined once for each lot of antigen by the technic to be described. One c.c. of antigen suffices for approximately 100 tests.

It is to be noted that the same basic extract, fortified by the addition of 0.6 Gm. of cholesterol, and 0.6 Gm. of corn germ sterol to each 100 c.c., is used in the Eagle flocculation technic previously described.

(*b*) *Anticomplementary and Hemolytic Titration of Antigen.*—Each lot of antigen should be tested once for its anticomplementary and hemolytic activity, to ensure that these undesirable properties are not so pronounced as to interfere with its use in the test. The technic of these titrations is given in Tables 1 and 2. Antigens prepared by the method just described are usually not anticomplementary in more than a 1:6 dilution, and are not significantly more hemolytic than is pure alcohol. The use of a 1:120 dilution in the test proper (vide infra) thus provides a wide margin of safety.

(*c*) *Determination of the Optimal Antigen Dilution.*—As in the case of the anticomplementary titration, the optimal dilution of the antigen need be determined only once with each lot of antigen. The importance of this titration is not generally realized. A typical titra-

[1] If the complement is pooled serum from many guinea-pigs and is uniformly of high activity, a 1 per cent concentration of cholesterol may be used with safety, with somewhat more sensitive results. With such a highly fortified antigen, three hours in the ice-box followed by half an hour at 37° C. is the recommended incubation period. Particular care must be paid to the complement and antigen controls.

TABLE 1

HEMOLYTIC TITRATION OF ANTIGEN

	Dilution of antigen.*						
	1	1:2	1:3	1:4	1:6	1:8	1:12
Dilution of antigen, c.c.	0.4	0.4	0.4	0.4	0.4	0.4	0.4
0.85 per cent salt solution, c.c.	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Cell suspension,† c.c.	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Hemolysis is read after ½ hour at 37° C.							
Example of reading of hemolysis	Complete	None	None	None	None	None	None

Conclusion: The antigen is not significantly hemolytic.

* These dilutions are readily prepared by the following procedure:

Whole antigen, c.c.	0.4	0.2	0.13	0.1	0.07	0.05	0.033
Salt solution, c.c.	0	0.2	0.27	0.3	0.33	0.35	0.37
Final dilution of antigen	1	1:2	1:3	1:4	1:6	1:8	1:12

† Can be sensitized or unsensitized, but should be 1.5 per cent by volume.

TABLE 2

ANTICOMPLEMENTARY TITRATION OF ANTIGEN

	Dilution of antigen.*						
	1	1:2	1:3	1:4	1:6	1:8	1:12
Dilution of antigen, c.c.	0.4	0.4	0.4	0.4	0.4	0.4	0.4
0.85 per cent salt solution, c.c.	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Complement, 1:10, c.c.	0.4	0.4	0.4	0.4	0.4	0.4	0.4
After 4 hours at 0° to 5° C., followed by ½ hour at 37° C., add 0.8 c.c. of sensitized cells to all the tubes.							
Example of reading of hemolysis after ½ hour at 37° C.	Complete	0	0	Partial	Complete	Complete	Complete

Conclusion: Antigen is anticomplementary up to a 1:4 dilution.

* These dilutions are readily prepared by the following procedure:

Whole antigen, c.c.	0.4	0.2	0.13	0.1	0.07	0.05	0.035
Salt solution, c.c.	0	0.2	0.27	0.3	0.33	0.35	0.37
Final dilution of antigen	1	1:2	1:3	1:4	1:6	1:8	1:12

tion illustrating the method is given in Table 3. In that example, the most sensitive, and thus the optimal dilution, is approximately 1:140. If the powdered beef heart used for the antigen is a pooled, uniform product, and if the method of preparation is adhered to rigidly, it is generally found that the optimal antigen dilution is also very uniform and does not deviate significantly from the 1:120 to 1:160 value (Table 3).

TABLE 3

DETERMINATION OF THE OPTIMAL DILUTION OF ANTIGEN

Dilution of antigen.*	Result of Wassermann test* on:							
	Strongly positive serum.	The same serum diluted* with salt solution.						
		1:2	1:4	1:8	1:16	1:32	1:64	1:128
1:40	+	+	±	+	0	0	0	0
1:80	+	+	+	+	±	0	0	0
1:100	+	+	+	+	+	0	0	0
1:120	+	+	+	+	+	±	0	0
1 160	+	+	+	+	+	±	0	0
1:200	+	+	+	+	+	0	0	0

+ = positive (no hemolysis) ± = doubtful (partial hemolysis) 0 = negative (complete hemolysis)

* The antigen dilutions to be used in the mentioned titration can be prepared as follows:

1:40 antigen dilution, c.c.....	8.0	4.0	3.2	2.7	2.0	1.6
Salt solution, c.c.....	0	4.0	4.8	5.3	6.0	6.4
Final dilution of antigen.....	1:40	1:80	1:100	1:120	1:160	1:200

Similarly, the serum dilutions can be readily prepared as follows:

Serum, c.c.....	4.0	2.0	1.0	0.5	0.25	0.125	0.062
Diluent, c.c.....	0	2.0	3.0	3.5	3.75	3.9	4.0
Final dilution of serum....		1:2	1:4	1:8	1:16	1:32	1:64

In preparing the antigen for daily use in the test, the correct volume of salt solution is slowly poured into one volume of antigen. The antigen dilution should be opalescent and homogeneous, and should not contain any visible granules.

2. *Preparation of Complement.*—At least five, and preferably more, guinea-pigs are bled from the heart; the clots are broken up with a glass rod and centrifuged. Bleeding large guinea-pigs (more

than 1 pound, 0.5 Kg., in weight) for comparatively small quantities (5 to 10 c.c.) at intervals of four to six weeks, is more economical than exsanguinating the animal.

The small laboratory which cannot pool the fresh serum of several guinea-pigs for each series, or which cannot obtain such pooled fresh serum from a central source of supply, may have recourse to three methods of preservation. 1. Multiple containers of complement serum may be vacuum-dried from the frozen state and vacuum-sealed on the Flosdorf-Mudd apparatus.[1] Such complement remains fully active for at least ten months if stored in the refrigerator. 2. Although complement retains its activity when frozen at very low temperature (< 20° C.), over a period of weeks and even months, such freezing is not feasible in the ordinary laboratory. 3. Complement may be preserved by the addition of salt. Numerous methods of "salting" complement have been recommended. As simple and as satisfactory as any is the addition of sodium chloride in bulk to the complement serum (8 mg. of sodium chloride per cubic centimeter serum, dissolved by shaking). For actual use in the test, the salted complement is diluted with nine volumes of water (for example, 1 c.c. of complement +9 c.c. of distilled water) to make a 1:10 dilution.

Perhaps the most satisfactory method of preserving complement in the small laboratory is a combination of 2 and 3. The container of salted complement is placed in the freezing compartment of a mechanical refrigerator. When the complement is stored in this manner it undergoes no demonstrable deterioration in two weeks.

3. *The Preparation of the Serum or Spinal Fluid to Be Tested.*—The coagulated blood is separated from the sides of the tube with a clean glass rod, and centrifuged for five to ten minutes at 1000 to 1500 revolutions per minute. One c.c. of the clear serum is pipetted into a numbered tube, which is then placed in a water bath at 56° C. for fifteen to twenty minutes.[2] In an incompletely coagulated specimen, or in one prevented from coagulating by the addition of oxalate or citrate (neither of which should be used in the Wassermann reaction), a precipitate of coagulated fibrinogen will develop upon inactivation. Contrary to published reports, even large amounts of hemoglobin dissolved in the serum do not *per se* affect the results, save so far as they obscure the reading of hemolysis. Similarly, the only objection to blood older than twenty-four hours is that it tends to become anticomplementary with age, particularly if conditions

[1] The Flosdorf-Mudd apparatus is being manufactured by the F. J. Stokes Company, East Tabor Road, Olney, Philadelphia, Pennsylvania.

[2] In an emergency, the serum may be inactivated for three minutes at 60° to 62° C.

facilitate bacterial multiplication. Whenever feasible, the serum should be inactivated immediately before testing. If it is inactivated the day before the actual test, it should be reheated for five minutes at 56° C. before proceeding with the test on the following day.

Native amboceptor for sheep cells may be removed from the inactivated serum by adding 0.1 c.c. of a thick (20 to 40 per cent) suspension of washed sheep cells to each cubic centimeter of inactivated serum. The serum and cells are thoroughly mixed, and centrifuged five minutes later in order to remove the cells. An alternative method is to add the thick suspension of sheep cells to the whole cold blood, mix thoroughly with a clean rod, and centrifuge after it has been kept in the refrigerator for fifteen minutes. The latter procedure has the advantage of simplicity, for it eliminates the necessity of a double centrifugation for each serum; it has the disadvantage of causing slight hemolysis.

The removal of native amboceptor unquestionably results in an increased sensitivity. Nevertheless, it is perhaps an unnecessary complication if a flocculation test is carried out in parallel with the Wassermann reaction. The flocculation test ensures the detection of practically all sera which might be Wassermann-negative because of their native amboceptor content.

Spinal fluid contains neither complement nor amboceptor in significant quantities, and therefore requires neither inactivation nor the absorption of native amboceptor. The whole fresh fluid is used as such.

4. *Salt Solution.*—The salt solution used in diluting antigen, complement, serum, cells, and amboceptor is an 0.85 per cent solution of chemically pure sodium chloride in distilled water. It is advisable but not absolutely necessary to add a trace of alkaline buffer, as old distilled water may be sufficiently acid, because of dissolved carbon dioxide, to accelerate the deterioration of dilute complement. It also is desirable that all the tubes of the test be at approximately the same *p*H. A final concentration of 0.005 molar phosphate buffer at *p*H 7.4 is adequate. It is convenient to keep a 17 per cent stock solution of sodium chloride containing 0.1 M of phosphate buffer. This stoppered stock solution keeps indefinitely. A measured volume (50 c.c.) is diluted each day with twenty volumes (1000 c.c.) of distilled water to form the buffered, physiologically isotonic salt solution actually used in the test. The composition of the concentrated stock solution is as follows:[1]

[1] An alternative formula, which gives a similar stock solution, is as follows: Sodium chloride, 170 Gm.; monopotassium dihydrogen phosphate, 2.7 Gm.; sodium hydrogen phosphate, 11.3 Gm.; water, q. s. ad 1 liter.

Sodium chloride	170	Gm.
Monopotassium dihydrogen phosphate	13.6	"
Sodium hydroxide	3.0	" (30 c.c. of a 10 per cent solution)
Water, q. s. ad	1	liter

Dilute 50 c.c. of this stock solution with 1000 c.c. of water to form the buffered salt solution used in the test.

5. *Setting Up the Tests.*—(*a*) *Routine Qualitative Serum Tests.*—Three tubes are advisable, although only two are essential. Tube 1 is the serum control, containing serum and complement, but salt solution instead of antigen.

Tubes 2 and 3 are the test proper, each containing 0.4 c.c. of complement, 0.4 c.c. of antigen, and different quantities of serum (0.2 and 0.1 c.c.). The addition of salt solution to tubes 2 and 3 is not essential, but serves to bring the total volume in all three tubes up to 1.2 c.c., corresponding to 0.4 c.c. of each of the three reagents.

	Serum control	Test proper.	
Whole serum, c.c.	0.2	0.2	0.1
Complement, 1:10, c.c.	0.4	0.4	0.4
Antigen, 1:100	0	0.4	0.4
0.85 per cent solution of sodium chloride, c.c.	0.6	0.2*	0.3*

* May be omitted.

One antigen control suffices for the entire series of tests, and two complement controls are also set up, as here indicated:

	Antigen control.	Complement controls (in duplicate).				
Antigen, 1:100, c.c.	0.4	0.4	0.4	0.4	0.4	0.4
Complement, 1:10, c.c.	0.4	0.4	0.2	0.13	0.1	0
Salt solution, c.c.	0.4	0.4	0.6	0.7	0.7	0.8

(*b*) *Quantitative Serum Tests.*—If a quantitative determination of the reagin content of a known positive serum is desired, a series of dilutions of serum may be prepared by placing 0.4 c.c. of salt solution in each of a series of tubes. To the first tube is added 0.4 c.c. of serum, and 0.4 c.c. of the resulting mixture is transferred to the following tube; 0.4 c.c. is withdrawn from tube 2 and transferred to

tube 3, and the process is repeated with all the tubes of the series. The final set-up is then as follows:

Serum control.	Test proper.						
Serum, c.c., 0.2.....	0.2	0.1	0.05	0.025	0.0125	0.0062	0.0031
	in a total volume of 0.4 c.c., corresponding to a dilution of						
	1:2	1:4	1:8	1:16	1:32	1:64	1:128

It is a technically simpler procedure to prepare a single 1:20 dilution of the serum (0.1 c.c. of serum + 1.9 c.c. of salt solution) and distribute the serum as follows:

	Serum control.	Test proper.					
Whole serum, c.c.....	0.2	0.2	0.05				
Serum, 1:20, c.c.....	..	..	...	0.4	0.2	0.1	0.05
Salt solution, c.c.....	0.6	0.2	0.35	0	0.2	0.3	0.35

The dilutions so obtained are 1:2, 1:8, 1:20, 1:40, 1:80, 1:160, in a volume of 0.4 c.c. This is the recommended procedure. Antigen and complement are then added exactly as in the routine test (0.4 c.c. of each dilution).

(*c*) *Spinal Fluid Wassermann Test.*—The routine Wassermann test on spinal fluid is a quantitative test. Except for the quantities used, and the fact that the fluid is not inactivated, the set-up of the test is similar to that of the quantitative serum test:

	Spinal fluid control.	Test proper.						
Spinal fluid, c.c.....	1.0	1.0	0.6	0.4	0.2	0.1	0.05	0.025
Complement, 1:10, c.c...	0.2*	0.2*	0.2	0.2	0.2	0.2	0.2	0.2
Antigen, 1:100, c.c.....	0.0	0.2*	0.2	0.2	0.2	0.2	0.2	0.2

* The quantities of the reagents are halved in order to conserve spinal fluid. It is to be noted that the maximal amount of spinal fluid used in the Wassermann test relative to the other reagents is ten times that used in the tests on serum.

The entire series, serum and spinal fluid tests and controls alike, are then placed in the icebox for three to four hours.

While the tests are in the icebox, one proceeds to prepare and test the sensitized cell suspension.

6. *Preparation of the Sensitized Cell Suspension.*—Because this technic calls for the use of a fixed quantity of a pooled, standard complement, it becomes possible to set up the diagnostic tests as the first step in the daily performance of the Wassermann reaction, to place these tests in the refrigerator for their primary incubation of three to four hours, and then proceed to the preparation of the sensitized cell suspension. The cells can be washed, the amboceptor titrated, and the titration checked within two hours. This leaves ample time for other routine work before the tests are placed at 37° C. for their secondary incubation of thirty minutes prior to the addition of the cells.

(*a*) *Preparation of the Stock, 3 Per Cent Suspension of Sheep Cells.*—Citrated sheep blood (1 part of 5 per cent solution of sodium citrate and 5 to 10 parts of blood) or defibrinated blood is collected as aseptically as conditions permit. The method of choice is to bleed the animals from the jugular vein into a sterile vessel containing the citrate. The addition of sucrose in bulk to a concentration of 2.5 per cent (2.5 Gm. per 100 c.c. blood) serves to postpone spontaneous disintegration. Sterile sheep blood generally remains serviceable for one to three weeks if stored at 0° to 5° C.

For use in the test, one volume of blood is washed with ten to fifteen volumes of 0.85 per cent salt solution, the mixture is centrifuged, and the supernatant fluid carefully withdrawn. The sedimented cells are resuspended in a second portion of salt solution and again centrifuged, this time in a graduated tube, until the volume of sedimented cells becomes constant. Centrifuging for ten to fifteen minutes at 2000 to 2500 revolutions per minute will suffice. The supernatant fluid is carefully withdrawn, and the measured cell sediment is resuspended in thirty-two volumes of salt solution to form the stock 3 per cent suspension of unsensitized cells. This must be prepared fresh daily.

(*b*) *Titration of Amboceptor.*—The minimal hemolytic quantity (unit) of amboceptor must be determined daily for the particular cell suspension to be used that day, by the technic outlined in Table 4, p. 730. This hemolytic unit of amboceptor should be a 1:2400 dilution or higher, and an amboceptor should be discarded if it is so inactive that the hemolytic unit represents a 1:1000 dilution.

The unit of amboceptor in the example cited is a 1:3000 to 1:4000 dilution, or, interpolating, approximately a 1:3500 dilution. An amboceptor dilution is now prepared containing $2\frac{1}{2}$ units (in the

example cited, a 1:1400 dilution). This amboceptor dilution is poured into an equal volume of the 3 per cent suspension of cells to form the 1.5 per cent suspension of sensitized cells which is actually used in the test.

(*c*) *Check on the Amboceptor Titration.*—The sensitization of the cells should be completed within an hour. One set of complement controls previously placed in the refrigerator along with the tests, and containing 0.4, 0.2, 0.13, and 0.1 c.c. of complement in a total volume of 1.2 c.c., is now removed from the refrigerator, and 0.8 c.c. of the sensitized suspension of cells is added to each tube. The degree

TABLE 4

TECHNIC OF TITRATION OF AMBOCEPTOR

	Titration of amboceptor.*						
	1:1000	1:1500	1:2000	1:3000	1:4000	1:6000	1:8000
Dilution of amboceptor, c.c.	0.4	0.4	0.4	0.4	0.4	0.4	0.4
3 per cent cell suspension, c.c.	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Salt solutions, c.c.	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Complement, 1:10, c.c.	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Example of reading of hemolysis after ½ hour at 37° C.	Complete	Complete	Complete	Complete	Partial	Partial	None

* The following is a simple method of setting up these amboceptor dilutions:

1:1000 amboceptor, c.c.	0.4	0.27	0.2	0.13	0.10	0.067	0.05
Salt solution, c.c.	0.0	0.13	0.2	0.27	0.30	0.34	0.35
Final dilution of amboceptor	1:1000	1:1500	1:2000	1:3000	1:4000	1:6000	1:8000

of hemolysis is read after the set of controls has been kept at 37° C. for thirty minutes. If the titration of the amboceptor was correct, the 0.4 c.c. of 1:10 complement used in the test represents two to two and a half times the amount necessary to cause complete hemolysis in thirty minutes. Accordingly, the sheep cells in the first two tubes of the complement titration should be completely hemolyzed; tube 3 should show partial hemolysis, and tube 4 should show little or no hemolysis. Any error in the titration of the amboceptor becomes immediately apparent. If only the first tube shows complete hemolysis, the cells have been inadequately sensitized, and more amboceptor should be added. If complete hemolysis occurs in the three tubes, an excess of amboceptor has been used. The technician must reconcile himself to a sudden decrease in the sensitivity of the test, or a second portion of blood must be washed and sensitized with the correct amount of amboceptor, that is, somewhat less than that used in the first lot.

7. *Secondary Incubation of the Tests at 37° C., and the Addition of the Sensitized Cells.*—After three to four hours at 0° to 5° C., the tests and controls are placed in the 37° C. water bath for thirty minutes. Eight tenths (0.8) c.c. of the sensitized cell suspension is then added to all the tubes, which are vigorously shaken and replaced in an oven at 37° C. for their final incubation of twenty to thirty minutes.

This leeway of ten minutes is allowed the technician in order to compensate for any slight error in the sensitization of the cells as detected by the complement check just described. If tube 2 of the complement control, which contains half the amount of complement used in the test, is slow to show hemolysis, requiring, for example, thirty minutes for complete hemolysis, then the tests also are given thirty minutes. If, on the other hand, tube 2 shows complete hemolysis in twenty minutes, the results of the tests also should be read in twenty minutes after the addition of the sensitized cells. The futility of any stop-watch precision is apparent when one remembers that it requires five to fifteen minutes merely to add cells to a large series of test tubes.

8. *Reading of Results.*—(*a*) *The antigen controls* should be completely hemolyzed. Properly diluted, the antigen fortified with 0.6 or 1 per cent cholesterol is not demonstrably anticomplementary under the conditions of the test. Failure of hemolysis to occur indicates that the complement is defective, and is deteriorating under the conditions of the test.

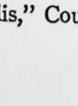
(*b*) *The complement controls* incubated along with the tests should show approximately the same degree of hemolysis as the first set used to check the titration of the amboceptor. Any pronounced differences, illustrated in the table (top of p. 732), indicate either that the complement is defective and is deteriorating under the conditions of the test, or that the antigen is significantly anticomplementary under the conditions of the test. Such deterioration with fresh complement indicates that the guinea-pigs are in poor condition, and necessitates the greatest caution in the reading of results.

(*c*) *Reading of Tests Proper.*—Tables 5 and 6, illustrating the reading of results, are self-explanatory. If the serum as such destroys

	Tube 1.	Tube 2.	Tube 3.	Tube 4.
Amount of complement referred to that used in the test	Same	½	⅓	¼
Hemolysis caused by freshly prepared dilutions	Complete	Complete	Partial	Trace
Hemolysis caused after storage at 0°–5° C. for four hours, followed by ½ hour at 37° C.	Almost complete	Trace	Trace	None

TABLE 5

READING OF RESULTS IN ROUTINE WASSERMANN TEST[1]

Serum Control	Test Proper 1:2 serum	Test Proper 1:4 serum	Reading of Result
Complete lysis	Complete lysis	 Complete lysis	Negative
Complete lysis	Partial lysis	Partial lysis	Doubtful
Complete lysis	Partial lysis	No lysis	Doubtful, unless flocculation test is clearly positive, in which case report may be made as positive
Complete lysis	No lysis	Partial lysis	
Complete lysis	No lysis	No lysis	Positive
Incomplete or no lysis in all three tubes			Anticomplementary

[1] Eagle: "The Laboratory Diagnosis of Syphilis," Courtesy of C. V. Mosby Co.

complement, in the absence of antigen, it is anticomplementary, a result which offers no evidence as to the presence or absence of syphilitic infection. If the serum control is completely hemolyzed, and the tubes containing serum, complement and antigen show no hemolysis, complement has been fixed by a lipoid-reagin compound, and the result is positive. If all three tubes show complete hemolysis, the complement has not been fixed and the result is negative. Finally, if the tests show partial hemolysis, the serum control being completely hemolyzed, only part of the complement has been fixed, and the result is doubtful.

TABLE 6

READING OF QUANTITATIVE SERUM TESTS[1]

Serum control	Test proper with serum dilutions 1:2	1:8	1:20	1:40	1:80	1:160	1:330	Reading of result
								Negative
Complete lysis in all tubes								
Complete lysis	No lysis	No lysis	No lysis	No lysis	No lysis	Complete lysis	Complete lysis	Positive up to a 1:80 dilution: Wassermann titer of 80
With a strongly positive serum (titer of 16 or higher), partial hemolysis in the 1:2 serum control can be safely disregarded; with less strongly positive sera, the reading in such a case is anticomplementary.								
No lysis	No lysis	No lysis	No lysis	No lysis	No lysis	Partial lysis	Complete lysis	Anticomplementary*

[1] Eagle: "The Laboratory Diagnosis of Syphilis." Courtesy of C. V. Mosby Co.

In the quantitative Wassermann test the result is expressed as the maximal dilution of serum which continues to give a positive result. In Table 6, the serum may be said to be positive up to a 1:80 dilution. Similarly, in the spinal fluid test, a Wassermann-positive fluid may be said to be positive down to, for example, 0.5 c.c.

Table 7, p. 734, is a condensed summary of the routine serum Wassermann test just described.

TABLE 7

OUTLINE OF ROUTINE SERUM AND SPINAL FLUID WASSERMANN TESTS

		Serum* or spinal fluid, c.c.	0.85% salt solution, c.c.	Dilution of antigen, c.c.	Complement, 1:10, c.c.		Sensitized sheep cells (1.5%), c.c.		Example of reading of hemolysis.	Result of test in the example cited.
Antigen control (no serum).		0	0.4	0.4	0.4	The entire series of tests are now placed at 3°–5° C. for 4 to 8 hours, followed by ½ hour at 37° C. Sensitized cells are then added as indicated in the next column.	0.8	The tests are now replaced at 37° C. for 20–30 minutes, when the results are read. The tubes may be removed from the bath as soon as the second tube of the complement control is completely hemolyzed.	Complete	
Complement controls.		0	0.4	0.4	0.4		0.8		Complete	Cells correctly sensitized with amboceptor.
		0	0.6	0.4	0.2		0.8		Complete	
		0	0.7	0.4	0.13		0.8		Partial	
		0	0.7	0.4	0.1		0.8		Trace	
		0	0.8	0.4	0.0		0.8		None	
Routine serum test.	Serum control.*	0.2	0.6	0	0.4		0.8		Complete	Serum positive.
	Test proper.	0.2	0.2†	0.4	0.4		0.8		None	
		0.1	0.3†	0.4	0.4		0.8		None	
Spinal fluid test.	Spinal fluid controls.	1.0	0	0	0.2		0.4		Complete	Spinal fluid positive down to 0.2 c.c.
		0.1	0.3†	0	0.2		0.4		Complete	
	Test proper.	1.0	0	0.2	0.2		0.4		None	
		0.6	0	0.2	0.2		0.4		None	
		0.4	0	0.2	0.2		0.4		None	
		0.2	0	0.2	0.2		0.4		None	
		0.1	0.1†	0.2	0.2		0.4		Partial	
		0.05	0.15†	0.2	0.2		0.4		Complete	
		0.025	0.20	0.2	0.2		0.4			

* The serum is inactivated; the spinal fluid is tested fresh.
† May be omitted.

F. KOLMER COMPLEMENT-FIXATION TEST

Glassware and Apparatus.—1. Pipets.
1 c.c. graduated in 0.01 c.c. to tip.
5 c.c. graduated in 0.10 c.c.
10 c.c. graduated in 0.50 c.c.

An automatic pipet is highly recommended for rapid work and saving of time for pipetting saline solution, complement, antigen and hemolysin in the conduct of the tests.

2. Test tubes: 85 by 15 mm. (inside diameter) with rounded bottoms and no lips.

3. Cylinders: glass-stoppered, graduated (50- or 100-c.c. capacity) to be used for measuring amounts over 50 c.c.

4. Test tube racks: galvanized wire racks carrying twelve rows of six tubes each.

5. Water bath: any easily regulated water bath is suitable, or a simple galvanized pan carrying water to a depth of 8 cm. can be used satisfactorily at 55° or 37° C.

6. Refrigerator: any refrigerator maintaining a temperature of 6° to 8° C. is satisfactory.

Method of Cleansing Glassware.—1. All glassware should be chemically clean and preferably sterile. To clean tubes and flasks, empty and rinse in running tap water; wash inside and outside in soapy water; rinse several times in running tap water and invert in wire baskets. Dry in the hot-air oven at about 160° C.

2. Pipets should be placed after use in a jar or cylinder of clean water with a pad of cotton in the bottom. To clean pipets, rinse thoroughly in running tap water, place in a metal box or wire basket and sterilize in the oven.

3. Flasks should be plugged with cotton and sterilized in the oven for thirty minutes at 160° C.

4. If glassware becomes cloudy, immerse in bichromate cleansing fluid (2 parts potassium bichromate, 3 parts commercial sulfuric acid, and 25 parts water) for twenty-four hours. Rinse thoroughly in running tap water and proceed with the washing as described.

Preparation of Saline Solution.—Dissolve 8.5 Gm. of dry, chemically pure sodium chloride in 1000 c.c. of tap or distilled water (former preferred in most localities). If the salt has absorbed moisture it should be dried in the hot-air oven for ten or fifteen minutes before weighing. Filter solution through paper into a flask fitted with a gauze-covered cotton stopper. Sterilize by heating in an Arnold sterilizer for one hour before use (not essential if immediately used).

A satisfactory saline solution should not of itself be hemolytic when 1 or 2 drops of washed corpuscles are added to 5 c.c. of the solution in a test tube followed by incubation in a water bath for one hour. Neither should it be antihemolytic as determined by the hemolysin or complement titrations. The antisheep hemolysin should give a unit of at least 0.5 c.c. of a 1:4000 dilution when titrated with 0.3 c.c. of 1:30 complement and 0.5 c.c. of a 2 per cent suspension of washed sheep corpuscles with incubation in a water bath for one hour. It is possible that failure of hemolysis may be due to defective hemolysin or complement or to the use of corpuscles of increased resistance to serum hemolysis, but whenever these factors may be excluded it is likely that the saline solution is defective.

If difficulties are experienced with saline solution prepared with distilled water, use ordinary tap water. If still unsatisfactory, add 0.1 Gm. of magnesium sulfate to each 1000 c.c. (Kellogg).

Preparation of Sheep Corpuscles (Indicator Antigen).—Sheep blood may be obtained at an abattoir or by bleeding a sheep from the external jugular vein. In a clean (but not necessarily sterile) quart sized Mason jar, place 30 c.c. of a 10 per cent solution of sodium citrate in saline solution and 2 c.c. of formalin. At the abattoir have the jar almost filled with *fresh* blood (blood kept over in buckets is unsatisfactory) screw on the top, mix well with the citrate-formalin solution and keep in a refrigerator. Ordinarily it is fit for use for two to three weeks at least, but as soon as the corpuscles become too fragile a fresh supply should be secured. Boerner and Lukens advised keeping blood in the refrigerator for forty-eight hours before using.

If preservation is not desired, glass beads alone may be placed in the jar, and after it is filled with blood, it should be thoroughly shaken to produce defibrination. Blood collected in this manner will keep at low temperature (jar placed on a block of ice) for about a week. If beef blood is used, it may be collected by either method.

Filter a small quantity of blood through cotton into a *graduated* centrifuge tube. Allow twice as much blood as the amount of cells required. Add two or three volumes of saline solution. Centrifuge at a moderate velocity until all the corpuscles are thrown down.

Remove the supernatant fluid with a capillary pipet or by suction. Add three or four volumes of saline solution; mix by inverting the tube and centrifuge again for the same length of time.

Repeat the process for a third time but centrifuge twice as long as in the first washing in order to pack the cells evenly and firmly.

Cells should be washed until the supernatant fluid is almost colorless. Three washings are usually sufficient. (If more than four washings are necessary, the cells are too fragile for use.)

Read the volume of cells in the centrifuge tube, carefully remove the supernatant fluid and prepare a 2 per cent suspension by washing the corpuscles into a flask with forty-nine volumes of saline solution. *Always shake before using to secure an even suspension, as the corpuscles settle to the bottom of the flask when not in use.*

Preparation of Antisheep Hemolysin.—Give a rabbit five or six intravenous injections of 5 c.c. of a 10 per cent suspension of washed sheep corpuscles every five days. Bleed the rabbit seven to nine days after the last injection if a preliminary titration gives a unit of 0.5 c.c. of 1:4000 dilution or higher. Separate the serum (it need not be inactivated) and preserve with an equal part of best grade neutral glycerin. Keep in a refrigerator.

Preparation of Complement.—The pooled sera of at least three healthy guinea-pigs should be used. Select large, well-nourished animals that have not been fed for twelve hours; avoid pregnant animals.

Anesthetize the guinea-pig lightly with ether or stun the animal with one or two sharp blows on the head. Sever the large blood vessels on both sides of the neck, being careful not to cut the esophagus or trachea. Collect the blood in a centrifuge tube by means of a large funnel. Place the blood in an incubator at 38° C. for one hour; break up the clot, and centrifuge. Separate the clear serum. *Keep in the refrigerator when not in use.*

If but a small amount of serum is required, 4 or 5 c.c. of blood may be obtained from each guinea-pig by bleeding from the heart with a 5-c.c. syringe fitted with a short needle, gage 20.

Complement serum may be preserved for several weeks by adding 0.3 Gm. of chemically pure sodium chloride to each cubic centimeter of serum. Keep in a dark glass bottle at or near the *freezing point.* To prepare for use, dilute 1 c.c. of serum with 29 c.c. of distilled water. This gives a 1:30 dilution in 1 per cent salt solution. As preserved complement loses first in fixability by syphilis antigen and antibody, it should not be kept for more than three or four weeks.

Lyophil Complement.—The most satisfactory method for preserving complement is by evaporation in the frozen state *in vacuo* by the method of Flosdorf and Mudd.[1] It retains both hemolytic activity and fixability for eight to twelve months and has proved satisfactory. It has the great advantage of being prepared of the pooled sera of a large number of guinea-pigs with uniform hemolytic activity and

[1] Jour. Immunol., *29*:389, 1935.

fixability by antigen and antibody. It may be obtained from Sharp and Dohme, Philadelphia, and is dispensed in vacules containing the equivalent of 5 c.c. of fresh serum. They should be kept in a refrigerator until used. By adding 5 c.c. of distilled water the material goes into immediate solution and is ready for use in the same manner as fresh serum. Preservation by the *cryochem process* is also recommended and more economical.

Preparation of Antigen.—A cholesterolized and lecithinized alcoholic extract of heart muscle is employed. "Bacto-Beef Heart" prepared by the Digestive Ferments Co. of Detroit, is recommended.

1. Place 30 Gm. of beef heart powder in a flask with 100 c.c. of chemically pure acetone. Stopper tightly. Keep at room temperature for five days with brief shaking each day.

2. Filter through fat-free paper or decant and discard the filtrate.

3. Dry the residue and extract with 100 c.c. of chemically pure absolute ethyl alcohol in a tightly stoppered flask for five days at room temperature, shaking each day.

4. Filter through fat-free paper with slight squeezing of the tissue.

5. Measure the filtrate and for each cubic centimeter add 0.002 Gm. of cholesterol. The total amount of cholesterol is dissolved in 10 c.c. of ether and added to the alcoholic filtrate. Shake thoroughly and place in a water bath at 55° C. for one hour to aid solution.

6. Allow to stand at room temperature for two or three days with brief shaking each day. Filter through fat-free paper.

7. Keep the antigen at *room temperature* in a tightly stoppered bottle. A new antigen of increased sensitiveness with practically no change in hemolytic or anticomplementary activity may be prepared in the same manner except that it is reinforced with acetone-insoluble lipoids as follows:

(*a*) Steps 1, 2, 3, and 4 as previously described.

(*b*) Save the first four ether extracts used in the preparation of Kahn or Eagle antigens. Concentrate to about a fifth of the volume in an evaporating dish and add three to six volumes of acetone. After mixing and setting aside overnight, the supernatant acetone is removed and the residue of acetone-insoluble lipoids kept in a refrigerator.

(*c*) Measure the alcoholic filtrate and for each cubic centimeter add 0.002 Gm. of cholesterol. Dissolve the cholesterol and 1 to 2 Gm. of the acetone-insoluble lipoids in 20 c.c. of ether and add to the alcoholic extract in a tightly stoppered bottle or flask.

(*d*) Shake thoroughly and place in a water bath at 55° C. for one hour to aid in the solution of the lipoids.

(*e*) Allow to stand at room temperature for two or three days with brief shaking each day. Filter through fat-free paper.

(*f*) Keep at *room temperature.* Do not disturb any sediment that may be present.

Preparation of Sera.—1. All specimens are lined up and properly labelled.

2. The sera are removed from the clots, with capillary pipets, to test tubes properly labeled. Great care is required to prevent errors in labeling and confusion of sera. Each serum should be free of corpuscles; otherwise, it is necessary to break up the clots with wooden applicators (one for each serum) and centrifuging for clear serum. Slight tinging with hemoglobin does no harm. Sera containing large amounts of hemoglobin are likely to be anticomplementary and unsatisfactory for both complement-fixation and precipitation tests.

3. It is not necessary to remove the natural antisheep hemolysins by absorption with *thoroughly washed sheep corpuscles* although this tends to increase the sensitiveness of complement-fixation reactions and especially in the case of sera containing small amounts of syphilis antibody. Kolmer recommended the routine removal of natural hemolysins when conditions permit in order to secure reactions of maximal sensitiveness, but when large numbers of sera require testing this procedure may be omitted. The method for removal of natural antisheep hemolysins from sera is as follows:

(*a*) To each is added *a drop of washed sheep corpuscle sediment for approximately each 2 c.c. of blood and serum* as gaged by inspection.

(*b*) Each specimen is then thoroughly mixed with a wooden applicator (one for each serum).

(*c*) All are then placed in a *refrigerator for fifteen minutes* to enable the sheep corpuscles to absorb the hemolysins with none or but a minimal and harmless amount of hemolysis.

(*d*) All specimens are now centrifuged and the sera separated into test tubes properly labelled.

4. The tubes of plain or absorbed sera are now placed in a water bath at 55° C. for fifteen to twenty minutes when they are ready for testing.

Preparation of Spinal Fluids.—These are usually tested without any preliminary preparation as they do not contain enough natural antisheep hemolysin to require removal or enough complement to require inactivation by heating at 55° C. If a specimen contains considerable blood which has not had time to settle out, it should be centrifuged. Otherwise, no preparation is required as spinal fluids are tested as delivered without preliminary heating unless they are more

than three days old, in which case they may be heated at 55° C. for fifteen minutes to remove thermolabile anticomplementary substances. As a general rule, however, spinal fluids are not anticomplementary unless heavily contaminated with bacteria (cloudy).

Titration of Hemolysin.—It is advisable (but not absolutely necessary) to make this titration each time the complement-fixation test is conducted.

Prepare a stock dilution of 1:100 hemolysin as follows:

Glycerinized hemolysin (50 per cent)	2.0 c.c.
Saline solution	94.0 "
Phenol (5 per cent in saline solution)	4.0 "

This may be kept in the refrigerator for several weeks.

For titration, dilute to 1:1000 (0.5 c.c. of 1:100 + 4.5 c.c. saline solution).

In a series of 10 tubes, prepare higher dilutions as follows:

No. 1. 0.5 c.c. hemolysin (1:1,000) = 1:1,000
No. 2. 0.5 c.c. hemolysin (1:1,000) + 0.5 c.c. saline solution = 1:2,000
No. 3. 0.5 c.c. hemolysin (1:1,000) + 1.0 c.c. saline solution = 1:3,000
No. 4. 0.5 c.c. hemolysin (1:1,000) + 1.5 c.c. saline solution = 1:4,000
No. 5. 0.5 c.c. hemolysin (1:1,000) + 2.0 c.c. saline solution = 1:5,000
No. 6. 0.5 c.c. hemolysin (1:3,000) + 0.5 c.c. saline solution = 1:6,000
No. 7. 0.5 c.c. hemolysin (1:4,000) + 0.5 c.c. saline solution = 1:8,000
No. 8. 0.5 c.c. hemolysin (1:5,000) + 0.5 c.c. saline solution = 1:10,000
No. 9. 0.5 c.c. hemolysin (1:6,000) + 0.5 c.c. saline solution = 1:12,000
No. 10. 0.5 c.c. hemolysin (1:8,000) + 0.5 c.c. saline solution = 1:16,000

Mix the contents of each tube thoroughly.

Prepare a 1:30 dilution of complement for hemolysin and complement titration by diluting 0.2 c.c. of complement serum with 5.8 c.c. of saline solution.

Prepare a 2 per cent suspension of sheep corpuscles.

In a series of ten tubes set up the hemolysin titration as shown in the following table:

Tube.	Hemolysin, 0.5 c.c.	Complement, c.c. (1:30).	Saline solution, c.c.	Corpuscles, c.c.
1	1:1,000	0.3	1.7	0.5
2	1:2,000	0.3	1.7	0.5
3	1:3,000	0.3	1.7	0.5
4	1:4,000	0.3	1.7	0.5
5	1:5,000	0.3	1.7	0.5
6	1:6,000	0.3	1.7	0.5
7	1:8,000	0.3	1.7	0.5
8	1:10,000	0.3	1.7	0.5
9	1:12,000	0.3	1.7	0.5
10	1:16,000	0.3	1.7	0.5

Mix the contents of each tube and incubate in the water bath at 38° C. for one hour. Read the unit of hemolysin. The *unit is the highest dilution of hemolysin that gives complete hemolysis.*

Two units are used in the titration of complement and antigen and in the complement-fixation tests. Hemolysis is so diluted that 0.5 c.c. contains two units. For example, if the unit equals 0.5 c.c. of 1:6000, two units equal 0.5 c.c. of 1:3000. Dilute just enough hemolysin for the complement titration and the complement-fixation tests. *Keep hemolysin and corpuscles in suspension in the refrigerator when not in use.*

The following table shows how the dilutions are made so that 0.5 c.c. carries two units:

1 unit 0.5 c.c. of	2 units would be 0.5 c.c. of	Prepared by diluting 1 c.c. of stock 1:100 solution with following amounts of saline solution.
1:1,000	1:500	4 c.c.
1:2,000	1:1000	9 c.c.
1:3,000	1:1500	14 c.c.
1:4,000	1:2000	19 c.c.
1:5,000	1:2500	24 c.c.
1:6,000	1:3000	29 c.c.
1:8,000	1:4000	39 c.c.
1:10,000	1:5000	49 c.c.

High titer hemolysin is recommended and the unit should be 0.5 c.c. of 1:4000 or higher.

In practice the hemolysin titration may be placed in the water bath at the same time as the complement titration; at the end of the first incubation of the complement titration the unit of hemolysin is available and two units added to all tubes of the complement titration, and so forth.

Complement Titration.—For the complement titration use 1:30 dilution of complement prepared as previously described. *Dilute antigen so that the dose employed in the main tests is contained in 0.5 c.c.* This dilution is made by placing the required amount of saline solution in a flask and adding antigen drop by drop. Shake the flask after each addition of antigen. Prepare enough antigen dilution for the complement titration and the complement-fixation tests.

In a series of ten-test-tube set-up the complement titrations are as follows:

The smallest amount of complement just giving complete sparkling hemolysis is the *exact unit.* The next higher tube is the *full unit* which contains 0.05 c.c. more complement. In conducting the antigen titra-

Tube.	Complement, c.c. (1: 30).	Antigen, dose, c.c.	Saline solution, c.c.		Hemolysin, c.c. (2 units).	Corpuscles, c.c. (2 per cent).	
1.....	0.1	0.5	1.4	Water bath 37° C. for one hour.	0.5	0.5	Water bath 37° C. for one hour.
2.....	0.15	0.5	1.4		0.5	0.5	
3.....	0.2	0.5	1.3		0.5	0.5	
4.....	0.25	0.5	1.3		0.5	0.5	
5.....	0.3	0.5	1.2		0.5	0.5	
6.....	0.35	0.5	1.2		0.5	0.5	
7.....	0.4	0.5	1.1		0.5	0.5	
8.....	0.45	0.5	1.1		0.5	0.5	
9.....	0.5	0.5	1.0		0.5	0.5	
10.....	None	None	2.5		None	0.5	

tion and complement-fixation tests, *two full units* are employed and so diluted as to be contained in 1 c.c. as in the following example:

Exact unit: 0.3 c.c.
Full unit: 0.35 c.c.
Dose (two full units): 0.7 c.c.

To calculate the dilution to employ so that 1 c.c. contains the dose of two full units, divide 30 by the dose:

$$\frac{30}{0.7} = 43 \text{ or } 1 \text{ c.c. of } 1{:}43 \text{ dilution of serum.}$$

The following table gives additional examples:

Exact unit, c.c.	Full unit, c.c.	Two full units, c.c.	Dilution to use.	Preparation.
0.2	0.25	0.5	1: 60	1 c.c. serum + 59 c.c. saline sol.
0.25	0.3	0.6	1: 50	1 c.c. serum + 49 c.c. saline sol.
0.3	0.35	0.7	1: 43	1 c.c. serum + 42 c.c. saline sol.
0.35	0.4	0.8	1: 37	1 c.c. serum + 36 c.c. saline sol.
0.4	0.45	0.9	1: 33	1 c.c. serum + 32 c.c. saline sol.
0.45	0.5	1.0	1: 30	1 c.c. serum + 29 c.c. saline sol.
0.5	0.55	1.1	1: 27	1 c.c. serum + 26 c.c. saline sol.

If, however, the complement is unusually sensitive to the anticomplementary effects of antigen and serum in the conduct of the complement-fixation tests, as is sometimes the case during the hot summer months, two and one-half exact units may be employed and so diluted that 1 c.c. contains this dose. Example:

Exact unit: 0.35 c.c.
$2\frac{1}{2}$ units: 0.88 c.c.

To calculate the dilution to use so that 1 c.c. contains the dose, divide 30 by the dose:

$$\frac{30}{0.88} = 34 \text{ or } 1 \text{ c.c. of } 1{:}34 \text{ dilution of serum.}$$

The following table contains additional examples:

Unit, c.c.	2½ units, c.c.	Dilution to use.	Preparation.
0.2	0.5	1: 60	1 c.c. serum + 59 c.c. saline solution
0.25	0.63	1: 47	1 c.c. serum + 46 c.c. saline solution
0.3	0.75	1: 40	1 c.c. serum + 39 c.c. saline solution
0.35	0.88	1: 34	1 c.c. serum + 33 c.c. saline solution
0.4	1.0	1: 30	1 c.c. serum + 29 c.c. saline solution
0.45	1.13	1: 27	1 c.c. serum + 26 c.c. saline solution
0.5	1.25	1: 24	1 c.c. serum + 23 c.c. saline solution

It is always advisable to dilute complement serum with cold saline solution instead of with saline solution kept at room temperature. Undiluted and especially diluted complement serum should always be kept in a refrigerator when not in use. Exposure of diluted complement to room temperature for more than an hour may result in some deterioration.

Occasionally, hyperactive complement yields a unit of 0.1 to 0.25 c.c. of 1:30 but when this occurs it is necessary arbitrarily to take 0.3 c.c. as the exact unit as less complement falls below the absolute minimum and is likely to be unsatisfactory.

Titration of Antigen.—It is not necessary to titrate for hemolytic and anticomplementary units as hitherto advised, because titrations of more than 280 antigens by Kolmer during the past fourteen years have never shown any to be hemolytic in 0.5 c.c. of 1:4 and the anticomplementary units have been uniformly from 0.5 c.c. of 1:6 to not higher than 1:10. Therefore, these titrations may be omitted providing the antigen is prepared by either of the methods described. It is, however, necessary to titrate for *antigenic activity* and the following method of Boerner and Lukens is recommended.

1. Prepare a 1:80 dilution of antigen by adding 0.1 c.c. of antigen, drop by drop with shaking between each, to 7.9 c.c. of saline solution in a large test tube or small flask. Higher dilutions are then prepared as follows:

4 c.c. of 1:80 + 4 c.c. saline solution = 1:160
4 c.c. of 1:160 + 4 c.c. saline solution = 1:320
4 c.c. of 1:320 + 4 c.c. saline solution = 1:640
4 c.c. of 1:640 + 4 c.c. saline solution = 1:1280
4 c.c. of 1:1280 + 4 c.c. saline solution = 1:2560

2. Arrange five rows of test tubes with six in each row. In the *first* tube of each row place 0.5 c.c. of antigen 1:80; to the *second* tube of each row, 0.5 c.c. of antigen 1:160; to the *third* tube, 0.5 c.c. of 1:320; to the *fourth*, 0.5 c.c. of 1:640; to the *fifth* 0.5 c.c. of 1:1280 and to the *sixth* 0.5 c.c. of 1:2560.

3. Heat 3 c.c. of a moderately to strongly positive syphilitic serum in a water bath at 55° C. for fifteen to twenty minutes and prepare five dilutions in large test tubes as follows:

1.0 c.c. serum + 4.0 c.c. saline solution = 1:5 (0.5 c.c. carries 0.1 c.c. serum)
0.5 c.c. serum + 4.5 c.c. saline solution = 1:10 (0.5 c.c. carries 0.05 c.c. serum)
0.5 c.c. serum + 9.5 c.c. saline solution = 1:20 (0.5 c.c. carries 0.025 c.c. serum)
2.0 c.c. serum 1:20 + 2.0 c.c. saline solution = 1:40 (0.5 c.c. carries 0.0125 c.c. serum)
1.0 c.c. serum 1:20 + 4.0 c.c. saline solution = 1:100 (0.5 c.c. carries 0.005 c.c. serum)

4. Add 0.5 c.c. of 1:5 dilution to each of the six tubes of the first row; 0.5 c.c. of 1:10 to each tube of the second row; 0.5 c.c. of 1:20 to each tube of the third row; 0.5 c.c. of 1:40 to each tube of the fourth row and 0.5 c.c. of 1:100 to each tube of the fifth row.

5. Add 1 c.c. of complement dilution carrying two full units to all tubes.

6. Put up a *serum control* carrying 0.5 c.c. of 1:15 serum and 1 c.c. of complement (two and one-half units); also a *hemolytic system control* carrying 1 c.c. of saline solution and 1 c.c. of complement (two full units).

7. Shake the tubes gently and place in refrigerator at 6° to 8° C. for fifteen to eighteen hours, followed by incubation in a water bath at 37° C. for ten minutes.

8. Add 0.5 c.c. of hemolysin (two units) and 0.5 c.c. of 2 per cent suspension corpuscles to all tubes.

9. Mix thoroughly and place in a water bath at 37° C. for one hour; make readings. The serum and hemolytic system controls should show complete hemolysis.

10. Chart the results according to the following example observed with a strongly positive serum.

Serum in 0.5 c.c.	Antigen in 0.5-c.c. amounts.					
	1:80.	1:160.	1:320.	1:640.	1:1280.	1:2560.
0.005	−	−	++	−	−	−
0.0125	−	+	++++	++++	++	+
0.025	+	++++	++++	++++	++++	+
0.05	+++	++++	++++	++++	++++	++
0.1	++++	++++	++++	++++	++++	+++

11. The dose of antigen to employ in the main tests is the *largest amount giving a ++++ reaction with the smallest amount of serum.* If three dilutions of antigen give ++++ reactions with the smallest amount of serum, the dose to use should be midway between the highest and lowest.

Choice of Complement-fixation Methods.—Two methods are available. One, the *quantitative test*, employs five doses of serum or spinal fluid and is generally preferred, especially in testing the sera in cases of syphilis in which the patients are undergoing treatment, in order to secure serologic evidence of improvement. The second method designated as the *qualitative test*, uses two doses of serum, 0.2 and 0.1 c.c., with an additional third tube as a control (0.2 c.c.). It is sufficient for diagnostic purposes as well as being more economical of materials and time required. Both are of equal sensitiveness and specificity.

The Quantitative Complement-fixation Test.—1. *For each serum:*

(*a*) Arrange six test tubes and place in them the following amounts of saline solution in the respective tubes: 0.9, 0.5, 0.5, 0.5, 2, and 0.5 c.c.

(*b*) To tube 1, add 0.6 c.c. of inactivated serum. Mix by drawing up in the pipet several times and transfer 0.5 c.c. to tube 2 and 0.5 c.c. to tube 6 (serum control).

(*c*) Mix the contents of tube 2 and transfer 0.5 c.c. to tube 3.

(*d*) Mix contents of tube 3 and transfer 0.5 c.c. to tube 4.

(*e*) Mix contents of tube 4 and transfer 0.5 c.c. to tube 5; mix contents of tube 5 and discard 2 c.c.

This leaves 0.5 c.c. in each of the first five tubes carrying the following amounts of serum, 0.2, 0.1, 0.05, 0.025, and 0.005 c.c.; tube 6 (serum control) carries 1 or 0.2 c.c. of serum since it receives no antigen and thereby makes the total volume in all tubes the same when the test is finished.

2. *For each spinal fluid:*

(*a*) Arrange six tubes and place 0.5 c.c. saline solution in tubes 2, 3, 4, 5, and 6.

(*b*) In tubes 1, 2, and 6 place 0.5 c.c. of spinal fluid. Mix contents of tube 2 and transfer 0.5 c.c. to tube 3. Mix contents of tube 3 and transfer 0.5 c.c. to tube 4. Mix contents of tube 4 and transfer 0.5 c.c. to tube 5. Mix contents of tube 5 and discard 0.5 c.c.

(*c*) Tubes 1 to 5 now contain 0.5 c.c., carrying 0.5, 0.25, 0.125, 0.0625, and 0.03125 c.c. of spinal fluid. Tube 6 (control) contains 1 c.c. carrying 0.5 c.c. of spinal fluid.

3. To the first five tubes of each set of tubes of serum or spinal fluid add 0.5 c.c. of diluted antigen carrying the proper dose.

4. After an interval of ten to thirty minutes, add 1 c.c. complement (two full units) to each tube.

5. Include the following controls:

Antigen control containing 0.5 c.c. of diluted antigen, 0.5 c.c. of saline solution and 1 c.c. of diluted complement (two full units).

Hemolytic system control containing 1 c.c. of saline solution and 1 c.c. of diluted complement.

Corpuscle control containing 2.5 c.c. of saline solution.

Positive and negative serum controls should be included.

6. Mix the contents of each tube by gently shaking and place tubes in the refrigerator at 6° to 8° C. for fifteen to eighteen hours.

7. Place tubes in the water bath at 37° C. for ten to fifteen minutes (not longer).

8. To all tubes, except the corpuscle control, add 0.5 c.c. of hemolysin (carrying two units) and to all tubes add 0.5 c.c. of 2 per cent corpuscle suspension (shaken up).

9. Mix the contents of each tube by gently shaking and place in the water bath at 37° C. for one hour, when the readings are made. *More sensitive readings may be made ten minutes after the antigen, hemolytic system and serum controls show complete hemolysis, and this time of reading is preferred.*

10. The following table shows the set-up for the quantitative complement-fixation test with serum:

Tube.	Patient's serum, in 0.5 c.c.	Antigen, c.c.	Interval of 10 to 30 minutes at room temperature.	Complement, c.c. (2 full units).	Primary incubation in refrigerator at 6° to 8° for 15 to 18 hours, followed by 10 to 15 minutes at 37° C.	Hemolysin, c.c. (2 units).	Corpuscles, c.c. (2 per cent).	Secondary incubation in water at 37° C.
1	0.2 c.c.	0.5		1.0		0.5	0.5	
2	0.1 c.c.	0.5		1.0		0.5	0.5	
3	0.05 c.c.	0.5		1.0		0.5	0.5	
4	0.025 c.c.	0.5		1.0		0.5	0.5	
5	0.005 c.c.	0.5		1.0		0.5	0.5	
6	0.2 c.c. (control).	None		1.0		0.5	0.5	
7	Antigen control: 0.5 c.c. saline solution.	0.5		1.0		0.5	0.5	
8	Hemolytic control: 1.0 c.c. saline solution.	None		1.0		0.5	0.5	
9	Corpuscle control: 2.5 c.c. saline solution.	None		None		None	0.5	

11. After the secondary incubation the readings may be made at once or after the tubes have been placed in the refrigerator for several hours to permit the settling of nonhemolyzed corpuscles. Read the degree of inhibition of hemolysis and record for each tube as: −

(complete hemolysis); + (25 per cent inhibition recorded as 1); ++ (50 per cent inhibition recorded as 2); +++ (75 per cent inhibition recorded as 3); ++++ (100 per cent inhibition recorded as 4). All serum, antigen and hemolytic controls should show complete hemolysis. The corpuscle control should show no hemolysis.

12. Reactions may be interpreted as follows:

(*a*) *Very strongly positive* when *complete fixation* (++++) occurs in the third, fourth or fifth tubes. Examples: 44444; 4444−; 4442−; 444−−; 344−−.

(*b*) *Strongly positive* when *complete fixation* (++++) occurs in the second tube. Examples: 4431−; 442−−; 342−−; 44−−−.

(*c*) *Moderately positive* when *complete fixation* (++++) occurs in the first tube. Examples: 431−−; 42−−−; 4−−−−.

(*d*) *Weakly positive* when *partial fixation* occurs in one or more tubes. Examples: 321−−; 21−−−; 1−−−−.

(*e*) *Doubtfully positive* when the reaction is ± in the first tube. Example ±−−−−.

(*f*) *Negative* when there is complete hemolysis in all tubes. Example −−−−−.

The method of recording and reporting the results of complement-fixation tests by this method is according to the following examples:

Quantitative reaction = strongly positive (442−−)
Serum 0.2 c.c. = +++++
Serum 0.1 c.c. = ++++
Serum 0.05 c.c. = ++
Serum 0.025 c.c. = −
Serum 0.005 c.c. = −
Serum 0.2 c.c. (control) = −

Quantitative reaction = strongly positive (42−−−)
Spinal fluid 0.5 c.c. = ++++
Spinal fluid 0.25 c.c. = ++
Spinal fluid 0.125 c.c. = −
Spinal fluid 0.0625 c.c. = −
Spinal fluid 0.03125 c.c. = −
Spinal fluid 0.5 (control) = −

The American Committee on Evaluation of Serodiagnostic Tests for Syphilis has recommended reporting reactions only as positive, doubtful, or negative.

The Qualitative Complement-fixation Test.—1. This test is conducted in exactly the same manner as described for the quantitative test except that three doses of serum (0.2 and 0.1 c.c. with 0.2 c.c. in the *serum control*) are employed. With *spinal fluid* a single dose of 0.5 c.c. in the control is employed.

2. For each *serum* arrange three test tubes and place in them the following amounts of saline solution in the respective tubes: 0.9, 0.5, and 0.5 c.c.

To tube 1 add 0.6 c.c. of inactivated serum. Mix by drawing up in the pipet several times and transfer 0.5 c.c. to tube 2 and 0.5 c.c. to tube 3 (serum control).

Mix the contents of tube 2 and discard 0.5 c.c.

This leaves 0.5 c.c. in each of the first two tubes carrying 0.2 and 0.1 c.c. of serum respectively. Tube 3 contains 1 c.c. (0.2 c.c. of serum) since it receives no antigen; this makes the total volume in all tubes the same when the test is finished.

3. For each *spinal fluid* arrange two tubes and place 0.5 c.c. of the fluid in each. The first tube receives antigen; the second does not and is the control. To tube 2 add 0.5 c.c. of saline solution.

4. Place 0.5 c.c. of proper dilution of antigen in tubes 1 and 2 of each serum and in tube 1 of each spinal fluid; also, in control tube carrying 0.5 c.c. of saline solution (antigen control).

5. Allow tubes to stand ten to thirty minutes. Then add two full units of complement (1 c.c.) to each tube; also to a control carrying 1 c.c. saline solution (hemolytic system control).

6. Put up corpuscle control: 2.5 c.c. of saline solution.

7. Mix contents of tubes gently and place tubes in a refrigerator at 6° to 8° C. for fifteen to eighteen hours. Keep hemolysin and corpuscle suspension in refrigerator.

8. Place tubes in water bath at 37° C. for ten to fifteen minutes (not longer).

9. To all tubes except corpuscle control add two units of hemolysin.

10. To all tubes add 0.5 c.c. of 2 per cent corpuscle suspension (shaken up).

11. Mix and place tubes in water bath for one hour, when the readings are made, or place the tubes in a refrigerator for an hour or two before making the readings. *More sensitive readings may be made ten minutes after the antigen, hemolytic systems and serum controls show complete hemolysis; this method of reading is preferred.*

12. The serum, hemolytic system and antigen controls should be completely hemolyzed; the corpuscle control should show no hemolysis.

13. Read and record the *serum* tests as follows:

(*a*) *Strongly positive:* complete fixation (++++) in first or second tube. Examples: 44; 43; 34.

(*b*) *Moderately positive:* complete fixation (++++) in first tube only. Examples: 42; 41.

(*c*) *Weakly positive:* partial fixation in one or both tubes. Examples: 31; 21; 3−; 2−; 1−.

(*d*) *Doubtfully positive:* ± in first tube. Example: ± −.

(*e*) *Negative:* complete hemolysis in both tubes.

14. Read and record the *spinal fluid* tests as follows according to first tube:

++++ = Strongly positive
+++ = Moderately positive
++ or + = Weakly positive
± = Doubtfully positive
− = Negative

The American Committee on Evaluation of Serodiagnostic Tests for Syphilis has recommended reporting reactions only as positive, doubtful, or negative.

Modified Technic for Small Amounts of Serum and Spinal Fluid.—It sometimes occurs that a sufficient amount of serum or spinal fluid is not available for conducting the quantitative test. In such cases the test can be conducted in exactly the same manner as described, *by using half of the usual amount of all reagents*. The hemolysin and complement are not titrated separately for this test as the same dilutions are employed in half the amounts. The same antigen dilution is also used but in half the amount.

1. For each *serum:*

(*a*) Arrange 6 test tubes and place the following respective amounts of saline solution in the tubes: 1.2, 0.5, 0.5, 0.5, 2, and 0.25 c.c.

(*b*) To Tube 1 add 0.3 c.c. of inactivated serum. Mix contents by drawing up in the pipet several times and transfer 0.5 c.c. to Tube 2 and 0.5 c.c. to Tube 6 (serum control).

(*c*) Mix contents of Tube 2 and transfer 0.5 c.c. to Tube 3.

(*d*) Mix contents of Tube 3 and transfer 0.5 c.c. to Tube 4.

(*e*) Mix contents of Tube 4 and transfer 0.5 c.c. to Tube 5; mix contents of this tube and discard 2 c.c.

This leaves 0.5 c.c. in each of the 5 tubes, carrying exactly half of the amounts of serum recommended for the regular test, namely; 0.1, 0.05, 0.025, 0.0125, and 0.0025 c.c.; Tube 6 (control) carries 0.1 c.c. of serum.

2. For each *spinal fluid:*

(*a*) Arrange 6 test tubes and place the following amounts of saline solution in the respective tubes: 0.75, 0.5, 0.5, 0.5, 0.5, and 0.25 c.c.

(*b*) To Tube 1 add 0.75 c.c. of spinal fluid. Mix the contents and transfer 0.5 c.c. to Tube 2 and 0.5 c.c. to Tube 6.

(*c*) Mix contents of Tube 2 and transfer 0.5 c.c. to Tube 3.

(*d*) Mix contents of Tube 3 and transfer 0.5 c.c. to Tube 4.

(*e*) Mix contents of Tube 4 and transfer 0.5 c.c. to Tube 5.

(*f*) Mix contents of Tube 5 and discard 0.5 c.c.

This leaves 0.5 c.c. in each of the 5 tubes, carrying exactly half the amounts of spinal fluid recommended in the regular test, namely; 0.25, 0.125, 0.0625, 0.0312, and 0.0156 c.c.

3. Proceed as directed on page 745, for the quantitative test, using exactly half the amount of each reagent. Start with Step 3.

4. Readings are reported in the same manner as described for the regular test.

5. A *qualitative* test may be conducted by placing 0.1, 0.05, and 0.1 c.c. (control) of serum in each of 3 test tubes. With spinal fluid, place 0.25 c.c. in each of 2 tubes (the second being the control). Add sufficient saline solution to make the total volume 0.5 c.c. in each tube of the test, with 0.75 c.c. in the control.

6. Proceed as directed for the qualitative test, using exactly half the amount of each reagent. Start with Step 4.

7. Readings are reported in the same manner as described for the regular test.

Analysis of Difficulties.—Defective Complement.—In the great majority of instances difficulties are due to complement supersensitive to the anticomplementary effects of antigen, serum, or both. This is especially likely to occur during the hot months of the year. Sometimes complement may be defective in hemolytic activity and whenever the unit is higher than 0.5 c.c. of 1:30 dilution it should not be used. But sometimes the complement is satisfactory from this standpoint but yet defective in the tests because supersensitive to antigen and prone to give prezone reactions. With negative or normal sera the reactions are likely to be −12−− or −1244 with perfect serum controls. Since $2\frac{1}{2}$ units of complement have been used instead of 2 full units as originally advised, this difficulty has been greatly reduced. Furthermore, under these conditions lyophil or cryochem complement is recommended since the complement is a mixture of the sera of a large number of guinea-pigs and may be prepared during the cold months of the year. When fresh serum is used it is important to prepare it from several *full grown*, healthy and *previously* unused guinea-pigs. As a general rule, the trouble is first thought to be due to defective hemolysin but, since this reagent keeps very well, it is seldom responsible.

Giordano and Carlson[1] reported that the serum of some guinea-pigs contains a substance which fixes complement in the presence of antigen at refrigerator temperature. This substance is thermostable and does not react at 37° C. Since this substance will not be detected in the daily titrations, they suggested that each guinea-pig serum employed as complement be subjected to a preliminary test for this factor.

Defective Saline Solution.—When trouble is experienced with the hemolytic system when first using these methods, it is likely that the saline solution is at fault. If it has been prepared with distilled water, try a saline solution prepared by dissolving 8.5 Gm. of chemically pure sodium chloride in 1000 c.c. of ordinary tap water; it is sometimes advantageous to add 0.1 Gm. of magnesium sulfate. If, however, trouble is experienced after a saline solution has been previously used with success it is unlikely to be the cause. Compressed tablets of salt should not be used.

Defective Hemolysin.—This is probably least likely to be a cause of trouble although usually first suspected, especially if the hemolysin has been previously used with success. The unit of antisheep hemolysin should be at least 0.5 c.c. of 1:4000 and sera of this and higher strengths are so easy to prepare that it is a mistake to use weaker products. If the saline solution and complement are satisfactory, a good hemolysin is rarely responsible even when shipped over long distances.

Defective Corpuscles.—When blood is obtained from an abattoir one is almost sure sooner or later to encounter the corpuscles of occasional animals possessing *increased resistance* to serum hemolysis. The cause of this phenomenon is unknown; fortunately it is rare. The remedy is to discard the corpuscles and secure a fresh supply of blood.

Anticomplementary Antigen.—Providing no mistakes have occurred in dilution and dosage, this is very rarely a cause of trouble. When the antigen control shows incomplete hemolysis it is almost surely due to some component of the hemolytic system, especially the complement.

Anticomplementary Sera.—Sera and spinal fluids may be found to be anticomplementary, as shown by incomplete hemolysis of the serum

[1] Giordano, A. S., and Carlson, Bonita: Occurrence of a Non-specific Substance in Guinea-pig Serum Fixed by Antigen in the Wassermann Test, Amer. Jour. Clin. Path., 9:130–135 (Mar), 1939.

controls. After experience has been gained some of these reactions may be safely read, but as a general rule, it is safer and wiser to repeat the tests with fresh serum, especially in the case of those technicians lacking experience in complement-fixation work. It is infinitely better to repeat the tests than to run the slightest chances of error, especially the regrettable and almost unpardonable error of rendering a falsely positive report. Sometimes the majority of sera of a day's work show incomplete hemolysis of the serum controls, but this trouble is not due to anticomplementary effects on their part but rather to the use of a defective supersensitive complement. Under these conditions the tests must be repeated and for this reason the *unused portions of all sera should be routinely kept in a refrigerator until the tests are completed, in case repetitions are required.*

Modified Sachs' Method for Anticomplementary Sera.—Sachs has described a very useful method for testing anticomplementary sera. Sera very deeply tinged with hemoglobin do not respond quite as well to this method. Sera of rabbits and dogs and the sera of other of the lower animals may be treated in the same manner since it likewise removes the anticomplementary substances from most of these, but not the substances responsible for the nonspecific complement-fixation reactions sometimes yielded by the normal sera of rabbits, dogs, and mules. The method has not been applied to spinal fluids.

1. Heat 0.5 c.c. of serum at 55° C. for fifteen minutes.
2. Add 4.1 c.c. of accurately titrated N/300 hydrochloric acid and mix.
3. After the tube has stood one-half hour at room temperature, centrifuge thoroughly and discard the sediment.
4. To the supernatant fluid add 0.4 c.c. of 10 per cent solution of sodium chloride. The acid is fixed by the precipitate of globulin; hence, neutralization is unnecessary.
5. This gives a 1:10 dilution of original serum ready for testing.
6. Arrange 2 rows of 5 test tubes (the rear row are serum controls and receive no antigen).
7. Place 1 c.c. of normal saline solution in Tubes 3 and 4 and 2 c.c. in Tube 5 of the first row; place 0.5 c.c. in each of the 5 tubes of the second row.
8. Place 1 c.c. of serum diluted 1:10 in the first and third tubes of the first row and 0.5 c.c. in the second tube. Mix the contents of Tube 3 and transfer 1 c.c. to Tube 4 and 0.5 c.c. to Tube 3 of the second row. Mix the contents of Tube 4; transfer 0.5 c.c. to Tube 5; 0.5 c.c. to Tube 4 of second row and discard 0.5 c.c. Mix the contents of Tube 5, transfer 0.5 c.c. to Tube 5 of the rear row and discard 1.5 c.c.

9. Place 1 c.c. of serum diluted 1:10 in Tube 1 and 0.5 c.c. in Tube 2 of the second row.
10. Add antigen (0.5 c.c. of proper dilution as used in the regular test) to each tube of the front row. Allow to stand at room temperature for ten to thirty minutes when 2 full units of complement are added to all tubes of both rows and the balance of the test is completed in the usual manner.
11. The tubes of the front and rear rows carry 0.1, 0.05, 0.025, 0.0125, and 0.0025 c.c. of serum respectively.
12. Upon completion of the test all of the tubes of the second row should show complete hemolysis. However, the first tube carrying 0.1 c.c., and sometimes the second carrying 0.05 c.c. of serum, may not show complete hemolysis. With negative sera the corresponding front tubes show the same degree of inhibition of hemolysis and under these conditions a negative report may be rendered. With positive sera inhibition of hemolysis is much more marked in the tubes of the front row. It is advisable to report the reactions as positive, doubtful, or negative.

Prevention of Nonspecific and Prezone Reactions.—Kolmer[1] recommended that egg albumin complement mixtures be used routinely in both the simplified and quantitative Kolmer tests with spinal fluid to prevent nonspecific and prezone reactions. He also recommended that, if quantitative Kolmer tests with nonsyphilitic serums give nonspecific reactions, the egg albumin complement mixture be used.

Carefully separate the white from the yolk of an egg. Make a 10 per cent solution of egg albumin in sterile salt solution. Measure the amount of egg albumin and beat it before adding to the salt solution. Use this 10 per cent solution of egg albumin instead of plain saline solution in preparing the 1:30 dilution of complement for the preliminary titration. Also use this same albumin solution in making the dilution of complement to contain two full units.

IV. MISCELLANEOUS SEROLOGIC TESTS

A. Autohemolysis

In paroxysmal hemoglobinuria there is destruction of erythrocytes, which results in hemoglobinemia and in the excretion of hemoglobin in the

[1] Kolmer, J. A.: The Prevention of Non-specific and Prezone Reactions in the Wassermann Test with Sera and Spinal Fluids by the Addition of Egg Albumin to the Complement, Amer. Jour. Clin. Path., *11*:402–413 (May), 1941.

urine. This reaction occurs when the patient becomes chilled. Rosenbach has demonstrated that the phenomena of a typical paroxysm of the disease may be produced by placing the patient's hands or feet in ice water. Donath and Landsteiner demonstrated in vitro that the hemolysis is caused apparently by an autohemolysin which is thermolabile and will not act at body temperature, but which is often very active at a temperature slightly above 0° C. MacKenzie[1] has reviewed the literature and reported his studies, in which he employed a simple technic for the demonstration of the presence of this unusual substance in the blood serum. As a rule, but not always, the patient's serum may disclose a strongly positive Wassermann reaction. MacKenzie has demonstrated, however, that the complement-fixing syphilitic body is entirely independent of the autohemolysin, which may also be an isohemolysin if it acts on erythrocytes from the blood of another individual, but of the same blood group.

Donath-Landsteiner Test (*MacKenzie Method*).—Withdraw blood by venipuncture from the patient's arm. Place a portion of the blood in a tube which contains a solution of sodium citrate. Place the rest in a clean dry tube; allow it to clot; centrifugalize; and remove the serum immediately. (The lysin deteriorates rapidly and it therefore is advisable to set up the test as promptly as possible.) Centrifugalize the citrated blood and wash three times with 0.85 per cent solution of sodium chloride, and make a 5 per cent suspension of erythrocytes in physiologic saline solution. Prepare also as complement a 1:10 dilution of fresh guinea-pig serum. Set up the test in small tubes as follows:

Serum.	Five per cent suspension of erythrocytes.	Complement 1:10.	Saline solution 0.85 per cent.
0.25 c.c.	0.1 c.c.	0.1 c.c.	0.05 c.c.

Double these quantities may be used. Set up a control which consists of serum and a suspension of erythrocytes from a normal patient. Also set up a test for isohemolysin, using the patient's serum and a suspension of erythrocytes from another individual of the same blood group.

Plunge the tubes into melting ice for ten minutes and then incubate for two hours in a water bath of 37° C. Complete hemolysis of the patient's erythrocytes in his own serum will occur in a strongly positive test.

Marchiafava-Micheli Syndrome Test.—Ham and Horack[2] described the following technic for demonstrating the tendency to hemolysis of the corpuscles of patients with chronic hemolytic anemia and paroxysmal nocturnal hemoglobinuria. Defibrinate 10 c.c. of the patient's blood, and as a

[1] MacKenzie, G. M.: Observations on Paroxysmal Hemoglobinuria, Jour. Clin. Investigation, *7*:27–43, 1929.

[2] Ham, G. C., and Horack, H. M.: Chronic Hemolytic Anemia with Paroxysmal Nocturnal Hemoglobinuria: Report of a Case with Only Occasional Hemoglobinuria and with Complete Autopsy, Arch. Int. Med., *67*:735–745 (Apr.), 1941.

control also defibrinate 10 c.c. of normal blood. Centrifuge, wash the corpuscles three times with physiologic salt solution, and make a 5 per cent suspension of corpuscles in physiologic salt solution. Centrifuge 1 c.c. samples of the suspension of corpuscles and discard the supernatant salt solution from each sample. Resuspend the corpuscles of both the patient and the control in 1 c.c. of the patient's serum and make a similar suspension of the corpuscles of the patient and of the control in 1 c.c. of normal serum. Also set up another set of tubes, adding 0.05 c.c. third normal hydrochloric acid to 0.95 c.c. of the serum of both the patient and control before suspending the corpuscles. Incubate all the tubes at 37.5° C. for one hour. The tube should be shaken every ten minutes. Centrifuge at the end of one hour and note for hemolysis. The red blood corpuscles of a patient with the Marchiafava-Micheli syndrome should be hemolyzed both in the patient's own serum and in normal serum, and there should be no hemolysis of the control corpuscles.

B. Heterophile Antibody

Another serologic test, which is specific in its action, but for which there is little adequate explanation, is the so-called "heterophile antibody reaction." This has been studied in glandular fevers, or infectious mononucleosis, and also in serum sickness. This is sometimes called the Hangantziu-Deicher test because of their early independent investigations of the phenomenon in the fever and rash that appears after injections of horse serum. Davidsohn[1] has described the use of a method for diagnosis of glandular fever by the demonstration of agglutination of sheep cells by high dilutions of the serum of a patient who has infectious mononucleosis.

Detection of Heterophile Antibody (*Davidsohn's Method*).—*Materials Required.*—(*a*) A dozen test tubes; 75 mm. × 10 mm. placed in a suitable rack.

(*b*) Patient's blood serum inactivated for thirty minutes at 56° C. (Only 0.1 c.c. is required for the test.)

(*c*) A 2 per cent suspension of thoroughly washed sheep red corpuscles. These cells should be not less than twenty-four hours old, nor more than a week old, and must be washed the day of the test. Wash the corpuscles three times with physiologic solution of sodium chloride, mixing well and using two to three times as much salt solution as the measured volume of cells. The third centrifugalization should take twice as long as the others, or about fifteen minutes, and should concentrate the cells to about half the original volume. The supernatant fluid must be clear after the third centrifugalization.

Procedure.—The test is set up in 11 tubes, with a twelfth tube for the control. The dilutions of serum range from 1:5 to 1:5120. However, the final dilutions are 1:7 to 1:7168 and are used in reporting the titer. The technic is clearly summarized in the following table:

[1] Davidsohn, Israel: Test for Infectious Mononucleosis, Am. Jour. Clin. Path., Technical Suppl., *8*:56–60 (March), 1938.

Tubes.	Saline solution, c.c.	Serum, c.c.	Serum dilutions.	2 per cent sheep cells, c.c.	Titer (final dilutions of serum).	
1	0.4	0.1	1:5	0.1	1:7	Shake tubes well; keep at room temperature for two hours and read.
2	0.25	0.25 of 1:5	1:10	0.1	1:14	
3	0.25	0.25 of 1:10	1:20	0.1	1:28	
4	0.25	0.25 of 1:20	1:40	0.1	1:56	
5	0.25	0.25 of 1:40	1:80	0.1	1:112	
6	0.25	0.25 of 1:80	1:160	0.1	1:224	
7	0.25	0.25 of 1:160	1:320	0.1	1:448	
8	0.25	0.25 of 1:320	1:640	0.1	1:896	
9	0.25	0.25 of 1:640	1:1280	0.1	1:1792	
10	0.25	0.25 of 1:1280	1:2560	0.1	1:3584	
11	0.25	0.25 of 1:2560*	1:5120	0.1	1:7168	
Control 12	0.25			0.1		

* Discard 0.25 c.c. from last tube.

The results are read after shaking the test tubes until the sediment is suspended. Agglutination of the corpuscles indicates the presence of heterophile antibody in that tube. The highest dilution in which this can be detected either with the naked eye, or with the low power objective is taken as the end-point. The finding of a titer of 1:224, or more (agglutination in at least the first 6 tubes), is presumptive evidence of infectious mononucleosis in a person presenting the clinical picture and hematologic findings of this disease.

C. Complement-fixation Test for Gonorrhea

Either of the methods given for syphilis may be used. The antigen is prepared from cultures of a great number of strains of gonococci and is best purchased from a biologic supply house. For use it is generally diluted 1 in 10 with saline, and is titrated each time tests are made. The dose used for the test is generally one quarter to one half the smallest amount that is anticomplementary, provided that this gives a strongly positive reaction with a known positive serum, or with the antigonococcus serum marketed by biologic supply houses. The test is conducted with three different quantities of inactivated serum—0.05, 0.1, and 0.2 c.c.—together with corresponding controls in which no antigen is used. The primary incubation should be at least one hour in the water bath or fifteen hours in the refrigerator.

The reaction is negative during the acute stage of gonorrhea, but is useful in determining the presence of a focus of chronic infection. In ordinary chronic gonorrheal urethritis the reaction is positive in about 35 per cent of cases, while in gonorrheal arthritis the percentage is much higher, probably above 80 per cent. In general it

may be said that the reaction is highly specific if properly carried out, but not very delicate, owing to the small amount of antibody present in the blood. A definitely positive reaction, therefore, is practically diagnostic; but the reactions are usually weaker than in syphilis, and a negative reaction does not exclude gonorrhea. A fact of much importance is that it becomes negative in a short time, usually two to four weeks, after a cure is effected.

D. Tests for Echinococcus Disease

The complement-fixation test using cyst fluid as antigen, and the skin test (*Casoni test*) are described on page 551. The precipitin test as applied to the sera of patients infested with echinococcus is described on pages 662–665.

Craig's complement-fixation test for amebiasis (p. 509), and tests using various bacterial antigens are of academic interest, but are not very useful for clinical diagnosis.

CHAPTER XI

BACTERIOLOGIC METHODS

BACTERIOLOGY has become so important a part of medicine that some knowledge of bacteriologic methods is imperative for the present-day practitioner. It has been the plan of this book to describe the various bacteria and bacteriologic methods with the subjects to which they seemed to be particularly related. The tubercle bacillus and its detection, for example, are described in the chapters upon Sputum and Urine. There are, however, certain methods, notably the preparation of media and the study of bacteria by cultures, which do not come within the scope of any previous section, and an outline of these is given in the present chapter.

I. APPARATUS

Much of the apparatus of the clinical laboratory is called into use. Only the following need special mention:

1. **Sterilizers.**—Two are required.

The *dry*, or *hot-air*, *sterilizer* is a double-walled oven similar to the detached ovens used with gas and gasoline stoves. It has a hole in the top for a perforated cork with thermometer. The oven of any stove, even without a thermometer, will answer for many purposes. Ordinarily the heat should be sufficient to slightly brown but not char paper or cotton, and should be continued for one half to one hour.

The *steam sterilizer* may be of the Arnold type, opening either at the top or the side. An *autoclave*, which sterilizes with steam under pressure, is very desirable, but not absolutely required. An aluminum pressure cooker (Fig. 364) is a very satisfactory substitute for the autoclave. A small sterilizer made of polished aluminum with a lid of curved flexible steel is illustrated in figure 365. The simple pressure regulating valve is covered with a weight that allows steam to escape at 15 pounds' pressure.

2. **Incubator.**—This is the most expensive piece of apparatus which will be needed. It is made of copper, and has usually both a water and an air jacket surrounding the incubating chamber. It is provided with thermometer, thermoregulator, and some source of heat, usually a Koch safety Bunsen burner if gas be used. With a little

ingenuity one can rig up a drawer or a small box, in which a fairly constant temperature can be maintained by means of an electric light. The degree of heat can be regulated by moving the drawer in or out, or holes can be made in which corks may be inserted and removed as needed. A thermos bottle has been suggested as a temporary make-shift. Upon occasion cultures may be kept warm by carrying them in an inside pocket.

The gas-heated copper incubators are now fast being displaced by the cheaper and more satisfactory incubators in which electricity is the source of heat.

3. **Culture Tubes and Flasks.**—For most work ordinary test tubes, 125 × 19 mm. without flange, are satisfactory. For special purposes a few 100 × 13 mm. and 150 × 19 mm. tubes may be needed. Heavy tubes, which do not easily break, can be obtained, and are especially desirable when tubes are cleaned by an untrained assistant. The tubes are usually stored in wire baskets.

Fig. 364.—Aluminum pressure cooker, an efficient and comparatively inexpensive substitute for an autoclave.

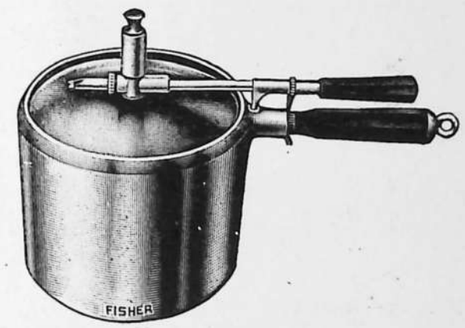


Fig. 365.—Midget sterilizer.

Flasks of various sizes are needed. The Erlenmeyer type is best. Quart and pint milk bottles and 2-ounce, wide-mouthed bottles will answer for most purposes.

4. **Platinum Wires.**—At least two of these are needed. Each consists of a piece of platinum wire about 8 cm. long, fixed in the end of a glass or metal rod. One is made of about 22-gage wire, and its end is curled into a loop 2 to 3 mm. in diameter. The other wire is somewhat heavier and its tip is hammered flat.

Lyon recommends the use of No. 20 nichrome wire as nearly equal to platinum and very much cheaper. He makes a handle of No. 8, or thicker, aluminum wire, sawing an oblique notch in

the end, inserting the nichrome wire, and hammering the aluminum over it.

5. **Pipets.**—In addition to the graduated pipets with which every laboratory is supplied, there are a number of forms which are generally made from glass tubing as needed. One of the simplest of these is the "capillary pipet," made as follows: A section of glass tubing, about 12 cm. long and 7 mm. in diameter, is grasped at the ends, and its center is heated in a concentrated flame. A blast lamp is best, but a Bunsen burner will usually answer, particularly if fitted with a "wing" or "fish-tail" attachment. When the glass is thoroughly softened it is removed from the flame, and, with a steady but not rapid pull, is drawn out as shown in Fig. 366. The slender portion is scratched

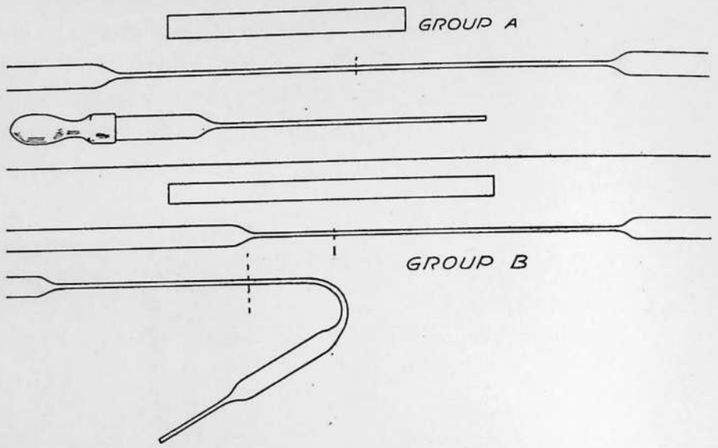


Fig. 366.—Process of making capillary pipets (Group A) and Wright's capsule (Group B). The dotted lines indicate where the glass is to be broken.

near the middle with a file, and is broken to make 2 pipets, which are then fitted with rubber nipples. Two conditions are essential to success: the glass must be thoroughly softened, and it must be removed from the flame before beginning to pull.

A nipple can be made of a short piece of rubber tubing, one end of which is plugged with a glass bead.

This pipet has many uses about the laboratory. With a grease pencil mark about 2 cm. from its tip (Fig. 377); it is useful for measuring very small quantities of fluid, as in making dilutions for the Widal test and in counting bacteria in vaccines. Mett's tubes for pepsin estimation may be made from the capillary portion. The capillary portion also makes a very satisfactory blood lancet if the center is heated in a low flame and the two ends pulled quickly apart.

Another useful device is the Wright capsule, which is made as shown in Fig. 366. Its use is illustrated in Fig. 351. After the straight end is sealed, the curved portion may be hooked over the edge of the metal tube of the centrifuge, and the contained blood or other fluid sedimented; but the speed should not be so great as to break the capsule.

II. STERILIZATION

All apparatus and materials used in bacteriologic work must be sterilized before use.

Glassware and metal are heated in the hot-air sterilizer at 150° C. for one hour, at 180° C. for half an hour. Flasks, bottles, and tubes are plugged with cotton before heating. Petri dishes may be wrapped in paper in sets of three, or better yet in covered copper

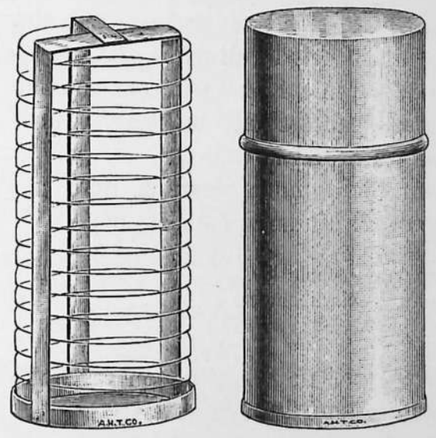


Fig. 367.—Petri dish holder.

receptacles, made to hold a number of assembled dishes (Fig. 367). Pipets and all-glass hypodermic syringes are placed in cotton-stoppered test tubes.

Culture media and other fluids must be sterilized by steam. Exposure in an autoclave to a temperature of 110° C. (6 pounds' pressure) for one half hour or of 121° C. (about 15 pounds' pressure) for fifteen minutes is generally sufficient. Sometimes 20 pounds for twenty minutes will be required to kill resistant spores. In using the autoclave it is extremely important that all the air be allowed to escape, so that the sterilizing chamber may be filled with superheated steam. With the Arnold sterilizer the intermittent plan must be adopted, since steam at ordinary pressure will not kill spores. This consists in steaming for thirty to forty-five minutes on three or four suc-

cessive days. Spores which are not destroyed upon the first day develop into the vegetative form and are destroyed at the next heating. Gelatin media must not be exposed to steam for more than twenty minutes at a time, and must then be removed from the sterilizer and cooled in cold water, otherwise the gelatin may lose its power to solidify. In preparing sugar fermentation tubes it is preferable to sterilize the sugar solutions by filtration, and to add the appropriate amount to the sterile blanks which have been previously prepared, since great heat tends to break down the sugar.

Cotton and gauze are sterilized by either hot air or steam, preferably the latter.

III. PREPARATION OF CULTURE TUBES

New tubes should be washed in a very dilute solution of nitric acid, rinsed in clear water, and allowed to drain dry.

Tubes which contain dried culture media are cleaned with a test-tube brush after boiling in a 1 or 2 per cent solution of washing soda. They are then rinsed successively in clear water, acidulated water, and clear water, and allowed to drain.

The well-known **bichromate cleaning fluid** is very valuable for cleaning glassware of all kinds. It consists of:

Potassium bichromate, technical	100 Gm.
Concentrated sulfuric acid, technical	250 c.c.
Water	750 "

Glassware may be placed in this solution for one day or longer and then rinsed thoroughly and dried.

The tubes are now ready to be plugged with raw cotton—the "cotton batting" of the dry-goods stores. This is done by pushing a wad of cotton into each tube to a depth of about 3 cm. with a glass rod. The plugs should fit snugly, but not too tightly, and should project from the tube sufficiently to be readily grasped by the fingers. The tubes are next placed in wire baskets and heated in an oven for about one half hour at 150° C. in order to mold the stoppers to the shape of the tubes. The heating should not char the cotton, although a slight browning does no harm. The tubes are now ready to be filled with culture media.

IV. CULTURE MEDIA

For a careful study of bacteria a great variety of culture media is required, but only a few—bouillon, agar, and solidified blood serum—are much used in routine work. A great deal of work can be done with a single medium, for which purpose Huntoon's "hormone agar"

is probably best. The ordinary culture media, put up in tubes ready for use, can be purchased through any pharmacy. A long list of media, including practically all formulae in general use, and many others, is now offered in powder form under the name Bacto Dehydrated Media. These have only to be dissolved in a specified amount of water and sterilized. Most of them, at least, are very satisfactory, and their use greatly facilitates the work of a small laboratory.

Preparation of Culture Media.—

Beef Infusion

Hamburger steak, lean	500 Gm.
Tap water	1000 c.c.

Mix well; let soak about twenty-four hours in an ice-chest and squeeze through cheesecloth. This infusion is not used by itself, but forms the basis for various media. "Double strength" infusion, used in making agar-agar, requires equal parts of the meat and water.

Infusion Bouillon

Beef infusion	1000 c.c.
Peptone (Witte)	10 Gm.
Salt	5 "

Boil until dissolved; bring to original volume with water; adjust reaction, and filter.

Beef Extract Bouillon

Liebig's extract of beef	3 Gm.
Peptone	10 "
Salt	5 "
Tap water	1000 c.c.

Heat until all ingredients are dissolved, cool, and beat in the whites of 2 eggs; bring slowly to the boiling point again, boil briskly for five minutes, and filter. It is not usually necessary to adjust the reaction unless the peptone has an acid reaction.

Meat Mash

Lean chopped beef	1 lb.
Distilled water	1000 c.c.

Boil for twenty minutes. Make up to 1000 c.c. and adjust the reaction to *p*H 7.6. Boil again for ten minutes. Filter through gauze to separate the meat and broth. Fill tall tubes (8 × $\frac{5}{8}$-inch tubes) about $1\frac{1}{2}$ inches from the bottom of the tubes with the ground meat and add broth to fill the tubes about two thirds full. Place in the autoclave for twenty minutes at 20 pounds' pressure. This medium

is excellent for keeping stock cultures of streptococci viable for long periods of time. Other organisms will grow luxuriantly in this medium.

Dextrose Brain Broth

This is an excellent fluid medium for growing streptococci. It is used to advantage in making blood cultures, the indicator at times showing growth before colonies appear on plates. Dissolve 8 Gm. dehydrated "Bacto" nutrient broth (Difco Laboratories, Detroit), and 8 Gm. of salt in 1 liter boiling water. Cool, add 2 Gm. C. P. dextrose, and 1 c.c. Andrade indicator (p. 772). The medium is tubed in tall tubes (8 × $\frac{5}{8}$ inches). Three small pieces of calf's brain and two or three pieces of marble are added to each tube, and the medium is then sterilized in the autoclave at 20 pounds' pressure for twenty minutes.

Kracke's Blood Culture Medium

This medium like many others is most easily prepared by using "Difco" dehydrated material. Five Gm. of the powdered culture medium makes 50 c.c. of broth medium, or 1 pound will make 4.5 liters. The ingredients in the revised formula prepared by the Digestive Ferments Co. were mixed in the following proportions:

Beef heart tissue, fresh	400 Gm.
Beef brain tissue, fresh	55 "
Peptone	10 "
Dextrose	10 "
Sodium chloride	4 "
Sodium citrate	1 "
Disodium phosphate	2 "

Suspend a weighed amount of the powder in ten times the amount of distilled water (measured in cubic centimeters) until the particles are thoroughly wetted. Divide into tubes or small flasks. Sterilize in an autoclave for fifteen to twenty minutes at 15 pounds' pressure. The final reaction will be *p*H 7.4. It is claimed for this medium that the complement in the patient's blood is immediately fixed by the brain and heart tissue; coagulation is prevented by the sodium citrate.

Beef Infusion Agar

Preparation of this medium usually gives the student much trouble. There should be no difficulty if the directions are carefully carried out.

Agar-agar, powdered or in shreds	15 Gm.
Tap water	500 c.c.

Boil until thoroughly dissolved and add

Peptone	10 Gm.
Salt	5 "

When these have dissolved, replace the water lost in boiling, cool to about 60° C., and add 500 c.c. double-strength beef infusion. Bring slowly to a boil, adjusting the reaction meanwhile, and boil for at least five minutes. Filter while *hot* through a moderately thick layer of absorbent cotton wet with *hot* water in a *hot* funnel. A piece of coarse wire gauze should be placed in the funnel underneath the cotton to give a larger filtering surface. This medium will be clear enough for ordinary work. If an especially clear agar is desired, it can be filtered through paper in an Arnold sterilizer.

Wilson and Blair Medium

(Thompson modification for C. welchii)

Thompson[1] has found this medium the most satisfactory for demonstrating *C. welchii* in clinical material. Prepare beef infusion agar, with 2 per cent of agar, and 0.5 per cent of glucose added. Set the reaction at *p*H 7.0. Bottle in 100 c.c. amounts, add 3 Gm. of marble chips to each bottle and autoclave. For use, melt a bottle of the medium, add 10 c.c. of freshly prepared 20 per cent solution of sodium sulfite and 1 c.c. of 8 per cent solution of ferric chloride. Pour 20 c.c. in a sterile Petri dish, inoculate, and when solidified pour 20 c.c. more of the medium on top of the inoculated layer. *Clostridium welchii* develops a black color in the medium about each colony after incubation for about six or seven hours.

Liver Infusion Agar

This medium is used chiefly in the culture of *Brucella abortus* according to Huddleson's method. It may be prepared from fresh liver but is most easily prepared from "Difco" material which represents

Beef liver, infusion from	500 Gm.
Proteose-peptone	10 "
Sodium chloride	5 "
Agar	20 "

Use 55 Gm. in 1 liter of distilled water. Dissolve with heat, tube and sterilize. The final reaction is *p*H 6.6. This medium, to which has

[1] Thompson, Luther: The Medium of Wilson and Blair for the Determination of Clostridium welchii, Amer. Jour. Clin. Path. (Tech. Suppl.), *9*:173–176 (July), 1939.

been added 3 Gm. of dibasic sodium phosphate, has been used as a base for the Cleveland and Collier medium for amebic culture described on page 508.

Beef Extract Agar

This is made by boiling 15 Gm. of powdered agar in 1000 c.c. of beef extract bouillon until dissolved, replacing the water lost in boiling, and filtering through paper in a sterilizer. It can be cleared with egg if desired. This makes a 1.5 per cent agar. For some purposes, as when beef extract agar is used as a base for Endo's medium, twice the amount of the powdered agar is required.

Glycerol Agar

To 1000 c.c. melted agar add 60 to 70 c.c. glycerol, and mix well.

N. N. N. Medium

Mix 14 Gm. agar, 6 Gm. salt, and 900 c.c. water. Prepare as plain agar medium, tube and sterilize in the autoclave. For use melt the agar and when cooled to 48° C. add one third volume of defibrinated blood.

Petroff's Medium

This medium is now very generally used for growing tubercle bacilli. Mix 500 Gm. chopped lean beef, or veal, with 500 c.c. of 15 per cent glycerol solution. Place in the icebox for twenty-four hours; filter through gauze, and to each 100 c.c. of filtrate add 1 c.c. of 1 per cent alcoholic solution of gentian violet. Sterilize the shells of 6 eggs by placing them in 70 per cent alcohol for ten minutes. Carefully crack the eggs, and mix the whites and yolks in a sterile beaker. Add an equal amount (about 200 c.c.) of the glycerol beef extract and mix well. Tube, placing 4 c.c. in each tube. Inspissate at 85° C. until medium is solidified, and on the two succeeding days heat for one hour to 75° C.

Corper and Uyei's[1] Medium

Cut large clean potatoes free from surface defects into cylinders about 3 inches long by $\frac{5}{8}$ inch in diameter, using a cork borer. Halve

[1] Corper, H. J., and Uyei, Nao: Further Observations with a New Method for Cultivating Tubercle Bacilli: A Comparison with Guinea-pig Inoculation and Petroff's Method, Jour. Lab. and Clin. Med., *14*:393–411 (Feb.), 1929. Corper, H. J., and Uyei, Nao: A Simple Glycerol Water Crystal Violet Potato Cylinder Medium for Diagnostic Cultures of Tubercle Bacilli, Arch. Path., *7*:835–838 (May), 1929. Corper, H. J., and Uyei, Nao: Oxalic Acid as a Reagent for Isolating Tubercle Bacilli and a Study of the Growth of Acid-fast Nonpathogens on Different Mediums with Their Reactions to Chemical Reagents, Jour. Lab. and Clin. Med., *15*:348–369 (Jan.), 1930.

longitudinally and immediately soak for from one to two hours in 1 per cent anhydrous sodium carbonate solution containing 1/75,000 crystal violet added just before using. One hundred sixty individual halves should be used for 1 liter of the sodium carbonate dye solution. Wipe the pieces of potato carefully with a clean towel, and place them in sterile culture tubes (6 by $\frac{3}{4}$ inch size) containing 1.5 c.c. of 6 per cent glycerol in water. Plug the tubes with cotton and sterilize them in the autoclave for thirty minutes at 15 pounds' pressure. It may be necessary to sterilize them at only 10 pounds' pressure if the potato tends to become friable. Incubate the tubes at 37° C. for two days to test for sterility.

Egg-Yolk-Agar Mixture

(Herrold's modification of Cappaldi medium)

Feldman[1] recommended the following medium which he has slightly modified by making a 1.5 per cent agar medium, instead of 1 per cent. It will grow avian, bovine, and human strains of tubercle bacilli.

Solution 1.

Liebig's beef extract	6 Gm.
Peptone	20 "
Sodium chloride	10 "
Distilled water	1000 c.c.

Boil the mixture for fifteen minutes and add distilled water to restore to the original volume. (Glycerin may be added if desired; 60 c.c. will make a 3 per cent glycerin medium.) Adjust the *p*H to 7.5 and filter through paper.

Solution 2.

Granulated agar	30 Gm.
Distilled water	1000 c.c.

Place the agar and the water in a Florence flask, and autoclave at 15 pounds' pressure for thirty minutes. When the flask has cooled sufficiently to be handled, pour the agar solution into a graduate, leaving the undissolved débris at the bottom of the flask. Add distilled water to make up to the original volume. Heat solutions 1 and 2, mix, and filter through two layers of fine mesh cheesecloth. Place in Clean-Easy bottles in 150 c.c. amounts, stopper, and sterilize in an autoclave for thirty minutes at 15 pounds' pressure, and store for future use.

[1] Feldman, W. H.: Avian Tuberculosis Infections. Baltimore, Williams and Wilkins Company, 1938, pp. 89–90.

To complete the medium, the yolk of one fresh egg must be added to each 150 c.c. of agar, which must be melted in a hot water bath and sufficiently cooled to remain just liquid. Thoroughly wash the eggs, and immerse in 80 per cent alcohol for fifteen minutes. Dry in the air. Flame one end of an egg, break the shell and underlying membrane with sterile forceps. Discard the white, and break the yolk with sterile scissors. Carefully pour the yolk of one egg into one of the bottles of melted agar. Since the medium is not sterilized after the egg yolk is added, this part of the procedure should be carried out under a hood in a room free from draft. Replace the cotton stopper, and shake the bottle to effect a homogeneous mixture. Put the medium into sterile tubes, slant, and then incubate for two or three days. Discard any contaminated tubes.

Sabouraud's Agar for Fungi

This is a widely used standard medium for cultivation of yeasts and molds, particularly the fungi of the parasitic skin diseases. Ashford recommends it for isolation of *Monilia psilosis* from the feces. By virtue of its acid reaction it retards the growth of bacteria.

Maltose or glucose	4.0 Gm.
Peptone	1.0 "
Agar	1.5 "
Water	100 c.c.

Adjust reaction by titration to +2 (p. 776).

Tartaric Acid Medium for Fungi

Another medium recommended for fungi because it inhibits the growth of ordinary bacteria is made as follows:

Tartaric acid	25 Gm.
Dextrose	50 "
Water, to make	100 c.c.

Sterilize in the autoclave twenty minutes at 20 pounds' pressure. Add 10 c.c. of this sterile tartaric acid-dextrose solution to 190 c.c. of melted agar, and pour plates.

Blood Agar

Blood agar is now widely used for culturing sputum and other material in which the pneumococcus or streptococcus may be expected.

Sterile agar in a flask or a series of tubes is melted, cooled to 45° to 50° C., and maintained at this temperature in a water bath. Sterile human or rabbit blood which may be citrated or defibrinated is then

added in the proportion of 1 c.c. of blood to 4 or 5 c.c. of agar, and well mixed by rotating. For the study of hemolysis by the streptococcus the proportion of blood should not exceed 1 in 10. The medium is then poured into Petri plates, or, if in tubes, is cooled in a slanted position, and is incubated to make sure of its sterility. When extreme economy is essential the blood agar may be poured upon the surface of plain agar slants.

Human blood may be obtained from an arm vein by means of the device shown in Fig. 94, page 195, which has been charged with 1 or 2 c.c. of 10 per cent sodium citrate before sterilization. If defibrinated blood be preferred, a number of small pieces of broken glass tubing are substituted for the citrate, and the tube is gently shaken. Rabbits' blood is secured from the heart.

Sauer's Potato Blood Agar for Whooping Cough Bacillus

Sauer and Hambrecht[1] have reported on the "cough plate" method for the early diagnosis of whooping cough. They used a medium which is made in the following manner:

Place 500 Gm. of peeled, sliced potatoes, 40 c.c. of glycerin, and 1000 c.c. of distilled water in a kettle. Weigh the container and contents, and with the kettle covered, boil the mixture until the potatoes are soft. Replace the water which has been lost, by adjusting the mixture to the original weight. Strain the mixture through gauze. To 500 c.c. of filtrate add 1500 c.c. of 0.6 per cent saline solution and 60 Gm. of agar. Weigh the mixture, melt the agar, and adjust to the original weight. Bottle the mixture in 150 c.c. amounts and sterilize in the autoclave. This stock medium may be kept for months in the refrigerator. For use, melt the medium, cool to 45° C. and add 30 c.c. of defibrinated blood to each 150 c.c. of medium. Pour the melted potato blood agar medium into sterile Petri dishes, using about 20 c.c. for each dish. Use freshly prepared plates for cultures. In collecting the material, expose the medium in front of the patient's mouth only when there is a true expulsive cough that will bring material from the bronchi.

"Chocolate"-Blood Agar

This medium is especially valuable for growing gonococci, the colonies producing an oxydase reaction which is demonstrated easily with a solution of tetramethyl-p-phenylenediamine hydrochloride.

[1] Sauer, L. W., and Hambrecht, Leonora: Whooping Cough; Early Diagnosis by the Cough Plate Method, Jour. Am. Med. Assn., *95*:263–264 (July 26), 1930.

The technic for using this medium is described on page 734. The medium is prepared as follows:

Dissolve 10 Gm. of peptone and 2 Gm. of dibasic sodium phosphate (Na_2HPO_4) in 1 liter of distilled water. Heat to 60° C. Add 1 pound of ground lean beef. Keep the temperature at 60° C. for forty-five minutes; then steam in the autoclave for thirty minutes. Filter, and adjust the reaction to *p*H 7.4. Dissolve 12 Gm. of agar (or just enough to make a "soft" agar medium) with as little boiling as possible. Put 100-c.c. amounts in 6-ounce bottles; place in autoclave at 15 pounds for fifteen minutes and store in the refrigerator. For use, melt the stock medium and *while still very hot*, add 10 c.c. of citrated human blood for each 100 c.c. of medium. Mix thoroughly. The mixture will become dark brown at once, instead of the red color of blood agar which is prepared at lower temperatures. Pour it into sterile Petri dishes and use on the same day that it is prepared.

Hormone Agar (Huntoon)

This has now become a very popular stock medium for general bacteriologic procedures. The modification proposed by Sadie Bailey is given: Thoroughly wash 15 Gm. of agar-agar shreds in running water, and dissolve by boiling in 1 liter of distilled water. Cool to 50° or 60° C. Add 500 Gm. ground lean beef or beef heart, heat to boiling, and cook slowly for from fifteen to twenty minutes. Filter through an ordinary round flour sieve, about 16 mesh. Add peptone, 10 Gm., and sodium chloride, 5 Gm., and boil for five minutes. Correct the reaction to *p*H 7.5. Allow the precipitate to settle out and decant the clear supernatant medium. Add from 0.25 to 1 per cent dextrose as desired. Also add three or four small pieces of marble to each tube to keep the reaction at the proper point. Tube and sterilize either by heating for one hour in the Arnold sterilizer on three successive days, or in the autoclave for twenty minutes at 15 pounds' pressure. When citrated blood is added to this medium it is especially useful in culturing pneumococci and streptococci. Gonococci will also grow on hormone-blood agar.

Eosin-Methylene Blue Agar

This medium is useful for differentiating *E. coli* from *A. aerogenes*. The colonies produced by *E. coli* are flat, almost black, with a metallic sheen, while *A. aerogenes* grows with large, mucoid, light blue colonies. The typhoid-dysentery group of organisms produce colorless colonies on this medium.

Mix peptone 10 Gm., dibasic potassium phosphate 2 Gm., and agar 15 Gm., in 1000 c.c. of distilled water. Boil until the ingredients are dissolved. Add distilled water to make up any loss due to evaporation. Adjustment of the reaction and filtration of the medium are not required. Place 100-c.c. quantities in flasks and sterilize in the autoclave at 15 pounds' pressure for fifteen minutes.

Just prior to use melt the stock agar and to each 100 c.c. add 5 c.c. of a sterile 20 per cent solution of lactose, 2 c.c. of a 2 per cent aqueous solution of yellowish eosin, and 2 c.c. of a 0.5 per cent solution of methylene blue. Mix thoroughly and pour into sterile Petri dishes.

A modification of this medium uses half as much lactose, with an equal amount of saccharose, and half of the quantities of eosin and of methylene blue.

Endo's Agar

Prepare a 3 per cent beef-extract agar (p. 766). Neutralize to phenolphthalein, sterilize, and store in 100-c.c. quantities.

To make Endo's medium melt 100 c.c. of this agar, add 1 Gm. chemically pure lactose, and 0.5 c.c. of fuchsin-sulfite solution prepared as follows: To 10 c.c. of a 10 per cent solution of anhydrous sodium sulfite add 2 c.c. of a 10 per cent alcoholic solution of basic fuchsin, and heat for a few minutes.

The finished medium may be sterilized in the Arnold, but too great heat is to be avoided because of the danger of breaking up the sugar. The medium is red when hot, faintly pink or colorless when cold.

Lactose fermenters (colon bacilli, and so forth) give red colonies on Endo's medium, although the color may not appear in twenty-four hours' incubation; others (typhoid, paratyphoid, and dysentery bacilli) give colorless or gray colonies.

Lactose Litmus Agar

Melt 100 c.c. of nutrient agar such as is used for Endo's medium, add enough azolitmin solution to give a distinct lilac color, and 5 c.c. of hot freshly prepared 20 per cent solution of pure lactose. Tube and sterilize, preferably in the Arnold.

Russell's Double Sugar Agar

Make a 2 to 3 per cent beef extract agar, adjust reaction by titration to about +0.7 (p. 776), and add azolitmin solution to a purple

violet color. Now neutralize to azolitmin, and to each 100 c.c. add 1 Gm. pure lactose and 0.1 Gm. pure dextrose. The sugars are dissolved in a little water before adding. Mix well, tube, sterilize in the Arnold, and cool in a slanted position. There should be a deep butt below the slant.

This medium is used to differentiate organisms of the colon-typhoid group. It is customary to inoculate tubes both with a surface streak and with a stab made from the same colony. Colon bacilli turn both slant and butt red with gas bubbles in the butt. Typhoid and dysentery bacilli give a grayish growth on the violet slant and a deep pink in the butt without bubbles. The growth of paratyphoid bacilli, A and B, resembles that of typhoid except that there is gas production in the butt.

Instead of azolitmin most workers prefer using 1 per cent of Andrade's indicator. The final reaction of the agar should be about *p*H 7.2. If the reaction is satisfactory, the medium will have a red color when hot and will be practically colorless when cold. Andrade's indicator is made by adding a 4 per cent solution of sodium hydroxide to a 0.5 per cent solution of acid fuchsin until the color changes from red to orange or yellow. As the color change takes place slowly, some time must elapse between additions. The usual proportion is about 16 c.c. of the alkali to 100 c.c. of the dye solution.

Lead Acetate Agar

Melt ordinary agar culture medium; cool to 60° C.; and add enough of a 0.25 per cent basic lead acetate solution to produce a concentration of 0.05 per cent of lead acetate in the culture medium. Place in tubes. When the culture medium has become hard, inoculate with a fine needle. Bacillus paratyphosus A (*Salmonella paratyphi*) does not produce any change in this medium, while B. typhosus (*Eberthella typhosa*), B. paratyphosus B (*Salmonella schottmülleri*) and B. enteritidis (*Salmonella enteritidis*) blacken this medium.

Gelatin

Dissolve 100 to 120 Gm. "golden seal" gelatin in 1000 c.c. nutrient bouillon with as little heat as possible, adjust the reaction, cool, beat in the whites of two eggs, bring slowly to the boiling point, boil for a few minutes, and filter hot through filter paper wet with hot water. Sterilize in an Arnold sterilizer for twenty minutes upon three successive days and cool in cold water after each heating. Keep at room temperature between heatings.

Sugar Media

Any desired sugar may be added to bouillon, agar, or gelatin in proportion of 10 Gm. to the liter. It is best added in solution. Dextrose is most frequently required. When other sugars are added, media made from beef extract should be used, since those made from beef infusion contain enough dextrose to cause confusion.

The various sugars may also be added to Dunham's peptone medium and Hiss' serum-water-litmus.

For the study of gas production the sugar media are placed in U tubes or, better, in Dunham's fermentation tubes (Fig. 368). These consist of standard culture tubes with small tubes, about 8 by 25 mm., inverted inside of them. The culture medium is introduced to a depth of about 1½ inches. The air in the inner tube is driven off during sterilization. As a rule, sugar media are sterilized in the Arnold, as sugar does not resist heat well.

Fig. 368.—Dunham's fermentation tube, consisting of a small tube inverted in a standard culture tube. When a liquid culture medium is present the air in the inner tube is driven off during sterilization.

Löffler's Blood Serum

Dextrose-bouillon (1 per cent)..... 1 part
Blood serum..... 3 parts

Mix and tube. Place in an inspissator at the proper slant for three to six hours at 80° to 90° C. When firmly coagulated, sterilize in the usual way. A large "double cooker" makes a satisfactory inspissator. The tubes are placed in the inner compartment upon a layer of cotton at the proper slant, a lid with perforation for a thermometer is applied, and the whole is weighted down in the water of the outer compartment. An Arnold sterilizer with the door left ajar also makes a good inspissator.

Blood serum is obtained as follows: Beef or pig blood is collected in a bucket at the slaughter house and placed in an ice-chest until coagulated. The clot is then gently loosened from the wall of the vessel. After about twenty-four hours the serum will have separated nicely and can be siphoned off. It is then stored in bottles with a little chloroform until needed. Red cells, if abundant, darken the medium, but do no harm.

This has long been the approved medium for the diphtheria bacillus. For this purpose Greenspon has improved it by including

1 c.c. of 50 per cent sodium citrate solution in each 100 c.c. of mixture, and then adjusting the reaction with 3 per cent citric acid to *p*H 6.4, using bromthymol blue as an indicator. The advantages are that gram-positive cocci are inhibited and diphtheria baçilli grow more luxuriantly.

Hydrolyzed Serum Agar

Thompson[1] has combined the method of Klein and that of Greenspon to make a serum agar that promotes the growth of diphtheria organisms and inhibits the growth of certain other bacteria which are found in the throat. It offers the convenience of an agar medium for the plating and isolation of pure cultures B. diphtheriae (*Corynebacterium diphtheriae*). The medium is prepared as follows:

Dissolve 40 Gm. of Löffler's blood serum ("Bacto" dehydrated) in 250 c.c. of water at 40° C. Mix well and add 150 c.c. of normal solution of sodium hydroxide. Place in an incubator at 37° C. for forty-eight hours. Neutralize with 5 per cent hydrochloric acid to *p*H 7.0, using bromthymol blue. Add 2.5 Gm. of sodium citrate; when dissolved, adjust to *p*H 6.4 with 3 per cent citric acid. Make a 3 per cent solution of agar, and while still hot, add equal parts of hydrolyzed serum mixture and 3 per cent agar solution. Put in tubes or flasks and sterilize in the autoclave at 15 pounds' pressure for fifteen minutes. The medium may be used in culture tubes, or it may be melted and poured into Petri dishes for the isolation of pure cultures.

Egg Medium

This has been recommended as a substitute for solidified blood serum. In a mortar grind thoroughly the white and yolk of one egg with 10 to 15 c.c. of 1 per cent dextrose bouillon. Place in tubes, inspissate, and sterilize as described for solidified blood serum.

Cystine Medium for B. Tularense (Francis)

The stock medium is made of beef infusion to which is added 1 per cent peptone, 0.5 per cent salt, and from 1 to 1.5 per cent agar-agar. The adjusted reaction should be *p*H 7.6. To this stock medium is added 0.1 per cent cystine and 1 per cent glucose. It is necessary to heat the medium for fifteen minutes in streaming steam in an Arnold sterilizer to melt the agar and to sterilize the cystine. After cooling to 45° C. add 5 per cent sterile horse serum. The medium is tubed and incubated for twenty-four hours to insure sterility.

[1] Thompson, Luther: Hydrolyzed Serum Agar for the Isolation of Corynebacterium Diphtheriae, Jour. Infect. Dis., *45*:163–166 (Sept.), 1929.

Litmus Milk

Fresh milk is steamed in an Arnold sterilizer for half an hour, and placed in the ice-chest overnight. The milk is siphoned off from beneath the cream, and sufficient aqueous solution of litmus or, preferably, azolitmin is added to give a bluish-violet color. It is then tubed and sterilized. Recently bromcresol purple has been used instead of other indicators with satisfactory results. Enough of a 0.2 per cent alcoholic solution of the dye is added to give the desired color.

Potato

Cylinders about ½ inch in diameter are cut from potato and split obliquely. These wedge-shaped pieces are soaked overnight in running water and placed, broad ends down, in large tubes, in the bottom of which is placed a little cotton saturated with water. They are sterilized for somewhat longer periods than ordinary media.

Dunham's Peptone Solution

Peptone	10 Gm.
Salt	5 "
Water	1000 c.c.

Dissolve by boiling; filter, tube, and sterilize.

This medium is used to determine indol production. To a twenty-four- to forty-eight-hour-old culture is added 5 to 10 drops of concentrated, chemically pure sulfuric acid and 1 c.c. of 1:10,000 solution of sodium nitrite. Appearance of a pink color shows the presence of indol. A pink color before the nitrite is added shows the presence of both indol and nitrites.

Hiss' Serum Water Medium

Blood serum	1 part
Water, about	3 parts

Steam in the Arnold for fifteen minutes to destroy any diastase that may be present. Adjust reaction to +0.2 to +0.8. Add litmus or azolitmin solution to give a bluish-violet color. Finally, add 1 per cent of inulin or any desired sugar and sterilize in the Arnold. Should the medium solidify, too large a proportion of serum was used. Inulin should be added in solution, and, since it usually contains spores, should first be sterilized in the autoclave. The inulin medium is very useful in distinguishing between the pneumococcus and streptococcus.

Bile Medium

Ox or pig bile is obtained at the slaughter house, tubed, and sterilized. This is used especially for growing typhoid bacilli from the blood during life and, filtered as clear as possible, for the solubility test which distinguishes between pneumococci and streptococci. The following is probably as satisfactory as fresh bile and is more convenient:

Dehydrated ox bile ("Bacto")	30.0 Gm.
Peptone	2.5 "
Water	250.0 c.c.

Dissolve, place in tubes, and sterilize.

Reaction of Media.—The chemical reaction of the medium exerts a marked influence upon the growth of bacteria. It is adjusted after all ingredients are dissolved by adding sufficient caustic soda solution to overcome the acidity of the meat and other substances used. In general, the most favorable reaction lies between the neutral points of litmus and phenolphthalein, representing a very faint alkalinity to litmus. The reaction may be adjusted by titration, or by the newer colorimetric method. Small chips of marble, which are placed in tubes of fluid media before sterilization, act as a buffer.

Titration Method.—After all ingredients are dissolved and the loss during boiling has been replaced with water, 10 c.c. of the medium are transferred to an evaporating dish, diluted with 40 c.c. of water, and boiled for three minutes to drive off carbon dioxide. One c.c. of 0.5 per cent alcoholic solution of phenolphthalein is then added, and decinormal sodium hydroxide solution is run in from a buret until the neutral point is reached, indicated by the appearance of a permanent pink color. The number of cubic centimeters of decinormal solution required to bring this color indicates the number of cubic centimeters of *normal* sodium hydroxide solution which will be required to neutralize 100 c.c. of the medium. The standard reaction of the American Public Health Association for bacteriologic examination of water and milk is +1, which means that the medium must be of such degree of acidity that 1 c.c. of normal solution would be required to neutralize 100 c.c. This corresponds to faint alkalinity to litmus. Most pathogenic bacteria grow better with a reaction of +0.5 to +1. Example: If the 10 c.c. which were titrated required 2 c.c. of decinormal solution to bring the pink color, the reaction is +2, and 1 c.c. of normal sodium hydroxide must be added to each 100 c.c. of the medium to reduce it to the standard, +1.

Colorimetric Method.—With the growing recognition of the importance of exact adjustment of the true reaction or hydrogen ion concentration of media for the optimum growth of many bacteria, titration has been largely

discarded as failing to give a sufficiently accurate index, owing partly to the many buffer substances which are present, and partly to the difficulty in getting an exact and uniform end-point. Accurate determination of hydrogen ion concentration by means of the potentiometer is out of the question for clinical laboratories owing to the great cost of the apparatus. The colorimetric method is therefore now generally relied upon, and is entirely sufficient for practical purposes. This depends upon the fact that the indicator, bromthymol blue, gives a series of color tints at different hydrogen ion concentrations within the range required for this work, that is, from *p*H 6 to *p*H 7.6. With this indicator the color is yellow at *p*H 6; at *p*H 6.2 the color is yellowish-green up to *p*H 7; at *p*H 7 the color becomes bluish-green, and deepens to bright blue at *p*H 7.6.

Reagents Required.—(*a*) Bromthymol blue, 0.04 per cent alcoholic solution.

(*b*) Normal sodium hydroxide and decinormal sodium hydroxide, the latter made from the former by accurate dilution. These are best kept in flasks which have been lightly coated inside with paraffin.

(*c*) A series of standard phosphate solutions of varying hydrogen ion concentrations prepared according to Sørensen as follows:

One-fifteenth molecular acid or primary potassium phosphate. Dissolve 9.078 Gm. of the pure crystalline salt, KH_2PO_4, in freshly distilled water and make up to 1000 c.c.

One-fifteenth molecular alkaline or secondary sodium phosphate. Expose the pure recrystallized salt, $Na_2HPO_412(H_2O)$ to the air or from ten days to two weeks, protected from dust. Ten molecules of water are given off and a salt of the formula $Na_2HPO_42(H_2O)$ is obtained. Dissolve 11.876 Gm. of this in freshly distilled water and make up to 1000 c.c.

To make standard solutions of different hydrogen ion concentrations in a graduated series mix the two phosphate solutions in the proportions shown in the following table:

*p*H	6.4	6.6	6.8	7.0	7.1	7.2	7.3	7.4	7.5	7.6	7.7	7.8	8.0	8.2	8.4
Primary potassium phosphate, c.c.	73	63	51	37	32	27	23	19	15.8	13.2	11	8.8	5.6	3.2	2.0
Secondary sodium phosphate, c.c.	27	37	49	63	68	73	77	81	84.2	86.8	89	91.2	94.4	96.8	98.0

Place 10-c.c. quantities of these standard solutions in a series of Pyrex or Non-Sol glass test tubes, which must be of equal diameter as shown by the 10 c.c. of fluid reaching to the same height in each. Add 0.5 c.c. of the 0.04 per cent bromthymol blue indicator to each tube, and seal with a paraffined cork. Sets of standard color tubes covering any *p*H range can

be purchased ready prepared together with empty tubes of the same diameter.

Method.—1. Place the standard color tube representing the *p*H value desired in one of the end holes of the front row of the comparator shown in Fig. 369. If the desired value falls between two of the standards, place these in the end holes of the front row. Place a tube of the untreated medium back of each.

2. In a test tube of the same diameter place 10 c.c. of the medium to be adjusted, agar or other solid medium being liquefied by heat. Add 0.5 c.c. of the 0.04 per cent bromthymol-blue solution and mix.

3. From an accurate buret add decinormal sodium hydroxide solution a very little at a time, mixing after each addition, until the color matches that of the standard chosen, or falls between the standards next above or below. For comparison the tube is placed in the middle hole of the front row of the comparator with a tube of water back of it. Note the amount of decinormal solution required to bring the 10 c.c. of medium to the desired reaction. This amount is then the amount of *normal* sodium hydroxide solution which must be added to 100 c.c. of the medium to secure this reaction. After this has been added, the reaction should again be determined as a check. Most media, particularly those containing sugars, become slightly more acid during sterilization; and it is well, when great accuracy is desired, to make a check determination upon a 10-c.c. portion of the finished and sterilized product which has been kept out for the purpose.

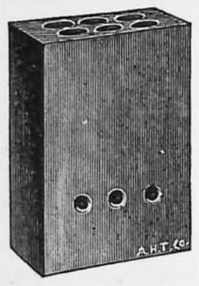


Fig. 369.—A color comparator (Clark's model) for use in adjusting reaction of culture media by the colorimetric method.

While the above technic is accurate, a much simpler method may be used for practical purposes with most culture media. Test a few drops of medium at a time on a white tile with bromthymol blue. Add sodium hydroxide solution, a very little at a time, to the entire amount of medium; mix thoroughly; and test the reaction. The end-point is reached when a sample turns the indicator dark green.

The optimum hydrogen ion concentration for growth of the more important bacteria, as determined by Fennel and Fisher, is as follows:

Pneumococcus	7.8
Streptococcus	7.6–7.8
Meningococcus	7.6
Gonococcus	7.5–7.6
Bacillus typhosus and B. paratyphosus	6.2–7.2
Bacillus dysenteriae	6.3–7.8
Bacillus influenzae	7.8–8.0

Tubing Culture Media.—The finished product is stored in flasks or distributed into test tubes. This is done by means of a funnel

fitted with a section of rubber tubing with a glass tip and a pinch cock. Great care must be exercised, particularly with media which solidify, not to smear any of them upon the inside of the mouth of the tube, otherwise the cotton stopper will stick. Tubes are generally filled to a depth of 3 or 4 cm. For stab cultures a greater depth is required.

After tubing all culture media must be sterilized, as already described. Agar tubes are cooled in a slanting position to secure the proper surface for inoculation.

Storage of Culture Media.—All media should be stored in a cool place, preferably an ice-chest. Evaporation may be prevented by covering the tops of the tubes with tin foil or with the rubber caps which are sold for the purpose; or the cotton stopper may be pushed in a short distance and a cork inserted.

V. STAINING METHODS

In general, bacteria are stained to determine their morphology, their reaction with special methods (for example, Gram's method), and the presence or absence of certain structures, as spores, flagella, and capsules. Staining methods for various purposes have been given in previous chapters and can be found by consulting the Index. The formulae of the staining fluids are given in the Appendix.

Method of Staining for Morphology.—The following method is used when one wishes to detect the presence of bacteria or to study their morphology. It is applicable both to films from cultures and to smears from pus or other pathologic material. Any simple bacterial stain may be used, but Löffler's methylene blue or Pappenheim's pyronine-methyl-green will generally be found more satisfactory:

1. Make a thin smear upon a slide or cover glass. Heavy wax-pencil marks across the slide will limit the stain to any portion desired.

2. Dry in the air, or by warming high above the flame, where one can comfortably hold the hand.

3. "Fix" by passing the preparation, film side up, rather slowly through the flame of a Bunsen burner: a cover glass three times, a slide about twelve times. One can learn to judge the proper temperature by touching the glass to the back of the hand at intervals. If the film takes on a brownish discoloration, most marked about the edges, it has been scorched and is worthless. Smears can also be fixed by flaming with alcohol, as described for blood films (p. 251), or by soaking for one to three minutes in a 1 per cent solution of mercuric chloride and rinsing well. The last avoids all possibility of spoiling the preparation by scorching.

4. Apply the stain for the necessary length of time, generally one-quarter to one minute.

5. Wash in water.
6. Dry by waving high above a flame or by blotting with filter paper.
7. Mount by pressing the cover, film side down, upon a drop of Canada balsam or immersion oil on a slide. Slides may be examined with the oil-immersion lens without a cover glass.

Gram's Method.—This is a very useful aid in differentiating certain bacteria and should be frequently resorted to. It is very easy and should not be the bugbear which it apparently is to many students. It depends upon the fact that when treated successively with gentian violet and iodine certain bacteria (owing to formation of insoluble compounds) retain the stain when subsequently treated with alcohol, whereas others quickly lose it. The former are called *gram-positive;* the latter, *gram-negative.* In order to render gram-negative organisms visible some contrasting counterstain is commonly applied, but this is not a part of Gram's method proper. The following modification of Gram's method, suggested by G. H. Ruhland, has been in daily use in The Mayo Clinic for many years:[1]

1. Make smears, dry, and fix by heat or mercuric chloride (p. 251).
2. Cover the preparation for half a minute with a 2 per cent solution of crystal violet in methyl alcohol of the highest purity. This stain is much more satisfactory than the unstable aniline gentian violet originally used.
3. Wash with water.
4. Apply Gram's iodine solution one-half minute.
5. Wash in alcohol until the purple color ceases to come off. This is conveniently done in a watch glass. The preparation is placed in the alcohol, face downward, and one edge is raised and lowered with a needle. As long as any color is coming off, purple streaks will be seen diffusing into the alcohol where the surface of the fluid meets the smear. If forceps be used, beware of stain which may have dried upon them. The thinner portions of smears from pus should be practically colorless at this stage. Microscopically, the nuclei of pus corpuscles should retain little or no color. *If, with proper technic, gram-positive organisms are decolorized, the fault probably lies in the iodine solution, which tends to become acid when long exposed to light. It can be corrected by adding a pinch of sodium bicarbonate to the bottle of solution.* Acetone, suggested by Lyon, has come into extensive use, in place of alcohol, as a decolorizer. It acts very quickly and is generally very satisfactory.
6. Apply a contrast stain for one-half to one minute. The stains formerly used for this purpose were an aqueous or alcoholic solution of Bismarck brown or a weak solution of fuchsin. A 1 per cent aqueous solution of safranin is very much better. Pappenheim's pyronine-methyl-green mixture if properly made is also satisfactory; it brings out gram-negative bacteria

[1] Sanford, A. H.: Modified Gram Stain (Ruhland), Jour. Lab. and Clin. Med., *10:* 668 (May), 1925.

sharply, and is especially desirable for intracellular gram-negative organisms like the gonococcus and influenza bacillus, since the bacteria are bright red and nuclei of cells blue.
7. Wash in water, dry, and mount.

The more important bacteria react to this staining method as follows:

Gram Staining (Deep purple).	Gram Decolorizing (Colorless unless a counterstain be used).
Staphylococcus.	Gonococcus.
Streptococcus.	Meningococcus.
Pneumococcus.	Micrococcus catarrhalis.
Bacillus diphtheriae.	Bacillus of influenza.
Bacillus tuberculosis.	Typhoid-dysentery-colon group.
Bacillus of anthrax.	Spirillum of Asiatic cholera.
Bacillus of tetanus.	Bacillus pyocyaneus.
Bacillus aerogenes capsulatus.	Bacillus of Friedländer.
	Koch-Weeks bacillus.
	Bacillus of Morax-Axenfeld.

Möller's Method for Spores.—Bodies of bacteria are blue, spores are red.

1. Make thin smears, dry, and fix.
2. Wash in chloroform for two minutes.
3. Wash in water.
4. Apply 5 per cent solution of chromic acid one-half to two minutes.
5. Wash in water.
6. Apply carbolfuchsin and heat to boiling.
7. Decolorize in 5 per cent solution of sulfuric acid.
8. Wash in water.
9. Apply 1 per cent aqueous solution of methylene blue one-half minute.
10. Wash in water, dry, and mount.

Huntoon's Method for Spores.—This method is simple and appears to be very reliable. Spores are deep red, bodies of bacteria are blue.

1. Make a rather thick smear, dry, and fix in the usual way.
2. Apply as much of the stain as will remain on the cover glass, and steam over a flame for one minute, replacing the stain lost by evaporation.
3. Wash in water. The film is bright red.
4. Dip the preparation a few times into a weak solution of sodium carbonate (7 or 8 drops of saturated solution in a glass of water). Too long application of the carbonate will cause the spores to be blue.
5. The instant the film turns blue, rinse well in water.
6. Dry, mount, and examine.

Preparation of Stain.—

1. Acid fuchsin (Grübler)	4 Gm.
Aqueous solution acetic acid (2 per cent)	50 c.c.
2. Methylene blue (Grübler)	2 Gm.
Aqueous solution acetic acid (2 per cent)	50 c.c.

Mix the two solutions, let stand for fifteen minutes, and filter off the voluminous precipitate through moistened filter paper. The filtrate is the staining fluid. It keeps several weeks, but requires filtration when a precipitate forms.

Löffler's Method for Flagella.—The methods for flagella are applicable only to cultures. Enough of the growth from an agar culture (which should not be more than eighteen to twenty-four hours old) to produce faint cloudiness is added to distilled water. A small drop of this is placed on a cover glass, spread by tilting, and dried quickly. The covers must be absolutely free from grease. To insure this they may be warmed in concentrated sulfuric acid, washed in water, and kept in a mixture of alcohol and strong ammonia. When used they are dried upon a fat-free cloth. Covers may be dried without touching them with the fingers by rubbing between two blocks of wood covered with several layers of lint-free cloth.

1. Fix by heating the cover over a flame while holding in the fingers.
2. Cover with freshly filtered mordant and gently warm for about a minute.

The mordant consists of:

Aqueous solution of tannic acid (20 per cent)	10 c.c.
Saturated solution ferrous sulfate, cold	5 "
Saturated aqueous or alcoholic solution gentian violet	1 "

3. Wash in water.
4. Apply freshly filtered aniline-gentian-violet, warming gently for one-half to one minute.
5. Wash in water, dry, and mount in balsam.

Capsule staining methods are given on pages 53 and 54.

VI. METHODS OF STUDYING BACTERIA

The purpose of bacteriologic examinations is to determine whether bacteria are present or not, and, if present, their species and comparative numbers. In general, this is accomplished by: (1) Direct microscopic examination; (2) cultural methods; (3) animal inoculation.

1. Direct Microscopic Examination.—Every bacteriologic examination should begin with a microscopic study of smears from the pathologic material, stained with a general stain, by Gram's method, and often by the method for the tubercle bacillus. This yields a great deal of information to the experienced worker, and in many cases is all that is necessary for the purpose in view. It will at least give a general idea of what is to be expected, and may determine future procedure.

2. Cultural Methods.—(1) **Collection of Material.**—Material for examination must be collected under absolutely aseptic conditions. It may be obtained with a platinum or nichrome wire loop—which has been heated to redness just previously and allowed to cool—or with a swab of sterile cotton on a stiff wire or wooden applicator. Such swabs may be placed in cotton-stoppered test tubes, sterilized, and kept on hand ready to use. Fluids which contain very few bacteria, and hence require that a considerable quantity be used, may be collected in a sterile hypodermic syringe or one of the pipets described on page 760. The method for blood cultures is given on page 788; for sputum, page 51; for urine, page 157; for feces, page 485.

(2) **Inoculating Media.**—The material is thoroughly distributed over the surface of some solid medium, Huntoon's hormone agar being probably the best for routine work. Blood agar is preferable for streptococci and pneumococci. Use plain nutrient agar without dextrose, to which oxalated or citrated blood has been added, for blood agar plates to differentiate hemolytic and nonhemolytic strains. Special media are required for the gonococcus, tubercle bacillus, and the hemophilic group. When previous examination of smears has shown that many bacteria are to be expected, a second tube should be inoculated from the first, and a third from the second, so as to obtain isolated colonies in at least one of the tubes. The platinum or nichrome wire must be heated to redness *before* and *after* each inoculation. When only a few organisms of a single species are expected a considerable quantity of the material is mixed with a fluid medium.

(3) **Incubation.**—Cultures are placed in an incubator which maintains a uniform temperature, usually of 37.5° C., for eighteen to twenty-four hours, and the growth, if any, is studied as described later. Gelatin will melt with this degree of heat, and must be incubated at about room temperature.

(4) **Study of Cultures.**—When the original culture contains more than one species, they must be separated, or obtained in "pure culture," before they can be studied satisfactorily. This must be done as soon as possible, since some pathogenic organisms quickly die

out in cultures. To accomplish this it is necessary so to distribute them on solid media that they form separate colonies, and to inoculate fresh tubes from the individual colonies. In routine work the organisms can be sufficiently distributed by drawing the contaminated wire over the surface of the medium in a series of streaks. If a sufficient number of streaks be made, some of them are sure to show isolated colonies. Another method of obtaining isolated colonies is to inoculate the water of condensation of a series of tubes, the first from the second, the second from the third, etc., and, by tilting, to flow the water once over the surface of the medium. One or more of these tubes will be almost sure to show nicely separated colonies.

In order to determine the species to which an organism belongs it is necessary to consider some or all of the following points:

(*a*) Naked-eye and microscopic appearance of the colonies on various media.

(*b*) Comparative luxuriance of growth upon various media. The influenza bacillus, for example, can be grown upon media containing hemoglobin, but not on the ordinary media.

(*c*) Morphology, special staining reactions, and the presence or absence of spores, flagella, and capsules. Staining methods for these purposes have been given.

(*d*) Motility. This is determined by observing the living organism with an oil-immersion lens in a hanging-drop preparation, made as follows: A small drop of bouillon culture or of water of condensation from an agar or blood serum tube is placed upon the center of a cover glass; and over this is pressed the concavity of a "hollow-ground slide" previously ringed with vaselin. The slide is then turned over so as to bring the cover glass on top. In focusing, the edge of the drop should be brought into the field. Great care must be exercised not to break the cover by pushing the objective against it.

It is not always easy to determine whether an organism is or is not motile, since the motion of currents and the brownian motion which affects all particles in suspension are sometimes very deceptive.

(*e*) Production of chemical changes in the media. Among these are coagulation of milk; production of acid in milk and various sugar media to which litmus has been added to detect the change; production of gas in sugar media, the bacteria being grown in fermentation tubes similar to those used for sugar tests in urine; and production of indol.

(*f*) Ability to grow without free oxygen.

(*g*) Effects produced when inoculated into animals.

(5) **Anaerobic Methods.**—Some bacteria, the "obligate anaerobes," will not grow unless free oxygen is excluded. This may be accomplished in various ways. Brewer[1] has developed a very satisfactory jar for growing anaerobic organisms on plates in an atmosphere entirely free of oxygen. He has utilized an old principle of Laidlaw, a jar devised by Fildes and McIntosh and improvements made by Brown, but none of these methods embodied the safety factors developed by Brewer as illustrated in Fig. 370. Hydrogen or illuminating gas is introduced into the jar in a steady stream. An electrically heated catalyst causes the oxygen to combine with the hydrogen to form water, or with illuminating gas to form water and carbon dioxide.

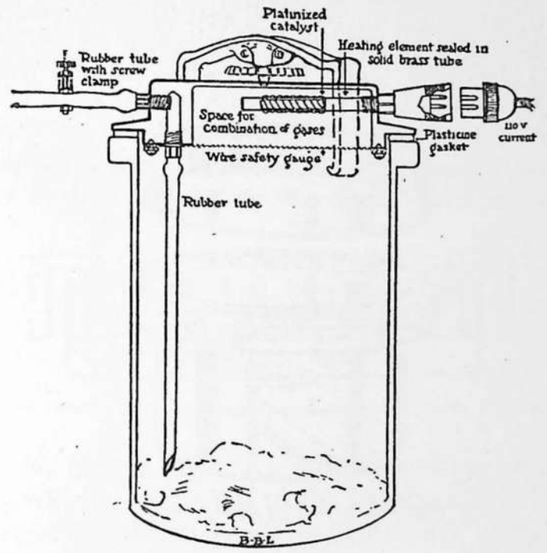


Fig. 370.—Brewer anaerobic culture jar.

Moisture collected on the walls on the inside of the jar indicates the proper operation of the apparatus.

The Spray[2] method eliminates the use of complicated apparatus and is particularly useful to isolate anaerobic organisms on a culture plate. The dish (Fig. 371) which is used in this method, is composed of two parts. The cover of an exceptionally well-made Petri dish, which measures 100 by 15 mm., serves as the lid. The inoculated agar medium is poured into this lid. The lower part of the dish has a rolled-up edge. This forms a moat which allows the dish to be sealed. The lower part of this dish is divided by an

[1] Brewer, J. H.: A Modification of the Brown Anaerobe Jar, Jour. Lab. & Clin. Med., *24*:1190–1192 (Aug.), 1939.

[2] Spray, R. S.: Improved Anaerobic Culture Dish, Jour. Lab. and Clin. Med., *16*: 203–206 (Nov.), 1930.

impressed ridge. Four c.c. of 40 per cent pyrogallic acid are placed on one side of this ridge, and 10 c.c. of a 20 per cent solution of sodium hydroxide are placed on the other side. When the culture medium in the upper dish has been inoculated, the upper part of the dish is inverted over the lower compartment, and is sealed air-tight with melted paraffin. The two chemicals are mixed by tipping the dish slightly and the oxygen in the entire chamber will be removed by the chemical reaction.

Brewer[1] has developed a clear, liquid medium which is placed in regular culture tubes without any type of seal, and which will remain capable of growing anaerobic bacteria for more than a month. Sodium thioglycollate, 0.1 per cent, maintains the oxidation reduction potential. The formula is pork infusion solids 1 per cent, peptone 1 per cent, sodium chloride 0.5 per cent, sodium thioglycollate 0.1 per cent, agar 0.05 per cent, which stiffens the medium slightly, dextrose 1.0 per cent (which may be omitted), and methylene blue 0.0002 per cent. The final *p*H is 7.5. The medium may be

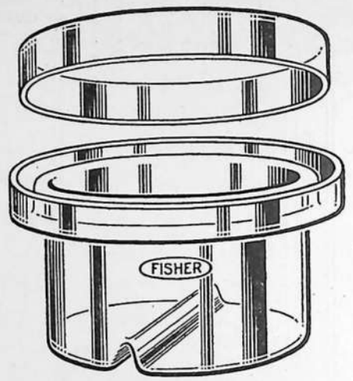


Fig. 371.—Dish for Spray method.

purchased in powdered form and is most easily prepared by dissolving 36.5 Gm. of the dry powder in 1,000 c.c. of distilled water. Boil the solution for a minute, and then place about 15 c.c. in 6 x $\frac{3}{4}$ inch culture tubes. Autoclave in the usual manner and store in a cupboard at room temperature, not in the refrigerator. The medium is clear, broth color, except at the surface where the methylene blue indicates aerobic conditions. The inoculation for anaerobes should be made deep in the medium.

Utilizing sodium thioglycollate in a solid medium, Brewer[2] has devised still another safety method for growing anaerobes. Add 2 gm. of sodium thioglycollate and 1 Gm. of sodium formaldehyde sulfoxylate to a liter of 1 per cent dextrose infusion agar containing 1 c.c. of 1:500 solution of methylene blue. Pour about 40 c.c. amounts of the liquefied medium into 100 by 15

[1] Brewer, J. H.: A Clear Liquid Medium for the "Aerobic" Cultivation of Anaerobes, Jour. Bact. *39:*10 (Jan.), 1940.

[2] Brewer, J. H.: A New Petri Dish Cover and Technique for Use in the Cultivation of Anaerobes and Micro-aerophiles, Science, *95:*587 (June 5), 1942.

mm. Petri dishes. The important part of this method is the use of a new Petri dish cover which is pressed onto the inoculated culture medium in such a way as to make an air seal and yet leave a slight air space above the surface as illustrated in figure 372. The reducing agent in the medium uses up the oxygen in this small amount of air and an anaerobic condition develops.

Tall tubes of dextrose-brain broth (p. 764), which are sealed tight with paraffin, may be used for growing many types of anaerobic organisms. The organisms will be found growing near the bottom of the tube.

(6) **Blood Cultures.**—Typhoid bacilli can be detected in the blood in practically every case of typhoid fever in the first week of the disease; in about 80 to 85 per cent of cases in the second week; and in decreasing percentages in the later weeks. The blood culture, therefore, offers the most certain means of early diagnosis. It is in a sense complementary to the Widal reaction, the former decreasing and the latter increasing in reliability as the disease progresses. The blood culture gives best results before the Widal appears, as one would

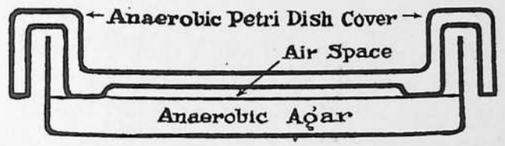


Fig. 372.—Cross section showing use of Brewer anaerobic Petri dish cover.

expect from the fact that the Widal test depends upon the presence of antibodies which are inimical to the bacilli. The two methods together will establish the diagnosis in practically every case at any stage. Bacilli disappear from the blood in convalescence and reappear in a relapse.

In general, blood cultures should be undertaken in all cases of long-continued fever of unknown etiology. Cultures may be more successful if made at a time when the temperature is rising than when it is falling. In septicemia and malignant endocarditis cultures are nearly always positive, the organisms most frequently found being streptococci, staphylococci, and pneumococci. Janeway[1] advocated the addition of p-amino benzoic acid, 5 mg. per 100 c.c., to medium that is to be used for blood cultures if the patient has been treated with sulfonamides. This acid neutralizes the inhibitory effect of these bacteriostatic drugs, without in itself inhibiting bacterial growth. Strep-

[1] Janeway, C. A.: Method for Obtaining Rapid Bacterial Growth in Cultures from Patients Under Treatment with Sulfonamides, Jour. Am. Med. Assn., *116:*941–942 (Mar. 8), 1941.

tococci, usually *Streptococcus viridans*, or more rarely, staphylococci, can be recovered in the great majority of cases of subacute endocarditis if repeated cultures be made. Among acute infectious diseases pneumonia and acute epidemic meningitis show bacteriemia in about half of the cases. Blood cultures are almost always negative in otitis media but usually become positive (showing streptococcus as a rule) when the inflammation extends to the lateral sinus or meninges. The author has seen a case of meningococcemia in an infant who never developed meningitis and who spontaneously recovered from the infection.

Method for Blood Cultures.—*Apparatus.*—It is necessary to have ready a sterile syringe and one or more flasks or tubes of citrate solution or of fluid culture media.

A high-grade all-glass syringe is best. The syringe and needle may be sterilized by boiling them for ten minutes, or by wrapping in a towel and sterilizing in the autoclave. When it is desired to keep a syringe on hand ready for use it is placed, with needle attached, in a large cotton-plugged test tube, in the bottom of which stands a short section of glass tubing into which the needle is inserted to protect its point; the top of the tube is covered with several layers of gauze which should be folded closely about its neck and tied with a string; and finally the tube and syringe are sterilized in the hot-air oven at 150° C. for one hour. In some laboratories it may be found more satisfactory to keep the syringes and needles on hand simply wrapped in towels and sterilized in the hot-air oven.

A number of small cotton-plugged flasks or large tubes are charged with 5 to 10 c.c. of citrate solution (2 Gm. sodium citrate, 0.6 Gm. sodium chloride, 100 c.c. distilled water), sterilized in the autoclave, and kept on hand ready for use. Before sterilization the tops of the flasks or tubes are covered with moderately heavy paper, which is folded neatly about the necks and held in place with rubber bands. Instead of citrate solution some prefer citrated broth or, in certain cases, a fluid medium without citrate, as dextrose-brain broth.

In private practice the use of the Keidel vacuum tube charged with citrated broth is very satisfactory (Fig. 93, p. 194).

Technic.—(*a*) Secure 5 to 20 c.c. of blood from a vein in a sterile syringe under strictly aseptic conditions (p. 193).

(*b*) Immediately remove the needle, place the blood in the flasks or tubes of citrate solution described, adding about as much blood as there is solution in the container, and mix by gently shaking. The citrate prevents clotting and facilitates handling in the laboratory later.

(*c*) At the laboratory mix the citrated blood with appropriate media and place in the incubator. Great care must be exercised to avoid contamination of media when the cotton stoppers are removed or replaced. There is less danger of contamination if the blood is transferred by means

of a sterile cotton-plugged pipet than if it is poured from flasks or tubes. A portion of the citrated blood is mixed with nutrient agar, which has been melted and cooled to 45° C., and is at once poured into Petri dishes. The usual proportion is about 1 part of citrated blood to 3 or 4 of agar. The remainder is distributed among flasks or tubes of fluid media, usually in the proportion of 1 c.c. of blood to 20 c.c. or more of medium.

(*d*) Examine the cultures daily. Do not discard any as negative for ten days or two weeks. Growth in fluid media containing an indicator is evidenced by change in color of the medium. Staphylococci in pyemia usually grow readily in the first twenty-four hours, and the colonies are deep in the plates as well as on the surface. Growth of a few surface colonies, only, commonly means contamination. On the other hand, streptococci and pneumococci do not appear for forty-eight hours or longer. Zones of hemolysis or of green-pigment production about the colonies of the various types of streptococci or pneumococci are readily noted on the plates.

Instead of using citrate solution as above described, it may be desirable in some cases to place all or part of the blood directly into fluid culture media at the bedside, but a relatively large volume of the medium must then be used, usually no less than 30 c.c. for 1 c.c. of blood.

Culture Media.—The choice of media depends on the organisms expected. The nutrient-agar plates described are generally prepared routinely. A medium containing bile is preferred for bacilli of the typhoid-paratyphoid group. For streptococci and pneumococci and for general purposes, a good medium is nutrient broth to which one fourth its volume of sterile ascitic fluid has been added. A still better medium is dextrose-brain broth (p. 764), or Kracke's medium (p. 764). Streptococci and pneumococci grow well in this, especially in the deeper portion. Growth is indicated by a pink color due to formation of slight acidity, as well as by the usual change in turbidity.

Enumeration of Bacteria in Blood.—When it is desired to count the bacteria, a definite amount of blood from a vein, measured as accurately as can be done with a graduated syringe, is mixed at the bedside with a measured amount of the citrate solution described above. At the laboratory definite amounts of this citrated blood are added to tubes of melted nutrient agar by means of a sterile graduated pipet and poured into Petri dishes. When colonies appear, the number of colonies for each plate is counted and the number of bacteria for each cubic centimeter of blood can then be calculated.

Blood Cultures for Typhoid Bacilli.—These are mentioned separately because they offer the most valuable means of early diagnosis of typhoid and paratyphoid fevers, and are so simple, owing to the large number of bacilli usually present in the blood in the first week or two of the disease and to the rapidity with which they grow, as to require little bacteriologic experience or equipment. The blood is obtained from a vein as described and placed at once into a small flask or large tube containing three or four times its volume of ox-bile medium (p. 776) or nutrient broth with 2 per

cent of sodium citrate. Both media prevent coagulation of the blood, but the bile medium is preferable in private practice because it is very easily prepared, and because it favors the growth of the typhoid group of bacilli while it retards that of other bacteria such as contaminating skin cocci. In every case a small amount of the blood should be reserved for the Widal test.

Cultures are placed in the incubator as soon as possible after the blood is added, and at the end of twelve and twenty-four hours are examined for motile bacilli by the hanging-drop method. If none is found transfers are made to slants of nutrient agar, or better, litmus-lactose agar, and incubated for twelve or twenty-four hours longer. On litmus-lactose agar colonies of contaminating staphylococci are pink. If gram-negative bacilli are found they are presumably typhoid or paratyphoid bacilli. Further study is, however, necessary to identify them with certainty, and appropriate media (p. 798) should be inoculated from the growth, or if there be contamination, from isolated colonies obtained as described on page 784. The agglutination test (p. 662) should be used if agglutinating serum is at hand, but it must be remembered that freshly isolated bacteria do not agglutinate well.

3. Animal Inoculation.—In clinical work this is resorted to chiefly to detect the tubercle bacillus. The method is described on page 607.

For the study of bacteria in cultures a small amount of a pure culture is injected subcutaneously or into the peritoneal cavity. The animals most used are guinea-pigs, rabbits, and mice. For intravenous injection the rabbit is used because of the easily accessible marginal vein of the ear.

VII. CHARACTERISTICS OF VARIOUS IMPORTANT BACTERIA

Following is a brief description of those bacteria that are commonly encountered in the clinical laboratory. There is also included a group of important pathogenic bacteria that are not so common, but whose characteristics should be known to the laboratory worker. The common name of the organism is given, and also in parentheses the name as given in the new classification of bacteria in the latest edition of Bergey's Manual of Determinative Bacteriology (p. 809).

1. Staphylococcus pyogenes aureus (*Staphylococcus aureus*).—"Boils" and abscesses are usually caused by this organism. The morphology and staining reactions (p. 588) and the appearance of the colonies are sufficient for diagnosis. Colonies on solidified blood serum and agar are rounded, slightly elevated, smooth and shining, and vary in color from light yellow to deep orange. Young colonies are sometimes white.

2. Staphylococcus pyogenes albus (*Staphylococcus albus*).—This is similar to the preceding, but colonies are white. It is generally less virulent and is found on normal skin.

3. Staphylococcus pyogenes citreus (*Staphylococcus citreus*).—The colonies are lemon yellow; otherwise it resembles the white staphylococcus.

Chapman, Berens, Nilson, and Curcio[1] concluded that the coagulase test is the most reliable single test for distinguishing pathogenic from nonpathogenic staphylococci. Coagulase-positive strains are usually pathogenic regardless of color produced on a solid medium. The test is performed as follows:

Mix a loopful of culture from solid medium with 0.5 c.c. of fresh oxalated or citrated human plasma. After shaking thoroughly, allow the mixture to stand in the incubator at 37° C. over night. Any formation of fibrin is considered to be a positive test and usually means that the organism is pathogenic.

4. Streptococcus.—The morphology and staining reactions have been described (p. 588). The chains are best seen in the water of condensation and in bouillon cultures. The cocci are not motile. Colonies on blood serum are minute, round, grayish, and translucent. Litmus milk is usually acidified and coagulated, although slowly.

When cocci of the morphology of streptococci are found it is first necessary to distinguish them from pneumococci to which they are closely related. Their colonies on ordinary media are similar. The following points will probably be sufficient:

Capsule Formation.—The great majority of streptococci do not possess capsules. Pneumococci usually show them in pus or in other material fresh from the animal body, or when grown on a serum medium.

Fermentation of Inulin.—The pneumococcus generally coagulates and acidifies Hiss' serum-water-litmus-inulin within twenty-four hours. The streptococcus rarely ferments inulin and then only after four or five days. The writer believes this to be an extremely valuable differential point.

Solubility in Bile.—Pneumococci are soluble in bile, streptococci are not. The test is carried out as follows: To a twenty-four-hour broth culture of the organism add one tenth to one fifth its volume of sterile 10 per cent solution of sodium taurocholate in physiologic salt solution or of ox-bile medium (p. 776) which has been filtered as

[1] Chapman, C. H., Berens, Conrad, Nilson, Edith L., and Curcio, Lillian G.: The Differentiation of Pathogenic Staphylococci from Nonpathogenic Types, Jour. Bact., *35:* 311–334 (Mar.), 1938.

clear as possible and sterilized. Place in the incubator for one hour. If the organism is bile soluble the turbidity of the culture will clear up, and subcultures made from this culture will not show growth.

The streptococci have been much studied of late, and a great number of strains have been separated upon the basis of carbohydrate fermentation. From the clinical and pathologic point of view, it is doubtful whether such a classification offers any advantage over the simpler one based upon the appearance of the growth upon blood agar. This distinguishes three groups:

Hemolytic Streptococci.—The colonies on blood agar, after twenty-four or forty-eight hours, are surrounded by a clear colorless zone 2 to 4 mm. wide, due to hemolysis. This group includes most of the virulent strains, such as *Streptococcus pyogenes*, *Streptococcus scarlatinae*, *Streptococcus epidemicus*, and *Streptococcus anginosus*. In liquid media they tend to grow in long chains.

Green-producing Streptococci.—The colonies on blood agar are surrounded after twenty-four or forty-eight hours by a cloudy, greenish zone about a millimeter wide. These are commonly included under the name *Streptococcus salivarius* (*S. viridans; S. mitior*). They are less actively virulent than the hemolyzing type and often associated with mild, chronic inflammations, and also in subacute bacterial endocarditis in which it is isolated often from the blood stream. Certain nonpathogenic strains are also included. In liquid media they tend to grow in short chains.

Anhemolytic Streptococci.—These cause no change in the surrounding medium. The group is practically limited to nonpathogenic strains.

It is important to remember that these reactions on blood agar depend largely upon the use of a proper medium. The optimum reaction of the agar is about *p*H 7.4, and not more than one tenth its volume of blood should be added. The plates should be freshly poured in order that the surface may be moist.

5. Pneumococcus (*Diplococcus pneumoniae*).—The only organism with which this is likely to be confused is the streptococcus. The distinction, which is sometimes difficult, is described above.

The morphology and staining of the pneumococcus have been described (p. 52). In cultures it frequently forms long chains. Capsules are not present in cultures except upon special media. They show best upon a serum medium like that described for the gonococcus, but can frequently be seen in milk. Colonies on blood serum resemble those of the streptococcus. Colonies on blood agar show a green zone like those of *Streptococcus viridans*. The pneumococcus usually promptly acidifies and coagulates milk and acidifies and

coagulates Hiss' serum water with inulin. The very valuable "bile-solubility" test is described in the section upon the Streptococcus.

Upon the basis of immunologic reactions thirty-two types of pneumococci are now recognized. Types I and II are the typical pneumococci. Type III has large distinct capsules and viscid colonies, and is known as *Pneumococcus mucosus* (*Diplococcus mucosus*). It has some of the characteristics of the streptococcus and was formerly known as *Streptococcus mucosus capsulatus*. Strains have been found which hemolyzed blood, did not ferment inulin, and were not bile soluble. The pneumococci of the normal mouth are most frequently of this type. For Types I, II, and III specific immune sera can be prepared by injecting suspensions of killed pneumococci into appropriate animals (horses, rabbits). These sera are extremely useful in the laboratory for determining the type of newly isolated organisms, and Type I serum has great value in the treatment of pneumonia.

In 454 cases of lobar pneumonia Avery, Chickering, Cole, and Dochez found the incidence of the types to be as follows: Type I, 33.3 per cent; Type II, including three subtypes, 33.4 per cent; Type III, 13 per cent; other types, 20.3 per cent.

Determination of Pneumococcus Types.—When pure cultures of the organism are at hand, the type is readily determined by means of agglutination tests with immune sera of Types I, II, and III. Encapsulated gram-positive cocci which are bile soluble and are not agglutinated by any of the three were assigned to Type IV. Type IV has now been subdivided serologically into twenty-nine other groups. Clinically, typing is generally undertaken in order that the patient may have early benefit of curative serum. Time is lacking for careful culturing and isolation of the organism by the usual methods. Several methods are available, three of which are given below. In the first of these inoculation of mice is resorted to. Pneumococci are very pathogenic for mice and outgrow practically all other organisms, and appear in the blood in pure culture. Another method, which is not available in all cases, extracts the antigen from the sputum, and this is at once identified by precipitin tests without need of isolation of the pneumococcus. The third method is a rapid, staining method.

Method of Avery, Chickering, Cole, and Dochez.—A small mass of the sputum, as fresh as possible, is washed gently in several changes of sterile saline. The mass is then ground in a sterile mortar with 1 c.c. of sterile saline and injected into the peritoneal cavity of a white or gray mouse. The mouse will appear sick within five to twenty-four hours. A small drop of peritoneal exudate is then obtained by peritoneal puncture with a sterile capillary pipet through a slight incision in the skin, and

smears are prepared. Should these show only a moderate growth of pneumococci, or if other organisms are present in considerable numbers, time should be allowed for further growth. If there is an abundant growth of pneumococci, the mouse is killed, tied out upon a board, and the body opened aseptically. Cultures upon blood agar are made from the heart's blood and peritoneal exudate, and also smears from the latter to be stained by Gram's method and a capsule stain. The peritoneal cavity is then thoroughly washed out with 4 or 5 c.c. of sterile saline by means of a capillary pipet and the washings are collected in a centrifuge tube. The agglutination test and precipitin tests are then applied to these washings as described in the following paragraphs. The cultures from the heart's blood and the peritoneal exudate are carried through in the usual way, the organism is isolated, and confirmatory agglutination tests are applied.

Agglutination Test.—This may be applied to the peritoneal washing mentioned above or to any pure culture of the pneumoccocus in broth. The fluid is centrifugalized for a few minutes at low speed to throw down leukocytes and débris, and the supernatant fluid which contains the bacteria is pipeted into another centrifuge tube. This is centrifugalized for a long time at very high speed to throw down the pneumococci. The supernatant fluid, which should be as clear as possible, is pipeted into another tube to be used for the precipitin test, and the sediment, which consists largely of bacteria, is mixed with just enough saline to give a turbid suspension. Agglutination[1] and bile-solubility tests are then set up in a series of five small test tubes (about 8 × 50 mm.), as follows:

Tube 1—Bacterial suspension, 0.5 c.c. + Type I serum, diluted 1:20, 0.5 c.c.
Tube 2—Bacterial suspension, 0.5 c.c. + Type II serum, undiluted, 0.5 c.c.
Tube 3—Bacterial suspension, 0.5 c.c. + Type II serum, diluted 1:20, 0.5 c.c.
Tube 4—Bacterial suspension, 0.5 c.c. + Type III serum, diluted 1:5, 0.5 c.c.
Tube 5—Bacterial suspension, 0.5 c.c. + Sterile ox bile or 10 per cent sodium taurocholate, 0.1 c.c.

The tubes are placed in the incubator for one hour. Agglutination is read macroscopically as has been described for the Widal test. Bile solubility is recognized by the clearing up of the turbidity in Tube 5. The tube in which agglutination occurs indicates the type of the pneumococcus under examination, since its type must correspond to that of the serum which agglutinates it. Strains which are agglutinated by Type II serum undiluted (Tube 2), but not by the same serum diluted 1:20 (Tube 3), belong to one of the subgroups (a, b, x) of Type II. Strains which are soluble in bile, but do not agglutinate in any tube, are placed in Type IV.

Precipitin Test.—This is applicable to the peritoneal washings mentioned above, and is undertaken when there are so many contaminating organisms as to make the agglutination test uncertain. It may fail if too much saline was used for the washings. The fluid must be cleared as much as possible by long centrifugation at high speed. The test is set up as follows:

[1] Immune sera for these tests may be obtained from the biological supply houses.

Tube 1—Peritoneal washings, 0.5 c.c. + Type I serum, diluted 1:10, 0.5 c.c.
Tube 2—Peritoneal washings, 0.5 c.c. + Type II serum, undiluted, 0.5 c.c.
Tube 3—Peritoneal washings, 0.5 c.c. + Type II serum, diluted 1:10, 0.5 c.c.
Tube 4—Peritoneal washings, 0.5 c.c. + Type III serum, diluted 1:5, 0.5 c.c.

The test is more delicate and the results more easily read if the serum is introduced into the tube with a capillary pipet so as to form a layer under the peritoneal washings. At the juncture of the two liquids a white cloud is formed.

A positive reaction in any tube is shown by the appearance of a white cloud. The interpretation is the same as that given above for the agglutination test.

Rapid Method of Oliver.—This method is applicable only when satisfactory samples of sputum, containing many pneumococci, can be obtained, which, however, is possible in nearly all typical cases of lobar pneumonia. The type of pneumococcus present can be determined within one-half to three-quarters of an hour. Briefly the method consists in treating the sputum with bile to dissolve the pneumococci, and thus liberate its specific precipitable substance, and then in applying the precipitin test with the several specific antisera.

1. Select the portion of sputum most likely to contain many pneumococci. This is usually the most purulent or blood-streaked portion. Make a smear and stain by Gram's method to confirm the presence of the pneumococcus.

2. Place 1 to 2 c.c. of this sputum in a conical centrifuge tube, and add 3 to 5 drops of 10 per cent sodium taurocholate or of undiluted sterile ox bile.

3. Thoroughly break up and mix the mass by stirring with a rod or grinding in a small mortar, meanwhile adding, a few drops at a time, just enough sterile physiologic saline to insure sufficient fluidity to allow of centrifugation. This should require less than 1 c.c. of saline.

4. Place the tube in a water bath at 42° to 45° C. for twenty minutes.

5. Centrifugalize at high speed until the supernatant fluid is as clear as possible.

6. Carefully pipet 0.3 to 0.5 c.c. of the supernatant fluid to each of 3 small absolutely clean test tubes (about 8 × 50 mm.). Label tubes 1, 2, and 3.

7. To tubes 1, 2, and 3 add 1 to 2 drops of Types I, II, and III pneumococcus antiserum, respectively. A positive test is evidenced by an almost immediate clouding and flocculation, which is increased by heating in a water bath at 42° C. for fifteen minutes. The tube in which this occurs indicates the type of the pneumococcus present in the sputum. When no reaction occurs in any tube, the organism is assumed to be of Type IV, provided it is, in reality, a pneumococcus.

8. When only a very small quantity of sputum is available it is desirable to centrifugalize in correspondingly narrow tubes. The three precipitin tests may be carried out with as little as 0.3 c.c. of supernatant fluid.

Sabin's Rapid Method (*Neufeld Reaction*).—This method which was described by Sabin[1] uses rabbit serum which can now be obtained from biologic supply houses. The method is so simple that it can be used by anyone who is familiar with the use of the microscope. The method is based on the observation of Neufeld that when pneumococci are mixed with specific immune serum there is a swelling of the capsules of the organisms. Fresh sputum with an abundance of pneumococci must be examined as soon as possible. Place two flecks of sputum on a cover glass for each type serum available. Add two loopfuls of serum to the sputum; mix; and add a loopful of Löffler's methylene-blue stain. Smear the edges of a deep hanging-drop slide with petrolatum and invert the cover glass. After two minutes examine the preparation with an oil-immersion objective. The

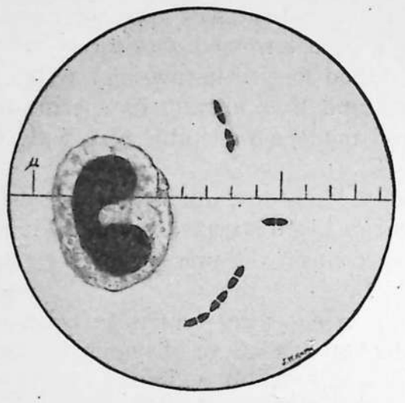


Fig. 373.—Sabin's rapid method. Type II pneumococcus in sputum mixed with Type I antiserum (rabbit); no capsular swelling. (Albert B. Sabin, in Jour. Am. Med. Assn.)

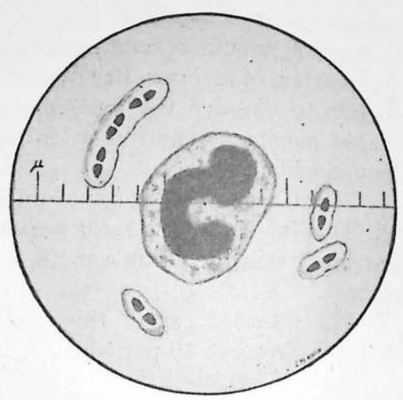


Fig. 374.—Sabin's rapid method. Type II pneumococcus in sputum mixed with Type II antiserum (rabbit); capsular swelling (Neufeld reaction). (Albert B. Sabin, in Jour. Am. Med. Assn.)

type reaction is a swelling of the capsule (Figs. 373 and 374). The diplococci are stained blue and in a positive reaction they lie within a clear zone of refractile substance. Type II pneumococci generally present the larger zone. Sabin emphasizes that agglutination plays no part in this reaction. He claims that the characteristic appearance of a single diplococcus is sufficient to make a diagnosis. Group IV pneumococcus has now been subdivided into 29 types, and there are now available for routine use rabbit serums for Types I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XII, XIII, XIV, XV, XVI, XVII, XVIII, XIX, XX, XXI, XXII, XXIII, XXIV, XXV, XXVII, XXVIII, XXIX, XXXI, XXXII. Types XXVI and XXX are apparently duplicates of other types serologically, and serums for these two types are not supplied.

[1] Sabin, A. B.: Immediate Pneumococcus Typing Directly from Sputum by the Neufeld Reaction, Jour. Am. Med. Assn., *100*:1584–1586 (May 20), 1933.

6. Micrococcus catarrhalis (*Neisseria catarrhalis*) grows readily at room temperature and on ordinary media, where it forms large, white, dry colonies with irregular edges and elevated centers. This readily distinguishes it from the gonococcus and meningococcus, which it closely resembles in morphology and staining reactions.

7. Gonococcus (*Neisseria gonorrhoeae*).—Its morphology and staining peculiarities are given on page 589. These usually suffice for its identification. In cultures the chief diagnostic point is its failure to grow on ordinary media. A satisfactory medium to produce growth is ascitic or hydrocele agar, or blood agar made from human or rabbit blood. Colonies are minute, grayish, and translucent. Of all of the culture media and methods which have been proposed for growing gonococci, that of McLeod, Coates, Happold, Priestley, and Wheatley[1] is perhaps the most striking. The culture medium that is used is the "chocolate"-blood agar, which is described on page 769. The poured plates are inoculated with the fresh material from urethral, cervical, or vaginal discharges. Place the plates in an anaerobic jar, and add carbon dioxide until the jar has been filled with a mixture of 8 or 10 per cent carbon dioxide in air. Incubate at 36° C. for eighteen hours (occasionally followed by further incubation in the open incubator for twenty-four hours if it seems necessary). The plates will usually show a luxuriant growth of several different types of organisms. If small, dewdrop-like colonies of gonococci are present they may be recognized by the oxydase reaction. Gently pour on to the plate a small amount of a 1 per cent aqueous solution of tetramethyl-*p*-phenylenediamine hydrochloride which is poured off immediately. Colonies of gonococci will turn rapidly to a bright purple color. These colonies may be fished and identified as gram-negative diplococci. Furthermore, they may be subcultured at once as the organisms are still viable after the reaction. If such subcultures fail to grow on ordinary culture media, but growth occurs on special media suitable for the growth of gonococci, a positive diagnosis may be accepted.

8. Meningococcus (*Neisseria intracellularis*).—It grows poorly or not at all on plain agar. On Löffler's blood serum, upon which it grows fairly well, colonies are round, colorless or hazy, flat, shining, and viscid looking. Upon serum, ascitic, hydrocele, and blood agar meningococci form faint bluish colonies 1 to 2 mm. in diameter, distinctly

[1] McLeod, J. W., Coates, J. C., Happold, F. C., Priestley, D. P., and Wheatley, B.: Cultivation of the Gonococcus as a Method in the Diagnosis of Gonorrhea with Special Reference to the Oxydase Reaction and to the Value of Air Reinforced in its Carbon Dioxide Content, Jour. Path. and Bacteriol., *39*:221–231 (July), 1934.

larger than the usual streptococcus colony. It quickly dies out. Its morphology and staining reactions are given on page 604.

9. Bacillus typhosus (*Eberthella typhosa*) was first described by Eberth in 1880, and was first grown and proved to be the cause of typhoid fever by Gaffky in 1884. It is a small rod-shaped organism which measures 0.6 by 2 to 3 microns, and which is actively motile and gram-negative. It is aerobic and grows best at 37° C. on practically all culture media, but it is most quickly isolated on Endo's culture medium or on some similar differential medium (p. 771). In sugar media, acid is produced in dextrose, levulose, galactose, xylose, maltose, raffinose, dextrin, glycerol, mannitol, and sorbite. There is no action on lactose, sucrose, inulin, inosite, salicin, and there usually is not any change in arabinose, and dulcitol. Xylose may be fermented rapidly by some strains, and very slowly by others; this difference has been used for differentiation. Indol is not formed by typical strains. The organism may be grown from the blood stream during the early part of typhoid fever (p. 789). Later in the course of the disease, and from typhoid carriers, it may be isolated from the stools, urine, and duodenal contents, on differential media. Suppurative lesions may result as a late complication, and chronic infection of the long bones, and of the joints occasionally may result. The extensive use of typhoid vaccine has proved thoroughly efficacious. This accepted prophylaxis, coupled with greatly improved sanitation in all respectable communities, has resulted in a most amazing and gratifying decrease in the amount of typhoid infection. Preventive medicine has likely been more successful in the control of typhoid fever than it has in the control of any other disease.

10. Bacillus paratyphosus A (*Salmonella paratyphi*).—This is a gram-negative, motile, rod-shaped organism which measures 0.6 by 3 to 4 microns. It belongs to the "intermediate group"; all organisms of this group ferment dextrose, mannite, and rhamnose. It slowly ferments dulcite. No organism of this group ferments lactose and sucrose. Bacillus paratyphosus A is distinguished from the other intermediate organisms by the fact that it does not ferment xylose. It also fails to blacken culture media which contain lead acetate. Used as an antigen, it produces a specific antiserum, and is combined with Bacillus typhosus and Bacillus paratyphosus B in a prophylactic vaccine.

11. Bacillus paratyphosus B (*Salmonella schottmuelleri*) is gram-negative, motile, and measures 0.6 by 2 to 3 microns. It was first described by Schottmueller in 1900. This intermediate organism is culturally almost identical with the organisms of the enteritidis

group, which cause "meat-poisoning." It can usually be distinguished by agglutination tests with a specific antiserum. It turns lead acetate agar brown and ferments xylose, which distinguishes it from B. paratyphosus A, but not from organisms of the enteritidis group, from which it is identified by its production of acid and gas in inositol.

12. Bacillus dysenteriae Shiga (*Shigella dysenteriae*), which was described in 1898 by Shiga as the cause of Japanese dysentery, is the type species of the genus of dysentery-producing organisms. These organisms are nonmotile rods, which produce acid in certain carbohydrates, but which do not form gas. The organism is gram-negative and measures 0.4 to 0.6 by 1 to 3 microns. Acid is formed in dextrose, levulose, raffinose, dextrin, glycerol, and adonitol, but none is formed in arabinose, xylose, rhamnose, maltose, lactose, sucrose, dextrin, salicin, mannitol, or dulcitol. No indol is produced. A therapeutic antiserum has been made against this strain of Shigella.

13. Bacillus dysenteriae Flexner (*Shigella paradysenteriae*) is morphologically similar to the Shiga organism. Different varieties of this species have been described by Hiss and Russell, Strong, and Sonne. The differential varieties may be distinguished serologically from *B. dysenteriae* Shiga, and from each other. Culturally, they are characterized by fermenting dextrose, galactose, maltose, arabinose, raffinose, and mannitol. The Sonne Group III, and the Strong varieties also ferment sucrose, while the Hiss-Russell, and the Sonne Group III varieties do not ferment maltose. No acid is produced from lactose or xylose. B. dysenteriae Shiga is the usual cause of summer diarrhea of infants.

14. Bacillus coli (*Escherichia coli*).—Escherich described this organism in 1885. One of the commonest of bacteria, the "colon bacillus" is found as a commensal species in the intestinal canal of all vertebrates, including man. It is pathogenic when it infects the gallbladder, kidneys, and bladder, and it is in this pathogenic rôle that chief interest lies. It may occasionally invade the blood stream, but, wholly unlike the other organisms of the colon-typhoid-dysentery group, it is of no importance to find it in the feces. It is motile, gram-negative, and measures 0.5 by 1 to 2 microns. It grows readily on ordinary culture media, and can be isolated readily on Endo's culture medium (p. 771), as it ferments lactose. It also produces acid and gas in dextrose, levulose, galactose, arabinose, maltose, raffinose, dextrin, salicin, mannitol, dulcitol, and sorbite. Indol is formed. It does not ferment sucrose, while a very similar form, which was called *Bacillus coli communior* by Durham in 1900 (*Escherichia communior*), produces acid and gas in sucrose.

CHARACTERISTICS OF THE PRINCIPAL MEMBERS OF TYPHOID-DYSENTERY-COLON GROUP

	Fermentation of carbohydrates.							
	Dextrose.	Lactose.	Sucrose.	Maltose.	Mannite.	Xylose.	Indol formation.	Motility.
Bacillus typhosus	a	0	0	a	a	x	−	+
B. paratyphosus A	ag	0	0	ag	ag	0	−	+
B. paratyphosus B	ag	0	0	ag	ag	ag	−	+
B. dysenteriae, Shiga	a	0	0	0	0	x	−	−
B. dysenteriae, Flexner	a	0	0	a	a	x	+	−
B. dysenteriae, Hiss-Russell, "Y"	a	0	0	0	a	x	+	−
B. dysenteriae, Strong	a	0	a	0	a	x	+	−
B. dysenteriae, Sonne Group III	a	0	a	a	a	x	−	−
B. coli communis	ag	ag	0	ag	ag	x	+	+
B. coli communior	ag	ag	ag	ag	ag	x	+	+
B acidi lactici	ag	ag	0	ag	ag	x	+	−
B. lactis aerogenes	ag	ag	ag	ag	ag	x	+	−

Explanation: a, acid produced. +, present or positive.
ag, acid and gas produced. −, absent or negative.
0, no reaction. x, not required for identification.

15. Bacillus acidi lactici (*Escherichia coli* var. *acidilactici*) is nonmotile, gram-negative, and nonpathogenic. It grows best at 30° C., though it will grow at 37° C. It produces acid and gas in dextrose, levulose, galactose, arabinose, maltose, lactose, raffinose, dextrin, mannitol, sorbite, and adonitol. No acid or gas is formed in sucrose, salicin, or dulcitol.

16. Bacillus lactis aerogenes (*Aerobacter aerogenes*) is an encapsulate nonmotile, gram-negative organism which sometimes is confused with colon bacillus because it is commonly found in the intestinal tract of man and other animals. It is widely distributed in nature, and is often found in milk, being one of the chief causes of its souring. It produces acid and a large amount of carbon dioxide in the fermentation of dextrose, levulose, galactose, arabinose, lactose, maltose, sucrose, raffinose, dextrin, salicin, glycerol, mannitol, sorbite, inosite, and adonitol. It grows with a light blue colony on eosin-methylene blue medium (p. 770). It is also distinguished from *E. coli* by the Voges-Proskauer reaction. Isolate colonies and incubate in 10 c.c. of dextrose broth for four days at 37° C. Add 5 c.c. of 10 per cent solution of sodium hydroxide. A pink color indicates a positive reaction, depending upon the production of acetyl-methyl carbinol from dextrose by organisms of the aerogenes group. "Colon bacilli" do not give this reaction.

17. Bacillus enteritidis (*Salmonella enteritidis*).—This is usually referred to as "Gaertner's bacillus," as Gaertner described it in 1888. It is the chief cause of "meat poisoning." This disease often manifests itself in epidemic form, after a large group of people have partaken of a common meal at which contaminated food has been served. While usually not fatal, very severe gastro-intestinal symptoms, with fever, appear in twenty-four hours after the meal. The organism may at times be recovered from the blood stream in severe cases. It is motile, gram-negative, and measures 0.6 by 2 to 3 microns. It turns lead acetate agar brown, and produces acid and gas in dextrose, levulose, galactose, mannose, arabinose, xylose, maltose, trehalose, dextrin, glycerol, mannitol, dulcitol, and sorbite. No acid or gas is produced in lactose, sucrose, inulin, salicin, raffinose, adonitol, or inosite.

18. Diphtheria bacillus (*Corynebacterium diphtheriae*).—The diagnosis of the diphtheria bacillus is usually made from a study of stained smears from eighteen-hour cultures upon blood serum medium, as described in 1884 by Löffler, who first grew the organism and who found it in cases of diphtheria, and by Klebs in 1883. Its morphology and staining peculiarities are characteristic (p. 611). It is nonmotile and gram-positive. The colonies are round, elevated, smooth, and grayish. By means of specific agglutinating sera, it has been demonstrated that there are at least five distinct types of organism. The following table (p. 802) from Bergey's Manual of Determinative Bacteriology shows the fermentating characteristics of these serologic types.

19. Bacillus xerosis (*Corynebacterium xerose*).—In xerosis of the conjunctiva, a form of chronic conjunctivitis, Neisser and Kuschbert in 1884 found a bacillus, which was similar morphologically and tinctorially, to the diphtheria bacillus. It is known now that this organism may be found on normal conjunctivas, and it is considered

	Maltose.	Dextrin.	Glycerol.	Galactose.	Sucrose.
Type I American (No. 8)....	+	+	−	−	−
Type II Durand....	−	−	−	−	−
Type III Nodet.....	+	+	+	+	−
Type IV Benjamin.....	+	+	+	+	−
Type V Sirbeaux.....	+	+	+	+	−

to be nonpathogenic. It readily is distinguished culturally from the diphtheria bacillus. Bacillus xerosis does not grow on gelatin, or potato, but grows aerobically at 37° C. on agar, Löffler's blood serum, and broth. The colonies are thin and grayish. They are adherent on blood serum. In dextrose, levulose, galactose, maltose, and sucrose media, acid is formed. There is no growth in dextrin broth. It is apparent then that this organism can be distinguished from the diphtheria bacillus by using dextrin and sucrose broths. Nearly all types of diphtheria bacilli grow in, and ferment, dextrin, but do not grow in sucrose, while just the reverse condition, fermentation of sucrose and no growth in dextrin, pertains in the case of the nonpathogenic Bacillus xerosis.

20. Tubercle bacillus (*Mycobacterium tuberculosis*).—The methods of identifying this important acid-fast organism have been described (pp. 44 and 156). Cultivation is not resorted to in routine clinical work. The organism grows very slowly and only on certain culture media. It is gram-positive and nonmotile. It first was described by Koch in 1884.

21. Bacillus leprae (*Mycobacterium leprae*).—This organism was found in 1879 in the lesions of leprous patients. It is an acid-fast, gram-positive, rod-shaped organism. It measures 0.2 to 0.35 by 1.5 to 4.6 microns, and is considered to be the cause of leprosy (see also p. 615). All attempts to culture such an organism have met with varied success. The names of Clegg, Rost, and Duval are each associated with the cultivation of organisms from the lesions of lepers. Koch's postulates cannot be met and the results are inconclusive. In Bergey's Manual of Determinative Bacteriology, *Mycobacterium leprae* is described as an aerobic organism growing at 37° C. on gelatin, agar, glycerol agar slant, broth, glycerol potato, and blood serum. The growth usually is wrinkled and grayish or yellowish on solid media, and there is a wrinkled pellicle, and a thick powdery sediment on broth.

22. "Ray fungus" (*Actinomyces bovis*).—This organism is described among the fungi by mycologists but is generally classed among the higher bacteria by bacteriologists (see Bergey's classification, page 810). It is the cause of "lumpy jaw" in cattle and causes actinomycosis in man. Probably *Actinomyces hominis* and *Actinomyces bovis* are the same organism. Many bacteriologists prefer the name *Cohnistreptothrix israeli*. The "sulfur bodies" are readily seen in the pus from draining sinuses in various parts of the body. Sanford and Voelker[1] in their review of actinomycosis in the United States, found that nearly 80 per cent of the persons affected were males. It is chiefly a disease of young adults. In sixty per cent of the cases the lesions involved the head and neck and were about equally divided among the jaw, neck and face. Slightly more than 18 per cent were abdominal, and about 14 per cent were found in the thorax, in the thoracic wall or the lungs. The characteristic "sulfur bodies" may be found in the sputum if the lungs are involved. (See p. 42, Figs. 26 and 27.) No region of the body, however, is immune from this infection. Although there are a number of aerobic actinomycetes, *Actinomyces bovis* is anaerobic and is grown with difficulty.

23. Bacillus influenzae (*Hemophilus influenzae*).—Diagnosis will usually rest upon the morphology and staining peculiarities, which have been described on page 54, and upon the fact that the bacillus will not grow on ordinary media. It can be grown on blood-smeared agar slants. Before inoculation, these slants should be incubated to make sure of sterility. The colonies are difficult to see without a hand lens. They are very minute, discrete, and transparent, and resemble small drops of dew.

24. Bacillus of Ducrey (*Hemophilus ducreyi*).—This organism was described in 1889 as the cause of soft chancre, or chancroid which is an irregular, spreading ulcer with undermined edges. It usually is found on the genitalia, and is distinguished clinically from the hard chancre or initial lesion of syphilis. The organism is a small, nonmotile, gram-negative rod, which measures 0.5 by 1.5 microns, and which occurs singly and in chains. It is grown with difficulty, and best on media which contain rabbit, sheep, or human blood which has been heated to 55° C. for fifteen minutes. Aerobic growth at 37° C. on this medium is characterized by small, grayish, glistening colonies. A slight zone of hemolysis appears in three or four days.

25. Bacillus pertussis (*Hemophilus pertussis*).—The bacillus of whooping cough, which was discovered by Bordet and Gengou in

[1] Sanford, A. H., and Voelker, Minna: Actinomycosis in the United States, Arch. Surg., *11*:809–841 (Dec.), 1925.

1900, was first cultivated by them in 1906. In sputum, early in the disease, the organisms are found in great numbers, scattered among the leukocytes as small gram-negative, ovoid, nearly coccal, forms. This bacillus is slightly larger than the influenza bacillus and has a tendency to polar staining. It grows as an aerobe at 37° C. on blood agar, or on the special culture medium of Sauer and Hambrecht (see p. 769). In broth, the growth is turbid; there is a heavy ropy sediment and floating strands. Litmus milk shows an alkaline reaction and is decolorized in ten to twenty days. On potato, the growth produces a light yellow streak which becomes tan. There is no action on carbohydrate media. Two types of organism have been differentiated serologically.

26. Anthrax Bacillus (*Bacillus anthracis*).—In 1849 this organism was observed in the blood of infected animals, being the first bacterial organism to be described accurately as a cause of disease. It is a large straight rod-shaped organism which is 5 to 10 microns long by 1 to 3 microns wide, nonmotile, and gram-positive. It grows in a characteristic manner, forming tangles of long threads on any culture medium. It grows aerobically at any temperature from 12° to 45° C., and produces large spores which are very resistant. It is the cause of anthrax in cattle, sheep, and swine. When anthrax affects man, there is usually a primary malignant pustule, which is followed by general systemic infection.

27. Bacillus welchii (*Clostridium perfringens*).—This organism is the cause of gas gangrene. It is a spore-forming, anaerobic, nonmotile, encapsulated, gram-positive organism which measures 1 to 1.5 by 4 to 8 microns. Growth occurs in various culture media at 35° to 37° C., although it will grow in temperatures up to 50° C. Gelatin is liquefied and blackened. Circular, moist, slightly raised colonies are formed on agar. Thompson's modification of the medium of Wilson and Blair (p. 765) demonstrates the presence of this organism in clinical material after incubation for a few hours. There is a turbid growth in broth. Litmus milk is coagulated; acid is formed and gas is produced profusely in all of the usual sugar media by all types, and by Type I in inulin, amylum, glycogen, glycerol, and inosite. Three types have been described in addition to Type I. Type II does not ferment inulin, Type III does not ferment glycerol, and Type IV does not ferment either glycerol or inulin. An exotoxin is formed and an efficacious antitoxin has been prepared, and is on the market.

28. Vibrion septique (*Clostridium septicum*).—This organism was studied extensively in war wounds and was usually found associated with Welch's gas bacillus. It is occasionally found alone in the

tissues, and causes malignant edema. It is a gram-positive, spore-forming, rod-shaped organism, which measures 0.6 to 1 by 3 to 8 microns. The ends are rounded. It grows anaerobically, best at 37° C., and forms chains. It is motile and forms grayish spreading colonies on agar. It liquefies gelatin and produces gas bubbles. Turbidity, with later clearing, is produced in broth. There is a disagreeable odor. Litmus milk becomes acid, coagulated, and filled with gas bubbles. Acid and gas are formed in nearly all of the ordinary carbohydrate media, with the exception of sucrose and inulin. The organism is pathogenic for laboratory animals. It forms a powerful exotoxin, against which an antitoxin has been prepared.

29. Bacillus sporogenes (*Clostridium sporogenes*).—This organism, which has been isolated from the soil and intestinal contents, is probably nonpathogenic, though it is often found in wounds in which there has been much contamination. It is anaerobic, gram-positive, and forms central oval spores. As a result of its active proteolytic ability, it produces a foul odor in wounds. It grows well on various culture media under proper conditions; liquefies gelatin; produces gas and a putrid odor in broth; and ferments dextrose, levulose, galactose, maltose, dextrin, glycerol, mannitol, and sorbite to form acid and gas.

30. Bacillus tetani (*Clostridium tetani*).—The cause of tetanus or lockjaw is a rod-shaped, spore-forming, anaerobic organism which was described by Nicolaier in 1884. It forms a very poisonous exotoxin, against which a potent antitoxin is marketed by the commercial biologic laboratories. The organism is gram-positive and measures 0.4 to 0.6 by 4 to 8 microns, and occurs singly. It is motile and produces spherical terminal spores, which give the organism the appearance of a "drumstick." Gelatin is slowly liquefied; growth on a solid medium appears as small, thin, transparent colonies. Carbohydrates are not fermented by this organism. Optimum temperature for growth is 37° C. The characteristics of the growth of this most deadly bacterial organism in artificial media are also observed in the activity of the organism in the animal body. If it has been carried deep into the tissues by some piercing instrument such as a bullet or a nail, and if the surface of the wound has become healed over, there results the anaerobic condition which is ideal for the growth of the tetanus bacillus. This takes place comparatively slowly, and it is usually about fourteen days after the injury, which may have seemed trivial, and which because of the healing of the surface may be nearly forgotten when the signs of the deadly disease produced by the toxin appear. It is during this incubation period that the prophylactic dose of antitoxin must be administered.

31. Bacillus botulinus (*Clostridium botulinum*).—A rapidly fatal disease, which is known as botulism is produced by this anaerobic bacillus. The organism is motile, gram-positive, measures about 1 by 4 to 8 microns, and forms spores. It does not produce its toxins in the living tissues, as does the gas bacillus, or the tetanus bacillus, but in meat or canned foods. Growth occurs at 25° C. (slightly above room temperature) and in temperatures up to 50° C. This accounts for the production of toxin in food which is canned at home, and in which sterilization has not been carried out properly. Even in such contaminated food the toxin can be destroyed by boiling before using the canned corn, beans, meat, or other similar food. Bacillus botulinus liquefies and blackens gelatin, and grows in flat, grayish, irregular, mottled colonies on agar. Turbidity is produced in bouillon. Acid and gas are formed in culture media which contain dextrose, maltose, sucrose, glycerol, and starch. Lactose is not fermented. There are three types, A, B, C; each is distinguished from the others by the action of the specific antitoxin in neutralizing only its own toxin, and affording no protection against the toxins of the other types.

32. Vibrio cholerae (*Vibrio comma*).—Koch reported finding the Komma-bacillus in the stools of patients who had Asiatic cholera, in 1884. It is a short, bent, rod-shaped organism, which resembles somewhat the punctuation mark that suggested the name which was given to it by the discoverer. It measures 0.4 to 0.6 by 1.5 to 3 microns, and is motile and gram-negative. It grows singly or in chains which form spirals. It is aerobic and grows at 37° C., on all culture media. It forms indol and hydrogen sulfide. Most of the carbohydrates are fermented, but there is no acid formed with lactose, inulin, or dulcitol. There have probably been a million or more deaths from Asiatic cholera all told in the past century. A very severe epidemic of this disease appeared in Russia in 1908–1910, in which there were more than 140,000 deaths. The disease confers permanent immunity and is essentially a disease of man. The same sanitary management that is used in typhoid fever applies to the control of cholera, except that the carriers may be more of a problem. All reports indicate that prophylactic vaccination is of benefit in those countries in which cholera is more or less endemic. While it is uncommon in the United States, this disease appeared in the early days of Chicago, and also as far north as St. Paul, Minnesota.

33. Glanders bacillus (*Malleomyces mallei*).—Löffler described the "Rotzbacillus" in 1886. It is a small slender rod, which has round ends, and which measures 0.25 to 0.4 by 1.5 to 3 microns. Usually, this organism grows singly, though rods may be formed. It is non-

motile, gram-negative, and aerobic, and grows readily at 37° C. on all laboratory media. Old cultures show a reddish brown tinge. Glanders is most commonly found in horses, in either an acute or chronic form. The acute disease, which is manifested by high temperature and a nasal discharge, usually is rapidly fatal, while the chronic form, which is called "farcy," develops slowly, involves the lymphatics, forms swellings and abscesses, and may go on to complete recovery. It is transmissible to man, sheep, and goats, and to all laboratory animals except rats. Cattle, hogs, and birds do not contract the disease.

34. Bacillus pestis (*Pasteurella pesti*).—This is a nonmotile, gram-negative rod-shaped organism, which measures 1 by 2 microns and which was first described by Yersin in 1894. It is the cause of bubonic plague of man, rats, and ground squirrels. Small laboratory animals can be infected experimentally. The means of transmission to rats, and to man, is by infected rat fleas. The organism is readily isolated from the buboes, growing aerobically at 37° C., or better at 30° C., on all ordinary culture media. Lactose is not fermented. Swollen and vacuolated involution forms appear in twenty-four hours on agar that contains 3 to 5 per cent salt, which is used as a diagnostic culture medium. The organism is not readily killed by freezing temperature. A rapidly fatal form of the disease is known as pneumonic plague, in which the lungs are involved. During the middle ages, the "black death" swept time and again over Europe. The modern fight against the rat, and the prevention of the introduction into this country of rats which are infected with plague is a most important function of the U. S. Public Health Service. The student should familiarize himself with the various government publications on this subject, and with the highly efficient manner in which small epidemics of bubonic plague have been controlled by the U. S. Public Health Service.

35. Bacterium tularense (*Pasteurella tularensis*).—This organism is the cause of a new American disease, tularemia, described by Francis.[1] The disease occurs in nature as a fatal bacteremia of various rodents, especially rabbits. It is transmitted to man either by handling diseased carcasses or through the bite of a blood-sucking insect or tick. Characteristic symptoms are glandular enlargement, often with suppuration, and typhoid-like fever. The diagnosis is best made by a blood-serum agglutination test using *B. tularense* antigen, according to the method for the macroscopic Widal test given on page 659. The organism is a small, nonmotile gram-negative bacillus,

[1] Francis, Edward: Tularemia, Francis, 1921. A New Disease of Man, Washington, Government Printing Office, 1922, Hygienic Laboratory, Bulletin 130, 84 pp.

from 0.3 to 0.7 micron long; it also appears as a coccus. It does not grow on ordinary media, but will grow readily on special media such as that described on page 774.

36. Brucella abortus (*Brucella abortus*).—This organism was first described by Bang (*Bacterium abortus*) in 1897 as the cause of contagious abortion in cattle. The convincing work of Alice Evans, and also that of Carpenter, substantiates the claim that it is the cause of undulant fever. *B. abortus* is similar to, if not identical with, the organism described by Bruce in 1893 as the cause of undulant fever in the island of Malta, and called *Micrococcus melitensis*. It is a short, nonmotile, oval rod, gram-negative, growing slowly, and only in reduced oxygen tension, and possibly better in an atmosphere containing about 10 per cent of carbon dioxide, at 37° C. It is probable that the chief source of infection is unpasteurized cow's milk from herds that may or may not be suffering from contagious abortion. Besides this bovine source, there also may be a porcine organism that infects man through direct contact. The disease, as it appears among veterinarians and packing-house workers, may be due to this strain. Then there is the rarer Maltese type of infection with the caprine strain, from drinking infected goat's milk. The disease among goats does not produce abortion. It is likely that all strains are practically identical, and they are now classified by Bergey in the genus *Brucella*. The laboratory diagnosis of undulant fever is made by finding the organism by blood culture, or more usually by means of the macroscopic agglutination technic described on page 659. Huddleson[1] described a rapid macroscopic agglutination method using a concentrated antigen, and Giordano[2] described a skin test that may give confirmatory evidence of infection especially when the agglutinins are of low titer. The antigens for the usual macroscopic agglutination test, as well as that for Huddleson's method, may be purchased from the biologic supply houses.

VIII. BACTERIOLOGIC CLASSIFICATION

There has been much confusion in bacteriologic nomenclature since Nägeli, in 1857, first used the class name Schizomycetes. Various systems of classification have been proposed, until recently the Committee on Characterization and Classification of the Society of American Bacteriologists has proposed a new classification, which has not been adopted universally, but which is taught in many schools.

[1] Huddleson, I. F., and Abell, Elizabeth: Rapid Macroscopic Agglutination for the Serum Diagnosis of Bang's Abortion Disease, Jour. Infect. Dis., *42*:242–247 (Mar.), 1928.

[2] Giordano, A. S.: Brucella Abortus Infection in Man. The Intradermal Reaction as an Aid in Diagnosis, Jour. Am. Med. Assn., *93*:1957–1958 (Dec. 21), 1929.

CLASSIFICATION OF SCHIZOMYCETES

An abridged key adapted from Bergey[1]

In this abridged key only those genera of general medical interest are given. The numbers of orders, families, tribes, and genera are those given in the enlarged, new classification.

Order I. *Eubacteriales* Buchanan

Family II. *Rhizobiaceae* Conn

Genus III. *Alcaligenes* Castellani and Chalmers

Family III. *Pseudomonadaceae* Winslow *et al.*

Tribe I. *Spirilleae* Kluyver and van Niel

Genus I. *Vibrio* Müller

Tribe II. *Pseudomonadeae* Kluyver and van Niel

Genus V. *Pseudomonas* Migula

Family VI. *Micrococcaceae* Pribram

Genus I. *Micrococcus* Cohn

Genus I. *Staphylococcus* Rosenbach

Genus III. *Gaffkya* Trevisan

Genus IV. *Sarcina* Goodsir

Family VII. *Neisseriaceae* Prévot

Genus I. *Neisseria* Trevisan

Family VIII. *Parvobacteriaceae* Rahn

Tribe I. *Pasteurelleae* Castellani and Chalmers

Genus I. *Pasteurella* Trevisan

Genus II. *Malleomyces* Hallier

Tribe II. *Brucelleae* Bergey, Breed and Murray

Genus III. *Brucella* Meyer and Shaw

Tribe III. *Hemophileae* Winslow *et al.*

Genus IV. *Hemophilus* Winslow *et al.*

Family IX. *Lactobacteriaceae* Orla-Jensen

Tribe I. *Streptococceae* Trevisan

Genus I. *Diplococcus* Weichselbaum

Genus II. *Streptococcus* Rosenbach

Family X. *Enterobacteriaceae* Rahn

Tribe I. *Escherichieae* Bergey, Breed and Murray

Genus I. *Escherichia* Castellani and Chalmers

Genus II. *Aerobacter* Beijerinck

Genus III. *Klebsiella* Trevisan

Tribe IV. *Proteae* Castellani and Chalmers

Genus VI. *Proteus* Hauser

[1] Bergey, D. H., Breed, R. S., Murray, E. G. D., and Hitchens, A. P.: Bergey's Manual of Determinative Bacteriology. (A Key for the Identification of Organisms of the Class Schizomycetes.) Ed. 5, Baltimore, Williams and Wilkins Company, 1939, 1043 pp.

Tribe V. *Salmonelleae* Bergey, Breed and Murray
Genus VII. *Salmonella* Lignières
Genus VIII. *Eberthella* B chanan
Genus IX. *Shigella* Castellani and Chalmers

Family XI. *Bacteriaceae* Cohn
Genus I. *Listerella* Pirie
Genus VII. *Actinobacillus* Brumpt
Genus VIII. *Bacteroides* Castellani and Chalmers
Genus IX. *Fusobacterium* Knorr

Family XII. *Bacillaceae* Fischer
Genus I. *Bacillus* Cohn
Genus II. *Clostridium* Praznowski

Order II. *Actinomycetales* Buchanan

Family I. *Mycobacteriaceae* Chester
Genus I. *Corynebacterium* Lehmann and Neumann
Genus II. *Mycobacterium* Lehmann and Neumann
Family II. *Actinomycetaceae* Buchanan
Genus I. *Leptotrichia* Trevisan
Genus II. *Erysipelathrix* Rosenbach
Genus IV. *Actinomyces* Harz

Order III. *Spirochaetales* Buchanan

Family I. *Spirochaetaceae* Swellengrebel
Genus IV. *Borrelia* Swellengrebel
Genus V. *Treponema* Schaudinn
Genus VI. *Leptospira* Noguchi

(The genera *Borrelia*, *Treponema*, and *Leptospira* are claimed as animal parasites by zoologists, and are classified thus on page 498. They are also described briefly on pages 510 to 514.)

CHAPTER XII

VACCINES, BIOLOGICAL SKIN TESTS

BACTERIA vaccines, sometimes called "bacterins," which have come to play an important rôle in therapeutics, are suspensions of definite numbers of dead bacteria in normal salt solution. While in many cases ready prepared or "stock" vaccines are satisfactory, it is usually desirable and often imperative for best results to use vaccines which are especially prepared for each patient from bacteria which have been freshly isolated from his own lesion. These latter are called "autogenous vaccines." Only through them can one be certain of getting the exact strain of bacterium which is producing the disease.

I. PREPARATION OF VACCINE

1. Preparation of Materials.—A number of small bottles (Fig. 375) with medium wide mouths are thoroughly cleaned, plugged with cotton, and sterilized by baking. A number of rubber caps are kept in 5 per cent phenol solution. These should be removed with sterile forceps, after the vaccine is placed in the bottles, and should be fitted into the neck of the bottle with great care to avoid contamination.

Fig. 375. — Vaccine bottle with rubber cap.

A number of test tubes, each charged with 10 c.c. of physiologic salt solution and plugged with cotton, are also prepared and sterilized.

2. Obtaining the Bacteria.—A culture on an appropriate solid medium is made from the patient's lesion, and a pure culture is obtained in the usual way. This preliminary work should be carried through as quickly as possible in order that the bacteria may not lose virulence by long growth upon artificial media. If for any reason there is much delay, it is best to begin over again, the experience gained in the first trial enabling one to carry the second through more rapidly. When a pure culture is obtained, a number of tubes of blood serum or agar—10 or 12 in the case of streptococcus or pneumococcus, 4 or 5 in the case of most other organisms—are planted and incubated over night or until a good growth is obtained.

3. Making the Suspension.—A few cubic centimeters of the salt solution from one of the 10-c.c. salt tubes is transferred by means of a sterile capillary pipet to each of the culture tubes, and the growth thoroughly rubbed up with a stiff platinum wire or a glass rod whose tip is bent at right angles. The suspension from the different tubes, usually amounting to about 10 c.c., is then collected in one large tube (about 150 x 19 mm.). The upper part of the tube is drawn out in the flame of a blast lamp or Bunsen burner, as indicated in Fig. 376, *B*, a short section of glass tubing being fused to the rim of the tube to serve as a handle. It is then stood aside, and when cool the narrow portion is sealed off.

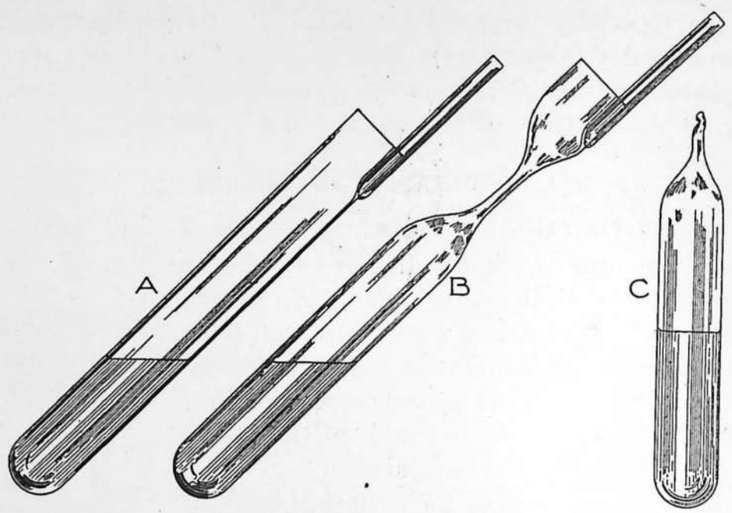


Fig. 376.—Process of making hermetically sealed capsules containing liquid: *A*, A short section of glass tubing has been fused to the top of the tube to serve as a handle; *B*, the upper part of the tube has been drawn out in a flame; *C*, the top of the capsule has been sealed.

The resulting hermetically sealed capsule is next thoroughly shaken for ten to twenty minutes to break up all clumps of bacteria. A few small sterile glass beads may be introduced to assist in this, but with many organisms it is not necessary.

Instead of the capsule described above, an ordinary sterile vaccine bottle may be used for the bacterial suspension. This is then covered with a rubber cap.

4. Sterilization.—The capsule is placed in a water bath at 60° C. for forty-five minutes. This can be done in an ordinary rice cooker, with double lid, through which a thermometer is inserted. When both compartments are filled with water it is an easy matter to maintain

a uniform temperature by occasional application of a small flame. The time and temperature are important: Too little heat will fail to kill the bacteria, and too much will destroy the efficiency of the vaccine. When sterilization is complete the capsule is opened, a few drops are planted on agar or blood serum, and the capsule is again sealed.

Sterilization is also readily effected by adding 0.03 c.c. of tricresol to 10 c.c. of the unheated vaccine. Shake thoroughly to emulsify. Most bacteria that are used in the preparation of vaccines usually are killed in twenty-four hours.

5. Counting.—When incubation of the planted tube has shown the suspension to be sterile it is ready for counting. Some prefer to do the counting as soon as the suspension is made and before sterilization. Of the methods given here the second is the most accurate, but the third will generally be found most convenient.

Wright's Method.—There must be ready a number of clean slides, a few drops of physiologic saline on a slide or in a watch glass; a blood lancet, which can be improvised from a spicule of glass or a pen; and two capillary pipets with squarely broken off tips and wax-pencil marks about 2 cm. from the tip (Fig. 377). These are easily made by drawing out a piece of glass tubing, as described on page 760.

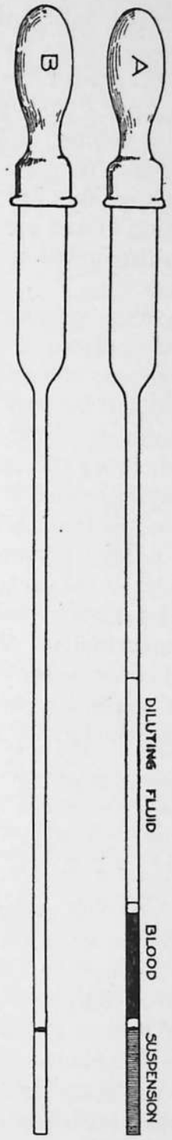


Fig. 377.—Capillary pipets: *A*, Filled for counting a vaccine by Wright's method; *B*, empty, showing wax-pencil mark. The slender portion should be narrower than here represented.

It is necessary to work quickly. After thorough shaking, the capsule is opened and a few drops forced out upon a slide. Any remaining clumps of bacteria are broken up with one of the pipets by holding it against and at right angles to the slide, and alternately sucking the fluid in and forcing it out. The pipet is most easily controlled if held in the whole hand with the rubber bulb between the thumb and the side of the index finger. A finger is

then pricked until a drop of blood appears; and into the second pipet are quickly drawn successively: 1 or 2 volumes physiologic salt solution (or, better, a 1 per cent solution of sodium citrate, which prevents coagulation); a small bubble of air; 1 volume of blood; a small bubble of air; and, finally, 1 volume of bacterial suspension. (A "volume" is measured by the distance from the tip of the pipet to the wax-pencil mark.) The contents of the pipet are then forced out upon a slide and thoroughly mixed by sucking in and out, care being taken to avoid bubbles; after which the fluid is distributed to a number of slides and spread as in making blood smears.

The films are stained with Wright's blood stain or, better, by a few minutes' application of carbol thionin, after fixing for a minute in 1 per cent mercuric chloride solution. With an oil-immersion lens both the red cells and the bacteria in a number of microscopic fields are counted. The exact number is not important; for convenience 500 red cells may be counted. From the ratio between the number of bacteria and of red cells it is easy to calculate the number of bacteria in 1 c.c. of the suspension, it being known that there are 5000 million red corpuscles in a cubic centimeter of normal human blood. If there were twice as many bacteria as red corpuscles in the fields counted, the suspension would contain 10,000 million bacteria per cubic centimeter.

Hemacytometer Method.—This is carried out in the same manner as a blood count, using any convenient dilution, usually 1:200. A weak carbolfuchsin or gentian violet solution, freshly filtered, may be used as diluting fluid, but the following solution, recommended by Callison, is better:

Hydrochloric acid	2 c.c.
Mercuric chloride (0.2 per cent) solution	100 "
Acid fuchsin (1 per cent aqueous solution), to color.	
The color should be just deep enough not to obscure the ruled lines.	

A very thin cover glass must be used; and, after filling, the counting chamber must be set aside for an hour or more to allow the bacteria to settle. Mallory and Wright advise the use of the shallow Helber chamber made for counting blood plates, but many 2-mm. oil-immersion objectives have sufficient working distance to allow the use of the regular counting chamber, provided a very thin cover is used. The heavy cover with central excavation is recommended.

Hopkins Method.—In this the number of bacteria is estimated from their bulk after centrifugation. The bacterial suspension is filtered through a small filter of sterile cotton to remove large clumps of bacteria and bits of agar. It is then placed in a special tube (Fig. 378) and centrifugalized for half an hour at about 2800 revolutions per minute. The tube may be covered with a sterile rubber cap, or plugged with sterile cotton held in place by adhesive tape. The high speed required necessitates a powerful centrifuge. It is too great for the ordinary medical centrifuge.

During centrifugation the bacteria collect in the narrow portion, which is graduated in hundredths of a cubic centimeter. The supernatant fluid and all of the bacterial sediment above the 0.05 mark are then removed with a sterile capillary pipet, and sterile salt solution containing 0.5 per cent phenol is added to the 5-c.c. mark. The bacteria are then well mixed with the fluid by means of a capillary pipet. This gives a 1 per cent bacterial suspension. In case the bacterial sediment does not reach the 0.05 mark a correspondingly smaller amount of the salt solution is added.

The number of bacteria in a 1 per cent suspension differs with the species as follows:

	Million in each cubic centimeter.
Staphylococcus albus and aureus	10,000
Streptococcus hemolyticus	8,000
Gonococcus	8,000
Pneumococcus	2,500
Bacillus typhosus	8,000
Bacillus coli	4,000

Turbidity Method.—The number of bacteria in a suspension may also be estimated by comparing the turbidity with that of standards which are used in the analysis of water. A standard that represents 500 parts per million is satisfactory for most vaccines.[1]

Fig. 378.—Hopkins centrifuge tube. Useful for approximate count of bacteria in vaccines. The narrow portion at the bottom is graduated in hundredths of a cubic centimeter.

The method of standardizing vaccines should be simple as it is more important to make them approximately alike than to try to set a different standard for each kind of organism. Vaccines are administered in graduated doses according to the tolerance of the patient rather than according to the number of bacteria.

6. Diluting.—The amount of the suspension which, when diluted to 10 c.c., will give the strength desired for the finished vaccine having been determined, this amount of salt solution is withdrawn with a hypodermic syringe from one of the bottles already prepared, and is replaced with an equal amount of suspension. Two tenths c.c. of 25 per cent phenol in water with just enough alcohol to maintain solution is then added with a tuberculin syringe and the vaccine is ready for use. If tricresol has been used to sterilize the bacterial

[1] A standard showing 500 parts per million may be prepared by sifting dry Pears' precipitated fuller's earth through a 200-mesh sieve, and suspending 0.5 Gm. in 1000 c.c. of distilled water. A portion of this suspension should be placed in vaccine vials that have the same diameter as those which are used in preparing vaccines. A small amount of mercuric chloride may be added to prevent algal or bacterial growths. The vial should be well shaken before comparing the turbidity with that of another suspension of any sort.

suspension it is not necessary to use any other preservative. The usual strengths are: Staphylococcus, 1000 million in 1 c.c., most other bacteria, 100 million in 1 c.c. Vaccines should be kept in the icebox, and, as a rule, should not be used when more than four months old.

II. METHOD OF USE

Vaccines are administered subcutaneously, usually in the arm or abdominal wall or between the shoulder blades. The technic is the same as for an ordinary hypodermic injection. The syringe is usually sterilized by boiling. The site of the injection may be mopped with alcohol, or may be touched with a pledget of cotton saturated with tincture of iodine or liquor cresolis compositus. The rubber cap of the container is sterilized by applying alcohol for some minutes, usually while the syringe is being sterilized, or simply placing a drop of liquor cresolis compositus upon it. The bottle is then inverted and well shaken, when the needle is plunged through the rubber and the desired quantity withdrawn. The hole seals itself. A satisfactory syringe is the comparatively inexpensive Luer 1 c.c. "Tuberculin" syringe graduated in hundredths of a cubic centimeter.

III. DOSAGE

Owing to variations, both in virulence of organisms and susceptibility of patients, no definite dosage can be assigned. Each patient is a separate problem. Wright's original proposal was to regulate the size and frequency of dose by its effect upon the opsonic index, but this is beyond the reach of the practitioner, and is now very rarely resorted to by any one. The "clinical method" consists in beginning with a very small dose and cautiously increasing until the patient shows either improvement or some sign of a "reaction," indicated by headache, malaise, fever, exacerbation of local disease, or inflammatory reaction at the site of injection. The reaction indicates that the dose has been too large. The beginning dose of staphylococcus is about 50 million; the maximum, 1000 million or more. Of most other organisms the beginning dose is 5 million to 10 million; maximum, about 100 million. Ordinarily, injections are given once or twice a week; very small doses may be given every other day.

IV. THERAPEUTIC INDICATIONS

The therapeutic effect of vaccines depends upon their power to produce active immunity: they stimulate the production of opsonins and other antibacterial substances which enable the body to combat the infecting bacteria. Their especial field is the treatment of sub-

acute and chronic localized infections, in some of which they offer the most effective means of treatment at our command. In most chronic infections the circulation of blood and lymph through the diseased area is very sluggish, so that the antibodies, when formed, cannot readily reach the seat of disease. Ordinary measures which favor circulation in the diseased part should, therefore, accompany the vaccine treatment. Among these may be mentioned incision and drainage of abscesses, dry cupping, application of heat, Bier's hyperemia, and so forth, but such measures should not be applied during the twenty-four hours succeeding an injection, since the first effect of the vaccine may be a temporary lowering of resistance. Vaccines are of little value, and, in general, are contraindicated in very acute infections, particularly in those which are accompanied by much systemic intoxication, for in such cases the power of the tissues to produce antibodies is already taxed to the limit.

Probably best results are obtained in staphylococcus infections, although pneumococcus, streptococcus, and colon bacillus infections sometimes respond nicely. Among clinical conditions which have been treated successfully with vaccines are furunculosis, acne vulgaris, infected operation wounds, pyelitis, cystitis, subacute otitis media, chronic gonorrhea and gonorrheal rheumatism, chronic bronchitis, bronchial asthma of bacterial origin, infections of nasal accessory sinuses, and so forth. Sometimes the principal organisms are put into a single vaccine in approximately the proportions in which their colonies appeared in the primary cultures. To avoid the delay and consequent loss of virulence entailed by study and isolation of the several varieties, many workers make the bacterial suspension directly from the primary cultures. The resulting vaccines contain all strains which are present in the sputum in approximately the same relative numbers. Although open to criticism from a scientific standpoint, this method offers decided practical advantages in some cases.

V. PROPHYLACTIC USE OF VACCINES

It has been shown that vaccines are useful in preventing as well as curing infections. Their value has been especially demonstrated in typhoid fever. Three doses of about 500 million, 1000 million, and 1000 million typhoid bacilli, respectively, are given about seven to ten days apart. Results in the army, where the plan has been tried on a large scale, show that such vaccination is effective, protecting the individual for one to two years, or longer. At present a triple vaccine, containing 1000 million typhoid bacilli, 500 million paratyphoid A, and 500 million paratyphoid B in 1 c.c., is used. The first

dose is 0.5 c.c., the second and third 1 c.c. Similar vaccines have been prepared from *Hemophilus pertussis* for prophylaxis against whooping cough. *Toxoids*, which are toxins in which the toxicity has been destroyed but which are antigenic, are employed in the same manner as bacterial vaccines. Alum precipitated toxoid is generally used as a prophylactic measure against diphtheria in children with positive Schick tests, and a similarly prepared toxoid is used as a prophylactic against tetanus.

VI. TUBERCULIN IN DIAGNOSIS

The tissues of a tuberculous person are sensitized toward tuberculin, and a reaction occurs when any but the most minute quantity of tuberculin is introduced into the body. Nontuberculous persons exhibit no such reaction. This is utilized in the diagnosis of obscure forms of tuberculosis, the test being applied in a number of ways. These tests have very great diagnostic value in children, especially those under three years of age, but are often misleading in adults, positive reactions occurring in many apparently healthy individuals. Negative tests are very helpful in deciding against the existence of tuberculosis.

1. **Hypodermic Injection.**—After first determining the patient's normal temperature variations, Koch's old tuberculin is used in successive doses, three or four days apart, of 0.01, 0.1, 1, 2, 5, and 10 mg. A negative result with the largest amount is considered final. The reaction is manifested by fever within eight to twenty hours after the injection. A rise in temperature of 1° F. is generally accepted as positive. The method involves some danger of lighting up a latent process, and has been largely displaced by safer, although perhaps less reliable, methods.

2. **Calmette's Ophthalmo-reaction.**—One or 2 drops of 0.5 per cent purified old tuberculin are instilled into one eye. Tuberculin ready prepared for this purpose is on the market. If tuberculosis exists anywhere in the body, conjunctivitis is induced within twelve to twenty-four hours. This generally subsides within a few days. *The method is now rarely used since it is not without some, though slight, risk of injury to the eye.* It is absolutely contraindicated in the presence of any form of ocular disease. A second instillation should not be tried in the same eye.

3. **Von Pirquet's Method.**—This is the most widely used of the tuberculin tests. Two small drops of old tuberculin are placed on the skin of the front of the forearm, about 2 inches apart, and the skin is slightly scarified, first at a point midway between them, and then

through each of the drops. A convenient scarifier is a piece of heavy platinum wire, the end of which is hammered to a chisel edge. A wooden toothpick with a chisel-shaped end is also convenient. This is held at right angles to the skin, and rotated six to twelve times with just sufficient pressure to remove the epidermis without drawing blood. In about ten minutes the excess of tuberculin is gently wiped away with cotton. No bandage is necessary. A positive reaction is shown by the appearance in twenty-four to forty-eight hours of a papule with red areola, which contrasts markedly with the small red spot left by the control scarification.

4. **Mantoux Intracutaneous Test.**—A more accurate skin test is that proposed by Mantoux. A 0.005 per cent solution of old tuberculin in 0.5 per cent phenol is used; also a control is made using 0.5 per cent phenol. The forearm is cleaned with alcohol, and 0.1 c.c. of the control solution is injected *intracutaneously* with a tuberculin syringe. In like manner, 0.1 c.c. of the 0.005 per cent, or 1/200 mg. of old tuberculin is injected a few inches above the control. The reaction is read in twenty-four hours, or, preferably, in forty-eight hours. No reaction is considered positive unless the area of hyperemia around the tuberculin is 5 mm. greater than the control.

5. **Tuberculin P. P. D.**—A new tuberculin, purified protein derivative (P. P. D.), has been placed on the market in tablet form. This product is sponsored by the research committee of the National Tuberculosis Association. Its advocates claim that it has greater accuracy in dosage, greater sensitivity, and greater convenience, and costs less than tuberculin preparations which formerly were available for the intradermal test. The tablets are supplied in sterile 1 c.c. rubber-capped vials together with a vial of sterile buffered diluent solution. There are tablets of two different strengths. The weaker tablet contains 0.0002 mg. of protein derivative, and the stronger tablet (*which is used only if no reaction occurs with the weaker tablet*) contains 0.05 mg. of purified protein derivative. Both tablets are made up with sterile lactose. To make a solution, transfer with sterile tuberculin syringe and with aseptic precaution, 1 c.c. of sterile diluent to a sterile vial, which contains a tablet, and shake the vial. Allow ten minutes for the complete solution of the tablet. Always use the weaker solution first. Inject intradermally on the forearm 0.1 c.c. from the vial that contains 0.0002 mg. of the purified derivative. This dose contains 0.00002 mg. of purified protein derivative. If no reaction occurs in forty-eight hours, inject intradermally on the forearm 0.1 c.c. of the stronger solution. This dose contains 0.005 mg. of purified protein derivative, or 250 times the amount which

was administered in the first dose. If no reaction occurs from this dose, the test is negative. A positive reaction consists of an area of swelling, which is 5 mm. or more in diameter. It is probable that because of its simplicity and accuracy this test may become a standard tuberculin test.

VII. SCHICK TEST FOR IMMUNITY TO DIPHTHERIA

By means of this test, which was introduced by Schick in 1913, it is possible to select from a group of individuals those who are immune to diphtheria by virtue of natural or artificial immunity. In an epidemic of diphtheria, therefore, it may be of very great value as a means of determining who shall and who shall not receive prophylactic injections of antitoxin. The reaction is not applicable to the diagnosis of diphtheria.

Material, which has been diluted with peptone solution, and which is specially prepared for the Schick test, may be purchased ready for use from the biologic supply houses. Inject 0.1 c.c. of the prepared dilution intradermally in the usual manner. This solution is very convenient, especially for the occasional test.

No reaction should occur in those who are immune to diphtheria. In those who are not immune a distinct red spot about 1 to 2 cm. in diameter appears at the site of injection of the toxin within twenty-four to thirty-six hours. This is followed by induration, reaching its height on or about the fourth day. The redness and induration gradually disappear, leaving a brownish area of pigmentation which sometimes persists for three or four months. The Schick test is very reliable if proper toxin be used and if the reaction be correctly interpreted. The percentage of positive reactions is higher in children than in adults.

VIII. DICK TEST FOR IMMUNITY TO SCARLET FEVER

A test which appears to bear the same relation to scarlet fever that the Schick test bears to diphtheria has been devised by George F. and Gladys H. Dick. This consists in injecting, intradermally, 0.1 c.c. of 1:1000 dilution of the Berkefeld filtrate of a culture of a streptococcus which has been isolated from a case of scarlet fever and which is capable of producing the disease in human beings. This filtrate can now be purchased, ready for use, from the biological supply houses.

The reactions are observed at the end of twenty-four hours, and are classified as *negative* when there is no more than a faint pink streak along the course of the needle; as *slightly positive* when there

is a faint red area less than 1 cm. in diameter with no swelling; as *positive* when the area is from 1 to 3 cm. in diameter, bright red, with some swelling of the skin; as *strongly positive* when the area is intensely red, from 3 to 5 cm. in diameter, and markedly swollen, with sharply raised edge.

Positive and strongly positive reactions may be interpreted as indicating susceptibility to scarlet fever; negative reactions as indicating immunity.

IX. TESTS FOR HYPERSUSCEPTIBILITY

Hypersusceptibility to various organic substances has of recent years come to be recognized as playing an important rôle in medicine. To it are ascribed such conditions as hay fever, asthma, shock following administration of antitoxins and therapeutic sera, the urticarias, and diarrheas which in some persons follow ingestion of certain foods. It is unnecessary to discuss the nature of the phenomena or the application of the special terms—allergy, anaphylaxis, and idiosyncrasy. The commonest cause of August hay fever is the pollen of low ragweed (*Ambrosia artimisiaefolia*). This ubiquitous weed is illustrated in Fig. 379.

Hypersusceptibility to any particular substance—pollen of certain plants, dandruff or serum of animals, drugs, articles of food—can be recognized by a simple skin test. This consists in applying an extract of the substance to a slight incision in the skin made with the point of a sharp knife only to such a depth as to introduce the substance below the epidermis, but drawing no blood.

A more accurate method is to inject *intradermally* definite amounts of the protein substance. The amount to be used may vary with the individual. In general, it is often the practice to inject 0.05 c.c. of an extract so diluted that 1 c.c. contains 0.001 mg. of the protein. Care should be taken to avoid injecting *under* the skin; otherwise unpleasant and even dangerous symptoms might be induced in an extremely sensitive individual.

Substances such as dandruff and pollens are extracted with decinormal sodium hydroxide solution, which is then used for the test, or the substance may be placed on the incision in the skin with a drop of the decinormal solution and very gently mixed and rubbed into the incision. Extracts of a long list of pollens, dandruff, and other substances, especially prepared for the tests, are available commercially. Drugs may be used in 10 per cent solution. In the case of antitoxin and the various therapeutic sera it is not the immune bodies which cause the dangerous reaction, but rather the horse

serum with which they are administered. Hypersensitiveness to horse serum is best recognized by intradermal injection of 0.2 c.c. of 1:10 dilution of normal horse serum in saline solution.

The arm is the usual site for the test, and a control test is made upon the other arm with omission of the specific substance. Tests with different substances may be carried out simultaneously. A positive reaction, indicating hypersusceptibility to the particular substance used in the test, is manifested by an urticarial wheal surrounded by a

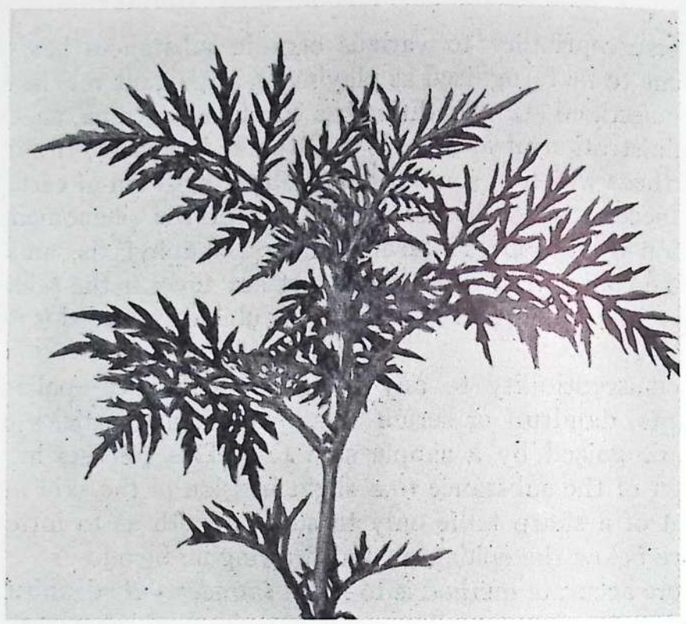


Fig. 379.—Low ragweed, the pollen of which is the most common cause of August hay fever. (Therapeutic Notes, Parke, Davis & Co.)

broad zone of redness. This appears within half an hour, often within five minutes, and begins to fade in an hour or two.

X. MISCELLANEOUS SKIN TESTS

The Casoni test (p. 551) uses the fluid of an Echinococcus cyst, which is injected intracutaneously in cases with evidence of cyst of the liver. The history should suggest the possibility of infestation—association with dogs that have run with sheep, or have eaten offal from slaughter houses. A marked erythema and wheal formation with pseudopods is a positive reaction and suggests the probability of infestation.

A similar skin test may be carried out in cases of suspected Trichinosis by using Trichinella extract. A positive reaction is illustrated in Fig. 380.

Frei Test.—There are two diseases which unfortunately have names so similar that confusion may arise in distinguishing between them, as they are both classed as venereal diseases. *Lymphogranuloma inguinale* (Nicholas-Favre's disease) is the result of a filtrable virus which causes an evanescent primary lesion, at times a urethritis, and involvement of the draining lymph nodes. A chain of these nodes becomes fused together, breaks down, and forms multiple fistulous

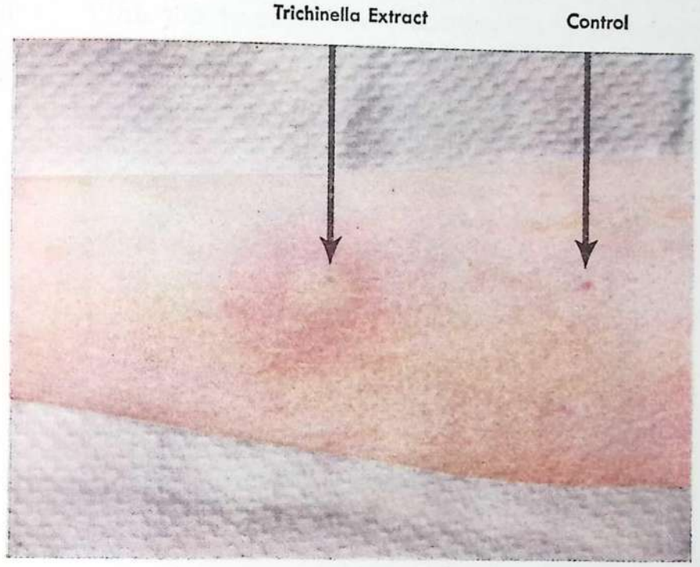


Fig. 380.—Positive intracutaneous test for trichinosis. (Eli Lilly and Co.)

openings. This disease is very different from *granuloma inguinale*, which is an ulcerating granuloma of the pudenda, a disease found in the tropics. It begins on the genitals, spreads by continuity to the thigh, and on up to the skin in the groin, but it does not involve the lymphatics. Scrapings from the ulcers in this disease show small, rod-shaped inclusion bodies, which are known as Donovan bodies, and which are seen in the mononuclear cells, when stained with Giemsa or Wright stain. (These "Donovan bodies" are not to be confused in the minds of students with Leishman-Donovan bodies of kala-azar.) In *lymphogranuloma inguinale*, a diagnosis may be confirmed by the so-called "Frei test." This is a specific intradermal test similar to a

tuberculin test, the antigen being pus that is taken from a bubo just before it has broken down. This antigen should be sterilized. This test material is used on other suspected cases by injecting 0.1 c.c. intradermally. In forty-eight hours there will be a raised, erythematous tubercle, from 0.5 to 1 cm. in diameter, in a positive reaction. An antigen may be purchased which is a saline suspension of killed virus in mouse brain. An antigen for this test has been prepared from virus grown in chick embryo yolk-sacs.

APPENDIX

I. OFFICE LABORATORY METHODS AND EQUIPMENT

It is not to be expected that a physician in active practice will make routine use of all the methods described in this book. Although he will need nearly all of them for the study of his more difficult cases, his daily laboratory work will probably be limited to a few simple procedures. With this in mind, the following list of laboratory procedures is suggested as the minimum with which a physician should be thoroughly familiar and upon which he may build as his practice requires. The methods are selected because of their simplicity and practical usefulness:

METHODS FOR OFFICE ROUTINE

Sputum

Careful inspection (p. 32).
Simple microscopical examination unstained (p. 35).
Examination for tubercle bacilli (p. 44).

Urine

Reaction (p. 67).
Specific gravity (p. 70).
Calculation of total solids (p. 72).
Phenolsulfonephthalein and Mosenthal's tests of kidney function (p. 162).
Albumin, qualitative:
    Robert's ring test (p. 92).
    Purdy's heat test (p. 93).
Albumin, quantitative:
    Tsuchiya's method (p. 94).
Sugar, qualitative:
    Benedict's test (p. 98).
Sugar, quantitative:
    Benedict's method (p. 101), or Einhorn's yeast method (p. 103).
Acetone, Lange's or Rothera's test (p. 108).
Diacetic acid, Gerhardt's test (p. 109).
Bile, Gmelin's test, (p. 111).
Indican, Obermayer's test (p. 77).
Microscopical examination (p. 126).

Blood

Coagulation time, any simple method (p. 197).
Bleeding time (p. 203).
Hemoglobin, some method estimating grams per cent (pp. 209–216).

Red corpuscles count (p. 217).
Color index calculation (p. 228).
Leukocyte count (p. 241).
Differential leukocyte count (p. 265).
Microscopical examination of stained films for pathologic red cells and malarial parasites (pp. 257, 532).

STOMACH CONTENTS

Careful inspection (p. 450).
Total acidity, Töpfer's method (p. 455).
Free hydrochloric acid, Töpfer's method (p. 457).
Lactic acid, Kelling's test (p. 453).
Microscopical examination (p. 459).

FECES

Careful inspection (p. 470).
Occult blood, benzidine test after extraction with ether (p. 475).
Microscopical examination:
(*a*) For parasites or their ova (p. 487).
(*b*) To ascertain state of digestion (p. 493).

SERUM METHODS

Widal test by macroscopical method, using one of the commercial outfits (p. 659).

MISCELLANEOUS

Microscopical examination of pus:
(*a*) Simple stain (p. 779).
(*b*) Gram's method (p. 780).
Puncture fluids:
(*a*) Careful inspection (pp. 590 and 595).
(*b*) Microscopical examination for bacteria and differential cell count (p. 592).
Syphilitic material for spirochetes, Giemsa's or Wright's stain or India-ink method (p. 643).
Milk:
Fat, Leffmann-Beam method (p. 637).
Protein, by calculation (p. 637).

EQUIPMENT

A list of equipment which is sufficient for all the above mentioned methods (and for many others in addition) is given below. There is no real economy in purchasing instruments of inferior quality.

A. FURNITURE

A table, with drawer, and a few shelves for bottles and glassware constitute the only really essential laboratory furniture even for fairly extensive work. Gas and running water are desirable.

The following **acid-proof wood finish** is extensively used for table tops in the laboratories of this country. It gives an ebony-black surface which resists practically all reagents. The wood must be new, or at least not painted, varnished, or waxed.

Solution No. 1:

Copper sulfate	125 Gm.
Potassium chlorate (or permanganate)	125 "
Water	1000 c.c.

Solution No. 2:

Anilin oil	120 c.c.
Hydrochloric acid, concentrated	180 "
Water	1000 "

Apply two coats of Solution No. 1, hot, and then two coats of Solution No. 2, without heating, allowing each coat to dry thoroughly before the next is applied. When the last coat is dry remove the excess of the chemicals by rubbing with a coarse cloth. Finally rub thoroughly with a mixture of equal parts of turpentine and linseed oil.

B. APPARATUS

Basin of white enameled ware.
Beakers with lip, about 50-c.c. capacity, preferably of pyrex glass.
Blood lancet or some substitute, as a glover's needle (p. 193).
Bunsen burner with rubber tubing.
Buret, 25-c.c. capacity, preferably with Schellbach stripe. An accurate 10-c.c. graduated pipet may be used for much work, but is not so satisfactory as the buret.
Centrifuge, electric. Metal shields with flat bottoms and rubber cushions are preferable to the ordinary conical aluminum shields because they allow the use of ordinary test tubes as well as conical centrifuge tubes.
Esbach tube.
Filter papers, round, about 15 cm. in diameter, good quality.
Funnels, glass, about 10 cm. in diameter.
Glass tubing, about 7 or 8 mm. outside diameter, of soft glass for making pipets. Should be of such size that rubber nipples may be fitted.
Graduated cylinders, 100 c.c., double graduations, used chiefly for making solutions.
Hemacytometer. Probably the most satisfactory outfit consists of an "open" counting chamber with Neubauer ruling, a "red pipet" and a "white pipet." It should be accompanied by the certificate of the U. S. Bureau of Standards.
Hemoglobinometer. The Sahli is recommended, or preferably a Newcomer disk for a colorimeter. A Tallqvist book may be carried in the hand bag.
Lens-cleaning paper.
Labels for bottles.
Labels for slides.
Mechanical stage, attachable (p. 25).
Medicine droppers: Two, labeled "Stain" and "Water," respectively;

to be reserved for use with Wright's blood stain; one, which delivers the proper sized drop, to be reserved for the quantitative sugar estimation (p. 98).

Eyepiece micrometer. The card-board micrometer made as described on page 20 will answer for most clinical work.

Microscope equipped as described on page 23.

Micro cover glasses, No. 2 thickness. The 22-mm. squares are most convenient for general purposes.

Micro slides, 75 x 25 mm., clear white glass, medium thickness, ground edges.

Pencils, wax, for writing on glass, red or blue.

Petri dishes with cover, about 15 cm. in diameter.

Pipets, graduated, various sizes.

Rulers, celluloid, 6 inches and 15 cm.

Stands for filter, buret, and so forth.

Stomach tubes. The Rehfuss type is required if fractional method of examination is employed and is best for all purposes.

Test tubes, various sizes.

Test-tube brush, bristle, with tuft at tip.

Test-tube racks.

Urinometer with cylinder. Must have wide graduations. Test with distilled water.

Wooden applicators.

C. REAGENTS AND STAINS

All staining solutions and many reagents are best kept in small dropping bottles, of which the "flat-topped T. K." pattern is most satisfactory. Other reagents may be kept in ordinary round prescription bottles of 4- to 8-ounce capacity. Bottles containing highly volatile reagents should be sealed with paraffin if not in constant use; while those containing strong caustic soda solutions should have rubber stoppers.

Most staining solutions and chemical reagents can be purchased ready prepared. If, however, his time permits the physician will find it more satisfactory and much more economical to prepare his own solutions, with exception of normal solutions and a very few stains.

Reagents

Acid, acetic, glacial, 99½ per cent. Other strengths can be made from this as desired.

Acid, hydrochloric, C.P., sp. gr., 1.16. Contains about 32 per cent HCl. An approximate decinormal solution for use with the Sahli hemoglobinometer can be made by adding 12 c.c. of this acid to 988 c.c. distilled water.

Acid, nitric, C.P. Yellow nitric acid can be made from this by adding a splinter of pine (match stick) or allowing it to stand in the sunlight for a short time.

Acid, sulfuric, C.P.

Alcohol, amylic, C.P. Used in the estimation of fat in milk.

Alcohol, ethylic (grain alcohol). This is ordinarily about 93 to 95 per cent, and other strengths can be made as desired. Whenever the word "alcohol" is used in the test without qualification, this alcohol is meant. The following is a simple rule for diluting alcohol to any desired strength: Take of alcohol a number of parts equal to the percentage desired and add of water a number of parts equal to the difference between the desired strength and the strength diluted. For example, to dilute 80 per cent alcohol to 50 per cent, take 50 c.c. of the alcohol and add 80 − 50 = 30 c.c. of water.

Alcohol, methylic, Merck's "Reagent," for making Wright's blood stain. May be omitted if the stain is purchased ready prepared.

Ammonium hydroxide (strong ammonia), sp. gr. 0.9.

Benedict's solution for qualitative sugar test (p. 98).

Benedict's solution for quantitative sugar estimation (p. 101).

Benzidine. Specify "for blood test."

Canada balsam in xylol. Necessary only if permanent mounts are to be made.

Chloroform, U. S. P.

Diluting fluid for red corpuscle count, Hayem's preferred (p. 221).

Diluting fluid for leukocyte count (p. 243).

Dimethyl-amino-azo-benzol, 0.5 per cent alcoholic solution.

Tsuchiya's solution (p. 94) or preferably Exton's reagent (p. 94).

Ether, sulfuric, U. S. P.

Ferric chloride, 10 per cent aqueous solution.

Formalin (40 per cent solution of formaldehyde gas). The expression "10 per cent formalin" means 1 part of this 40 per cent solution and 9 parts of water, making a 4 per cent solution of formaldehyde gas.

Hydrogen peroxide, U. S. P.

Litmus paper, red.

Litmus paper, blue.

Gram's iodine solution (p. 832).

Obermayer's reagent for indican (p. 77).

Oil of cedar for immersion. A sufficient quantity is usually supplied with the microscope when purchased.

Phenolphthalein, 1 or 0.5 per cent solution in alcohol.

Ampules of phenolsulfonephthalein.

Sodium chloride, C.P.

Sodium hydroxide, decinormal solution. The practitioner will find it best to purchase this solution ready prepared. Most chemical supply houses carry it in stock. For rough clinical work 4.1 Gm. of Merck's "Sodium hydroxide by alcohol" from a freshly opened bottle may be dissolved in 100 c.c. distilled water. This makes a normal solution and must

be diluted with 9 volumes of water to make the decinormal solution. The preparation of accurate normal solutions is described on pages 834, 835.

Sodium nitroprusside, C.P., crystals.

Talc, purified (*Talcum purificatum*, U. S. P.), or diatomaceous earth (*Kieselguhr*) for clearing urine.

Water, distilled. In some regions ordinary tap water answers for practically all purposes.

Stains

It will be found most satisfactory to have on hand a stock of dry stains (which keep well) and to make solutions as needed. Ordinarily the smallest quantity obtainable in an unbroken package should be purchased. The most reliable stains now bear a stamp on the bottle, showing that they have been approved by the Commission on Standardization of Biological Stains. This committee is composed of representatives of the leading societies in the United States interested in bacteriology, botany, dye chemistry, pathology, and zoology. The following dry stains make up a fairly complete stock for the clinical laboratory: Fuchsin, basic; crystal violet; methylene blue, B.X.; methyl green; pyronin, and Wright's stain. Wright's stain is obtainable in 1-Gm. vials, the others in 10-Gm. vials. The most frequently used solutions which can be purchased in 25-c.c. bottles are:

Carbolfuchsin (p. 831).

Carbol gentian violet (p. 831).

Giemsa's stain (p. 255). This is not necessary if Wright's stain or the India-ink method be used for spirochetes.

Löffler's alkaline methylene blue (p. 832).

Pappenheim's methylene-blue contrast stain for tubercle bacilli (p. 832).

Pappenheim's pyronine-methyl-green stain (p. 832).

Wright's blood stain (p. 253). Much of the solution on the market is unsatisfactory.

II. STAINING SOLUTIONS

In this section are given the formulae for staining fluids which have general use, particularly for identification of bacteria. Blood stains and others which are used only for special purposes are discussed in the body of the book and may be found by consulting the Index.

1. **Carbol Thionin.**—Saturated solution thionin in 50 per cent alcohol, 20 c.c.; 2 per cent aqueous solution phenol, 100 c.c.

This stain is especially useful in counting bacteria for standardization of vaccines (p. 813). It can be used as a general stain. In blood work it is sometimes used for the malarial parasite and for demonstration of basophilic degeneration of the red cells. The fluid is applied for one-half to three minutes, after fixation by heat, or about a minute in 1 per cent aqueous solution of mercuric chloride or 1 per cent formalin in alcohol.

2. **Crystal violet** is a powerful bacterial stain which may be substituted for gentian violet in all formulas and is more satisfactory. A solution of 2 Gm. crystal violet in 100 c.c. methyl alcohol of the highest purity is probably the best stain for Gram's method.

3. **Fuchsin.**—This dye should not be confused with acid fuchsin. Its solutions are generally made with phenol as a mordant, and they are then very powerful bacterial stains, with a strong tendency to overstaining. They are used chiefly for the tubercle bacillus.

Ziehl-Neelsen Carbolfuchsin.—Make a saturated solution of basic fuchsin in ethyl alcohol. Dilute 10 c.c. of this solution with 90 c.c. of a 5 per cent aqueous solution of phenol.

Czaplewski's Carbolfuchsin.—To 1 Gm. fuchsin and 5 c.c. liquefied phenol add 50 c.c. glycerol, with constant stirring; and finally add 50 c.c. water, mix well, and filter.

4. **Gentian Violet.**—This dye has long been widely used as a bacterial stain, especially for Gram's method, but is now being rapidly displaced by crystal violet and methyl violet. The combinations given below may be used interchangeably, but the solution with phenol is probably most serviceable. Formalin gentian violet remains good for years, but is less satisfactory for Gram's method than the others because it is not readily decolorized by alcohol.

Methyl violet, or crystal violet, may be substituted for gentian violet in these formulae, and either is preferable to it.

Aniline Gentian Violet.—Ehrlich's formula is the one generally used, but this keeps only a few weeks. *Stirling's solution*, which keeps much better and seems to give equal results, is as follows: Gentian violet, 5 Gm.; alcohol, 10 c.c.; aniline oil, 2 c.c.; water, 88 c.c.

Czaplewski's Carbol Gentian Violet.—To 1 Gm. gentian violet and 5 c.c. liquefied phenol add 50 c.c. glycerol with constant stirring; finally add 50 c.c. water, mix well, and filter.

Formalin gentian violet consists of 5 per cent solution formalin, 75 parts: saturated alcoholic solution gentian violet, 25 parts.

5. **Hematoxylin** is one of the best nuclear stains available. There are many combinations, most of which require weeks or months for "ripening." The following is a good solution which is ready for use as soon as made:

Harris' Hematoxylin.—Dissolve 1 Gm. hematoxylin crystals in 10 c.c. alcohol. Dissolve 20 Gm. ammonia alum in 200 c.c. distilled water with the aid of heat, and add the alcoholic hematoxylin solution. Bring the mixture to a boil, and add ½ Gm. of mercuric oxide. As soon as the solution assumes a dark purple color, remove the vessel from the flame and cool quickly in a basin of cold water.

6. **Iodine** is used as a part of Gram's method and as a special stain for various purposes. For starch a very weak solution is desirable; for *Leptotrichia buccalis* a strong solution such as Lugol's. The solutions deteriorate upon long standing.

Gram's Iodine Solution.—Iodine, 1 Gm.; potassium iodide, 2 Gm.; water, 300 c.c.

Lugol's solution (*Liquor iodi compositus*, U. S. P.) consists of iodine, 5 Gm.; potassium iodide, 10 Gm.; water, 100 c.c. Gram's iodine solution may be made from this by adding fourteen times its volume of water.

7. **Methylene blue** is a widely used basic dye which does not readily overstain. The following solutions are useful:

Gabbet's Stain.—This is used in Gabbet's method for the tubercle bacillus. It consists of methylene blue, 2 Gm.; water, 75 c.c.; concentrated sulfuric acid, 25 c.c.

Löffler's alkaline methylene blue is one of the most useful bacterial stains for general purposes. The solution is applied at room temperature for thirty seconds to three minutes, and is followed by rinsing in water. Fixation may be by heat or chemicals. The stain is composed of 30 parts of a saturated alcoholic solution of methylene blue and 100 parts of a 1 : 10,000 aqueous solution of potassium hydroxide. It keeps indefinitely.

Pappenheim's methylene blue solution is used as decolorizer and contrast stain in Pappenheim's method for the tubercle bacillus. Dissolve 1 Gm. corallin (rosolic acid) in 100 c.c. absolute alcohol; saturate with methylene blue, and add 20 c.c. glycerol.

8. **Methyl violet** is a useful bacterial stain which may be advantageously substituted for gentian violet in all formulas given on page 831.

9. **Pyronine.**—Used in strong aqueous solution this is useful as a contrast stain in Gram's method, but results are more satisfactory when the dye is combined with methyl green.

Pappenheim's Pyronine-methyl-green Stain.—This solution colors bacteria red and nuclei of cells blue. It is, therefore, especially useful for intracellular bacteria like the gonococcus and the influenza bacillus. It is a good stain for routine purposes, is a most excellent contrast stain for Gram's method, and is also used to demonstrate Döhle's inclusion bodies in the blood. It colors the cytoplasm of lymphocytes bright red, and has been used as a differential stain for these cells. The solution is applied cold for one-half to five minutes. It consists of saturated aqueous solution of methyl green, 3 to 4 parts, and saturated aqueous solution of pyronine, 1 to 1½ parts. It is a good plan to keep these solutions in stock and to mix a new lot of the staining fluid about once a month. If it stains too deeply with either dye, the proper balance is attained by adding a little of the other.

Owing to the fact that pyronine is now highly concentrated, the following formula, which has been proposed by the Commission on Biological Stains, of the Society of American Bacteriologists, may prove more useful: Methyl green (50 per cent dye content), 1 Gm., pyronine (Commission certified product), 0.25 Gm., ethyl alcohol (95 per cent), 5 c.c., glycerol, 20 c.c., 2 per cent aqueous solution of phenol, 100 c.c.

10. **Safranin** is widely used as a contrast stain for Gram's method, usually in 1 per cent aqueous solution.

11. **Simple Bacterial Stains.**—A simple solution of any basic aniline dye (methylene blue, basic fuchsin, gentian violet, and so forth) will stain nearly all bacteria. These simple solutions are not much used in the clinical laboratory, because other stains, such as Löffler's methylene blue and Pappenheim's pyronine-methyl-green stain, which serve the purpose even better, are at hand.

12. **Sudan III** is a valuable stain for fat, to which it gives an orange color. Scharlach R is a similar but stronger dye, and may be substituted to advantage. They may be used as a saturated solution in 70 per cent alcohol or in the following combination:

Herxheimer's sudan III consists of equal parts of 70 per cent alcohol and acetone saturated with sudan III (or Scharlach R).

III. NORMAL SOLUTIONS

A normal solution is one which contains in each liter enough of a chemical substance to replace or unite with 1 Gm. of hydrogen. The molecular weight of the substance divided by its valence and expressed in grams is dissolved in sufficient water to make 1 liter. In the case of a univalent substance like hydrochloric acid the molecular weight directly indicates the number of grams to the liter. Equal volumes of all normal solutions are equivalent, for example, 1 c.c. of a normal acid solution exactly combines with 1 c.c. of a normal alkali. The solutions are made up as normal solutions, and for use are generally diluted with water to one tenth, one twentieth, or one fiftieth the normal strength, giving "decinormal," "twentieth normal," and "fiftieth normal" solutions.

The normal solutions most frequently used are the following:

	Grams for each liter.
Hydrochloric acid	36.46
Oxalic acid	63.03
Sulfuric acid	49.04
Potassium hydroxide	56.12
Potassium permanganate	31.63
Silver nitrate	169.97
Sodium carbonate	53.05
Sodium chloride	58.50
Sodium hydroxide	40.06

Of the above, the only solutions ordinarily required in a small laboratory are normal sodium hydroxide and normal hydrochloric acid, from which decinormal solutions are made as needed, and only these will be described in detail. They can be purchased ready prepared from any reliable chemical supply house.

Normal Sodium Hydroxide.—Since sodium hydroxide absorbs water and unites with carbon dioxide of the air it is not of uniform strength, and accurate solutions cannot be made by weighing.

Dissolve 46 Gm. of sodium hydroxide in 1100 c.c. of distilled water. This solution will be of more than normal strength and must be standardized against a normal acid solution. For this purpose oxalic acid is used because it can be weighed accurately. From a bottle of chemically pure oxalic acid select the most nearly perfect crystals, weigh out 6.3 Gm. on a sensitive balance, place in a 100-c.c. volumetric flask, and make up to 100 c.c. with distilled water. Now pipet 10 c.c. of this normal oxalic acid solution into a beaker, add 3 drops of phenolphthalein indicator, and add the solution of sodium hydroxide from a buret until a faint but permanent pink color appears. If the sodium hydroxide solution were of correct strength exactly 10 c.c. would be required; but it will be too strong, and less than 10 c.c. will be used. The difference between 10 c.c. and the amount used indicates the amount of water which must be added to reduce it to the correct strength. If, for example, 9.5 c.c. were used, then 0.5 c.c. of distilled water must be added for each 9.5 c.c. of the sodium hydroxide solution. After this has been added and well mixed, refill the buret with the newly diluted solution and repeat the titration to check the accuracy of the work, using, preferably, 20 or 50 c.c. of the acid.

To make a decinormal solution add 1 volume of the normal solution to 9 volumes of distilled water.

In emergencies a decinormal solution of sufficient accuracy for rough clinical work may be made by dissolving 4.1 Gm. of Merck's "sodium hydroxide by alcohol" from a freshly opened bottle in 1000 c.c of distilled water.

Normal Hydrochloric Acid.—To 135 c.c. of concentrated chemically pure hydrochloric acid add 1 liter of distilled water, and mix well. This makes a solution which is slightly too strong and must be standardized by titrating with a known normal alkali. Pipet exactly 10 c.c. of the acid solution into a beaker, add 3 drops of phenolphthalein indicator, and titrate with an accurate normal solution of sodium hydroxide until a faint but permanent pink color appears. If the acid were accurately normal, exactly 10 c.c. of the alkali would be used; but it will, in fact, be too strong and more than 10 c.c. will be required. The excess over 10 c.c. indicates the amount of water which must be added to each 10 c.c. of the acid to reduce it to correct normal strength. If, for example, 10.6 c.c. of the normal sodium hydroxide are required, then 60 c.c. of distilled water must be added to 1000 c.c. of the acid solution. After the water has been added and well mixed the titration should be repeated, using 20 c.c. of the acid.

To make decinormal hydrochloric acid add 1 volume of the normal solution to 9 volumes of water.

Approximately decinormal hydrochloric acid for use with the Sahli hemoglobinometer can be made by adding 1.2 c.c. of concentrated hydrochloric acid to 98.8 c.c. of distilled water. A few drops of chloroform are added as preservative.

IV. PHYSIOLOGIC SOLUTIONS

Physiologic solutions are so made that they contain the same percentage of various salts as are found in the fluids of the animal body.

Physiologic or "normal saline" is made of sodium chloride (C.P.), 0.85 Gm.; distilled water, 100 c.c.

Locke's solution consists of sodium chloride, 0.9 Gm.; calcium chloride, 0.024 Gm.; potassium chloride, 0.042 Gm.; sodium carbonate, 0.02 Gm.; dextrose, 0.25 Gm.; distilled water, 100 c.c.

Ringer's solution (modified by Porter) consists of sodium chloride, 0.7 Gm.; calcium chloride, 0.0026 Gm.; potassium chloride, 0.035 Gm.; distilled water, 100 c.c.

V. SØRENSEN'S PHOSPHATE MIXTURES FOR BUFFER SOLUTIONS[1]

These buffer solutions are generally useful as the range of the mixtures is from *p*H 5 to 8.

Fifteenth Molar Primary Potassium Phosphate Solution.—Weigh exactly 9.078 Gm. of monobasic potassium phosphate, and dissolve in exactly 1 liter of distilled water. The solution must be absolutely clear and yield no test for chlorine or sulfates.

Fifteenth Molar Secondary Sodium Phosphate Solution.—Expose dibasic sodium phosphate containing 12 mols of water of crystallization to ordinary atmosphere for two weeks. It should then contain 2 mols of crystallization. Dissolve exactly 11.876 Gm. of this sodium phosphate in exactly 1 liter of distilled water. The solution should be absolutely clear and should yield no test for chlorine or sulfates.

SØRENSEN'S TABLE OF BUFFER MIXTURES

Secondary phosphate solution, c.c.	Primary phosphate solution, c.c.	*p*H.
0.25	9.75	5.288
0.5	9.5	5.589
1.0	9.0	5.906
2.0	8.0	6.239
3.0	7.0	6.468
4.0	6.0	6.643
5.0	5.0	6.813
6.0	4.0	6.979
7.0	3.0	7.168
8.0	2.0	7.381
9.0	1.0	7.731
9.5	0.5	8.043

VI. SOLUTIONS FOR INTRAVENOUS USE

The following information regarding solutions for intravenous use has been adapted from the directions for their preparation by Dr. A. E. Osterberg of the Section of Clinical Biochemistry of The Mayo Clinic. Intravenous therapy is a serious procedure. The preparation of solution, both with regard to the agents employed, and the solvent, requires careful chemical, biologic, and bacteriologic consideration. It is best to use only freshly prepared solutions for intravenous administration, and in some instances, especially in the administration of dextrose, it is absolutely necessary.

[1] From Clark: The Determination of Hydrogen Ions. Baltimore, Williams and Wilkins Company, 1928.

A few facts of prime importance in connection with the physiology of intravenous injection should be considered carefully by physicians when recommending this method of administering therapeutic agents. Whatever may be the mechanism for controlling the volume of circulating fluid and of total body fluid it is a very delicate one and depends on many factors, as evidenced by the following facts:

1. The plasma-corpuscle relationship remains fairly constant regardless of ingestion or excretion of fluid. Maintenance of this relationship is necessary for an efficient circulation.

2. The number of corpuscles remains constant under the same oxygen tension. This is necessary because a constant relationship between oxygen-carrying capacity and oxygen-utilization must be maintained.

3. In general, urinary secretion is in direct proportion in ingestion of fluid. Recognition of this fact is essential because the kidney can function only by means of a fluid medium.

4. The relationship between the amount of fluid in the cell and the fluid surrounding the cell is maintained.

5. Various constituents of the blood, absolutely essential in the bodily economy, for which blood is the transportation system, of necessity have a very limited range in concentration. Blood sugar is an example.

When an intravenous injection is given, apart from the substance injected, physiologic compensations inmediately take place in order that the relationships between the enumerated constituents may remain constant. Many other factors not mentioned are involved; for example almost instantaneous compensation occurs on the part of the heart and vasomotor system in order to take care of the increased volume of fluid without greatly changing blood pressure. Fortunately the normal body is able, quickly, and perfectly, to make these various compensations. There is no doubt, however, that there are many pathologic states in which such a compensation in all these phases cannot be made quickly enough to maintain the proper balance.

The important points in the process of intravenous injection are:

1. To procure the proper chemically pure substances from a reliable source.

2. To employ pure, sterile water.

3. Since the strength of the solution depends on the purpose of its administration, in general, not to administer hypotonic or markedly hypertonic solutions unless for a specific purpose it is desired to obtain the dehydrating effect of hypertonic solutions.

4. To use clean, sterile apparatus and to take proper precautions against toxic material in relation to the apparatus.

5. Thoroughly to sterilize both the solution and the apparatus.

6. To control the rate of injection, since this is important in connection with the proper physiologic compensations (this is shown by the foregoing enumeration of the various mechanisms disturbed by intravenous injection).

The following agents to be given intravenously have been recommended: Sodium chloride, sodium bromide, sodium iodide, glucose, calcium chloride, sodium citrate, sodium bicarbonate, quinine and urethane, sodium salicylate, magnesium sulfate, acacia, blood, and the various preparations of arsenic, mercury, antimony and dyes.

The best methods of preparing the solutions usually recommended are as follows:

Distilled Water.—Use freshly triple-distilled water and sterile pyrex glassware. For intravenous injection, never use water that has stood forty-eight hours after distillation. Distilled water in a small quantity should be redistilled from a glass flask connected, *without* a rubber stopper, to a block tin condenser. The side neck of a distilling flask bent in the form of a U makes a convenient water-seal entrance tube for water. For the preparation of large quantities of triple-distilled water such apparatus does not have sufficient capacity. In this case, use may be made of commercial tin-lined stills. Single-distilled water, redistilled twice through a single heavily tinned commercial still or through a set-up of two stills, operating in series, has been found satisfactory.

Distilled water, properly prepared, is free from chlorides and is neutral. To test for chlorides, place 10 c.c. of the water in a clean test tube and add 5 drops of 5 per cent solution of silver nitrate. If the water is turbid in the least it is not free from chloride.

To test for neutrality, place 10 c.c. of the water in a clean test tube and add 1 drop of 1 per cent solution of alizarin red. If the indicator turns pink, the color should be discharged by the addition of 1 drop of one fiftieth normal acid. If the indicator turns yellow it should be changed to pink by 1 drop of one fiftieth normal alkali.

New rubber tubing is an item of intravenous apparatus most likely to produce toxic material. Pure gum tubing, without bloom, should be used. To insure the removal of any toxic material the tubing is heated in 0.5 per cent sodium hydroxide solution, taking care the inside is well cleaned by kneading the tubing between the hands. The sodium hydroxide is removed by washing well in distilled water and allowing distilled water to run through the tubing for several hours.

Calcium Chloride Solution.—The most commonly employed calcium salts for intravenous injectiom are calcium lactate and calcium gluconate. The latter salt has the additional advantage that it may be administered intramuscularly. If one desires to use calcium chloride, however, the following procedure may be used for its preparation.

A 10 per cent solution of calcium chloride is suitable for injection, but is more advantageously given diluted with physiologic salt solution. All calcium chloride contains some water unless it has been recently fused; therefore, in weighing so-called "crystalline calcium chloride," allowance must be made for the water content in order to obtain a definite amount of calcium chloride in solution. Calcium chloride labeled crystalline, chemically pure, which gives a solution free from turbidity, should be satisfactory. The salt dissolves immediately and may be sterilized in the autoclave at a pressure of 18 pounds, or if sterile water is used, by boiling for a few minutes over a free flame.

A 10 per cent solution of calcium chloride is prepared by dissolving 13.24 Gm. of hydrated calcium chloride ($CaCl_2.2H_2O$), and making the solution to a volume of 100 c.c. The solution is sterilized by boiling or autoclaving at 18 pounds, or is made up into 5- and 10-c.c. ampules and sterilized by autoclaving at 18 pounds. Previous to injection the contents of the ampule are poured into 150 c.c. or more of physiologic salt solution and injected in this dilution.

If an exact percentage of calcium chloride is desired, weigh the equivalent amount of calcium carbonate and add the exact amount of dilute hydrochloric acid which will dissolve it. Add enough distilled water to the calcium chloride solution to produce the proper strength. One hundred Gm. of calcium carbonate, not produced from marble, but of highest purity, when added to just enough hydrochloric acid for solution produces 111 Gm. of calcium chloride, enough for 1110 c.c. of 10 per cent solution.

In dissolving calcium carbonate use hydrochloric acid solution that contains, by volume, about 50 per cent of concentrated acid; add it gradually and allow the carbon dioxide to escape between additions. After most of the carbonate has been dissolved, add the acid very cautiously; at the end allow a few particles to remain undissolved. This insures a neutral solution, which is boiled before it is made up to volume, to decompose any calcium bicarbonate which may have

been formed. The undissolved particles of carbonate are filtered out before adjusting the volume.

Sodium Chloride Solution.—Use only sodium chloride labeled chemically pure or highest purity; other samples may contain calcium or magnesium salts which cause turbidity, or in some cases precipitate after solution. In transfusions a 0.6 per cent solution of sodium chloride containing 3.46 Gm. of sodium citrate ($2Na_3C_6H_5O_7.11H_2O$) for each 100 c.c. is used. For intravenous purposes use a 0.9 per cent solution of sodium chloride. Weigh the salt and dissolve in triple-distilled water. Sterilize the solution either by three successive boilings or by one treatment in the autoclave. The solution keeps indefinitely but it is customary to discard any solutions more than one week old.

Sodium Bicarbonate Solution.—Solutions of sodium bicarbonate lose carbon dioxide if heated. It is, therefore, impossible to sterilize a solution of sodium bicarbonate with heat, unless it is done in a sealed tube. If sodium bicarbonate is dissolved in water and sterilized, only a very small fraction remains as bicarbonate and actually a solution of sodium carbonate is prepared. A method for preparing a solution of sodium bicarbonate is as follows: Weigh out either sodium bicarbonate or sodium carbonate; dissolve it in water and sterilize with heat; then saturate the solution with sterile carbon dioxide gas.

Sodium bicarbonate is usually administered in concentrations of 1 and 5 per cent.

Ampules of pyrex glass containing 70 c.c. of a 1 per cent solution are made and sterilized by autoclaving at 18 pounds. The ampule is allowed to cool and to stand for at least twelve hours to insure reabsorption of all carbon dioxide. The ampule is opened by heating the tip in a flame, and the contents are poured into a sterile flask.

Sodium bicarbonate solutions in greater quantities are prepared by dissolving the required amount of sodium bicarbonate in cold sterile water. No difficulty has ever been encountered in the sterility of these solutions.

Sodium Carbonate Solution.—The preparation of solution of sodium carbonate, as suggested by Fischer, is satisfactory.

FISCHER'S SOLUTION[1]

Sodium carbonate ($Na_2CO_3.10H_2O$)	10 Gm.
Sodium chloride	14 "
Distilled water, enough to make	1000 c.c.

[1] Fischer, M. H.: Oedema and Nephritis, New York, John Wiley and Sons, 1915, p. 557.

Sodium carbonate, U. S. P., may be used satisfactorily. The crystallized sodium carbonate containing 10 molecules of water of crystallization is recommended. Fischer's solution is a hypertonic alkaline solution. Use great care not to inject the solution subcutaneously because the tissue will slough following infiltration with it. This solution is not kept for more than a week.

Sodium Citrate Solution.—Sodium citrate crystallizes with several molecules of water of crystallization. The best grade comes in large transparent crystals containing approximately 5.5 molecules (28 per cent) of water ($2Na_3C_6H_5O_7.11H_2O$). Keep it stoppered in a glass bottle; do not weigh or keep in a paper package. Weigh enough salt to make a 2 per cent solution of anhydrous sodium citrate. Two and seventy-seven hundredths Gm. of crystalline sodium citrate will make 100 c.c. of 2 per cent solution of anhydrous sodium citrate. Sterilize it by three successive boilings or by one treatment in the autoclave at 18 pounds. Only fresh solutions should be used unless the preparation has been put into sealed ampules.

Magnesium Sulfate Solution.—The best grade of magnesium sulfate to use is the crystalline heptahydrate ($MgSO_4.7H_2O$). A 25 per cent solution of anhydrous magnesium sulfate is made by dissolving 51.19 Gm. of the heptahydrate for each 100 c.c. of solution. This is put into ampules of 2, 5, and 10 c.c. capacity; these are sterilized by autoclaving at 18 pounds.

Glucose Solution.—Glucose is administered intravenously with perfect safety, provided the important rules with regard to its preparation and administration are followed. The important points are: (1) The quality of the glucose, (2) the quality of the water, (3) the strength of the solution, (4) the preparation of the solution, and (5) the administration of the solution.

(1) *The Quality of the Glucose.*—Various preparations of glucose differ markedly in their reaction when injected intravenously. Certain preparations will not produce any untoward effects provided they are not given too rapidly. Others, seemingly just as pure, will cause death with as small an amount as 0.5 Gm. for each kilogram of body weight. It is not possible to determine which preparation will produce the toxic action except by direct tests of intravenous injection in an animal. Use only chemically pure preparations of glucose, and test each lot for toxicity by intravenous injection into an animal before it is injected intravenously into a human being.

Frequently it has been observed that in particular lots of dry glucose, even though kept in sealed tins, toxicity may develop after standing for a considerable time. Glucose of excellent quality pri-

marily intended for intravenous injection is now available commercially at a very reasonable price.

(2) *The Quality of the Water.*—Prepare sterile triple-distilled water as described.

(3) *The Strength of the Solution Administered.*—Solutions of glucose of from 5 to 50 per cent can be administered safely intravenously, provided the rate of administration is taken into consideration; the strength of the solution depends on the purpose of its administration. A 4.5 per cent solution is approximately isotonic with the blood, and if the amount of fluid injected is of prime importance, use a solution of this strength. For most purposes use a hypertonic solution. The strength of the hypertonic solution usually administered is 17 to 18 per cent. However, a 20 per cent solution is just as safe and much easier to prepare so far as computation of dosage is concerned. The solutions most generally used contain 10 or 20 per cent glucose. In special instances a 10 per cent glucose solution containing 1 per cent sodium chloride is desired. If the amount of glucose administered is the factor of prime importance, determine the desired amount for the patients and then make it into a solution approximately correct.

(4) *The Preparation of the Solution.*—Dissolve the weighed glucose in a small amount of hot, sterile water. Then filter the solution with suction, using a funnel of the Buchner type, to remove any impurities, and pour the filtrate into a sterile pyrex flask. Add a small excess of sterile water in addition to the amount necessary to make the solution of proper strength and concentrate to its proper volume by boiling for several minutes. This requires ten to fifteen minutes. Do not autoclave. Take care, while boiling the glucose solution, not to allow the glucose to dry on the sides or bottom of the container. Cool and inject at a temperature slightly less than that of the body. *Inject only freshly made solutions intravenously.*

(5) *Administration.*—The intravenous administration of glucose does not differ in any respect from the administration of other solutions. Inject at one time not more than 1 Gm. of glucose for each pound of body weight.

It is well to repeat several of the salient points regarding administration of glucose. *Toxic products are produced if solutions of glucose are autoclaved or boiled for too long a period of time.*

Slow injection should be the rule. Forty-five to sixty minutes for 1000 c.c. of 10 per cent glucose is satisfactory. Solutions of glucose that have stood more than eight hours, other than in sealed ampules, should not be used.

Gum Acacia Solution.—The acacia must be in the form of lumps (pearls), No. 1, extra quality. Prepare first a solution of twofold strength and then dilute to the required degree of concentration with freshly tripled-distilled water. Prepare 8 liters of this solution in the following manner: Weigh and place in a 4-liter beaker 480 Gm. of crushed acacia. Pour approximately 500 c.c. of hot triple-distilled water into the beaker containing the acacia and stir with a glass rod. A large portion of the acacia dissolves rapidly. Carefully pour off the thick solution thus obtained from the residual acacia into a 12-liter round-bottom pyrex flask and add to the residue about 500 c.c. of hot, triple-distilled water. The acacia is reextracted with hot water by stirring and pouring off the supernatant solution. This extraction is repeated until all the acacia is dissolved. It is important to add only a small amount of water at each stage, as the acacia goes into solution more readily when the lumps rub against each other freely and are not caked. To this solution is added 72 Gm. of sodium chloride, C.P., and the total volume made up to 8 liters contained in a 12-liter pyrex flask. This turbid solution containing some dirty impurities is sterilized by autoclave treatment. This solution is autoclaved at 18 pounds for periods of one hour on four consecutive days. This sterilization results in the precipitation of some flocculent material which is objectionable. Attempts to get rid of this precipitate by filtration and resterilization of the filtrate are of no avail since each period of heating results in further precipitation. It is best to siphon off the clear solution above the flocculent precipitate, under sterile conditions into sterile bottles. These bottles containing approximately 500 c.c. of the acacia solution are stoppered tightly with sterile plugs and gauze. Such a solution appears to be nontoxic. Convenient ampules containing 100 c.c. of a 30 per cent solution of acacia are now available on the market. These require dilution to 500 c.c. with sterile water previous to injection and have been found to be satisfactory.

Sodium Lactate Solution.—Sodium lactate may be administered with or without glucose but is most commonly administered with glucose. The solution may be prepared in the following manner: To 100 Gm. of glucose is added 10 or 20 c.c. (depending on the concentration desired) of commercial 85 per cent sodium lactate. This mixture is dissolved in slightly more than a liter of triple-distilled water and filtered. To the filtered solution is added an amount of normal sodium hydroxide to produce a *p*H value between 7 and 7.5. The *p*H value is determined colorimetrically by removing small samples during the alkalization. The solution is then sterilized by boiling down to a volume of 1 liter.

Dye Solutions.—The two commonly used dyes for intravenous injection are mercurochrome and gentian violet. Both of these may

be administered in doses of 5 mg. for each kilogram of body weight. Mercurochrome is generally given in a dosage of 2.5 mg. of the dye per kilogram of body weight.

(1) *Mercurochrome.*—This is prepared in a 1 per cent solution by adding the required quantity of boiling sterile water to the weighed amount of mercurochrome contained in a small, sterile flask. The 1 per cent solution of mercurochrome is diluted by pouring into approximately 750 c.c. of 0.9 per cent salt solution and is given intravenously in this dilution.

(2) *Gentian Violet.*—This is used in a concentration of 0.25 per cent in a 0.75 per cent solution of sodium chloride. The solution for injection is made by dissolving 2.5 Gm. of gentian violet in 500 c.c. of warm, sterile, triple-distilled water. When solution is practically complete 500 c.c. of a 1.5 per cent solution of sodium chloride prepared with sterile water is added. After standing for twenty-four hours, the solution is filtered through sterile gauze into a sterile flask. This preparation will keep for six months. Gentian violet may also be administered in a sodium phosphate buffer solution or simply in triple-distilled water. Prepared in this way, gentian violet is more soluble than in salt solution.

Solutions for Treatment of Varicose Veins.—The four most commonly used solutions are: (1) A solution of quinine hydrochloride and urethane, (2) glucose, (3) sodium salicylate, and (4) sodium morrhuate.

(1) *Quinine Hydrochloride and Urethane.*—Twelve Gm. of quinine hydrochloride and 6 Gm. of urethane are dissolved with the aid of heat in a small amount of sterile triple-distilled water and then made up to a volume of 100 c.c. The solution is filtered, put into 10-c.c. ampules, and sterilized by autoclaving at 18 pounds.

(2) *Glucose.*—A 50 per cent glucose solution is used. This is made by dissolving 50 Gm. of glucose in sterile triple-distilled water and making the volume up to 100 c.c. The solution is filtered, put into 10-c.c. ampules and sterilized by autoclaving at 18 pounds.

(3) *Sodium Salicylate.*—This solution, commonly used in a concentration of 20 per cent, is made by dissolving 20 Gm. of sodium salicylate, containing a trace of salicylic acid, in sterile triple-distilled water and making the volume up to 100 c.c. The solution is placed in 10-c.c. ampules, and sterilized by autoclaving at 18 pounds. Under some conditions a 30 or 40 per cent colution may be used. To prevent the decomposition of solutions of sodium salicylate and the resulting brown discoloration, the solution must not contain any free alkali. Hence it is best to add a small amount of salicylic acid in order to

insure an acid reaction of the solution. Some commercial samples of sodium salicylate crystals contain small amounts of salicylic acid. In these instances it is not necessary to add a further amount.

(4) *Sodium Morrhuate.*—The substance most commonly used for treatment of varicose veins at the present time is sodium morrhuate. This substance is available commercially in solution.

VII. WEIGHTS AND MEASURES, WITH EQUIVALENTS

METRIC

Meter (unit of length): Millimeter (mm.) = $\frac{1}{1000}$ meter.
Centimeter (cm.) = $\frac{1}{100}$ meter.
Kilometer = 1000 meters.
Micron (μ) = $\frac{1}{1000}$ millimeter.

Gram (unit of weight): Milligram (mg.) = $\frac{1}{1000}$ gram.
Kilogram (kilo.) = 1000 grams.

Liter (unit of capacity): Cubic centimeter = $\frac{1}{1000}$ liter = Same measure as milliliter (ml.).

1 Millimeter = 0.03937 ($\frac{1}{25}$ approx.) in.; 1000 microns.

1 Centimeter = 0.3937 ($\frac{2}{5}$ approx.) in.; 0.0328 feet.

1 Meter = 39.37 in.; 3.28 feet.

1 Micron (μ) = $\frac{1}{25000}$ in.; 0.001 millimeter.

1 Gram = 15.43 grains; 0.563 dram; 0.035 ounce; 0.0022 pound (Avoir.); 0.257 dram; 0.032 ounce; 0.0027 pound (Apoth.).

1 Kilogram = 35.27 ounces (Avoir.); 2.2 pounds (Avoir.); 1.056 (1 approx.) quarts; 61.02 cubic inches; 1000 cu. centimeters.

1 Liter =

1 Sq. Millimeter = 0.00155
1 Sq. Centimeter = 0.1550
1 Sq. Meter = 1550
1 Sq. Meter = 10.76 sq. feet.
} sq. in.

1 Cu. Millimeter = 0.00006
1 Cu. Centimeter = 0.0610
} cu. in.
1 Cu. Centimeter = 0.001 liter.
1 Cu. Meter = 35.32 cu. feet; 61025.4 cu. in.

1 Inch = 25.399 millimeters.
1 Sq. Inch = 6.451 sq. centimeters.
1 Cu. Inch = 16.387 cu. centimeters.

1 Foot = 30.48 centimeters.
1 Sq. Foot = 0.093 sq. meter.
1 Cu. Foot = 0.028 cu. meter.

AVOIRDUPOIS WEIGHT

1 Ounce = 437.5 grains; 16 drams.

1 Pound = 16 ounces.

1 Grain = 0.065 ($\frac{3}{50}$ approx.)
1 Dram = 1.77 ($1\frac{3}{4}$ approx.)
1 Ounce = 28.35 (30 approx.)
1 Pound = 453.59 (500 approx.)
} grams.
1 Pound = 27.7 cu. inches.
1 Pound = 1.215 lb. Troy.

APOTHECARIES' MEASURE

1 Dram = 60 minims.	1 Dram = 3.70	cu. centimeters.
1 Ounce = 8 drams.	1 Ounce = 29.57	
1 Pint = 16 ounces.	1 Pint = 473.1	
1 Gallon = 8 pints.	1 Gallon = 3785.4	
	1 Gallon = 231 cu. inches.	

APOTHECARIES' WEIGHT

1 Scruple = 20 grains.	1 Grain = 0.065	grams.
1 Dram = 3 scruples = 60 grains.	1 Dram = 3.887	
1 Ounce = 8 drams = 480 grains.	1 Ounce = 31.10	
1 Pound = 12 ounces.	1 Pound = 373.2	

To convert	*minims*	into	*cubic centimeters*	multiply by	0.061
" "	*fluidounces*	"	*cubic centimeters*	" "	29.57
" "	*grains*	"	*grams*	" "	0.0648
" "	*drams*	"	*grams*	" "	3.887
" "	*cubic centimeters*	"	*minims*	" "	16.23
" "	*cubic centimeters*	"	*fluidounces*	" "	0.0338
" "	*grams*	"	*grains*	" "	15.432
" "	*grams*	"	*drams*	" "	0.257

VIII. TEMPERATURE

Centigrade.	Fahrenheit.	Centigrade.	Fahrenheit
110°	230°	37°	98.6°
100	212	36.5	97.7
95	203	36	96.8
90	194	35.5	95.9
85	185	35	95
80	176	34	93.2
75	167	33	91.4
70	158	32	89.6
65	149	31	87.8
60	140	30	86
55	131	25	77
50	122	20	68
45	113	15	59
44	111.2	10	50
43	109.4	+5	41
42	107.6	0	32
41	105.8	−5	23
40.5	104.9	−10	14
40	104	−15	+5
39.5	103.1	−20	−4
39	102.2		
38.5	101.3	0.54° =	1°
38	100.4	1 =	1.8
37.5	99.5	2 =	3.6
		2.5 =	4.5

To convert Fahrenheit into Centigrade, subtract 32 and multiply by 0.555.

To convert Centigrade into Fahrenheit, multiply by 1.8 and add 32.

IX. PER CENT AND PER MILLE

The mark, %, signifying per centum, or per cent, is well known in American and European literature. In German, and in Scandinavian literature, another mark, ‰, signifying per mille is not so well known to American students. Mistakes in translating this sign as per cent have occurred.

1 ‰ equals 0.1%.

X. TABLE OF NORMAL VALUES

Albumin-globulin ratio, 1.5/1 to 3/1.
Amino-acids, blood, 5 to 8 mg. per 100 c.c.
Base (total), serum, 145 to 160 c c. of tenth normal base per 100 c.c.
Bilirubin, serum, up to 1.5 mg per 100 c.c.
Bleeding time, capillary, three minutes or less.
Calcium, serum, 9 to 11 mg. per 100 c.c.
Carbon dioxide combining power, plasma, adults, 53 to 65 volumes per 100 c.c.; children, 40 to 50 volumes per 100 c.c.
Chlorides, plasma, 570 to 620 mg. per 100 c.c.
Cholesterol, plasma, 160 to 200 mg. per 100 c.c.
Cholesterol esters, plasma, 110 to 145 mg. per 100 c.c.
Clot retractility, blood, complete in one to two hours.
Coagulation time (venous), six to ten minutes.
Congo red (for amyloidosis), 10 to 14 per cent disappearance in one hour; Paunz test, any dye in blood serum after one hour.
Creatinine, blood, 1 to 2 mg. per 100 c.c.
Diastase, in urine, less than 300 units.
Differential count: Leukocytes, total number 5000 to 9000 per cu. mm. of blood.
Neutrophils, 60 to 65 per cent.
Lymphocytes, 25 to 30 per cent.
Monocytes, 4 to 8 per cent.
Eosinophils, 0.5 to 4 per cent.
Basophils, 0 to 1.5 per cent.
Nonfilament forms, less than 16 per cent.
Enzymes, duodenal, trypsin, 7.5 Gm. or more N liberated by 100 c.c. duodenal contents; 2.5 Gm. or more of glucose liberated by 100 c.c. of duodenal contents.
Erythrocytes, 4,250,000 to 5,250,000 per cu. mm.
Fibrinogen, plasma, 300 to 600 mg. per 100 c.c.
Filament-nonfilament count, less than 16 per cent nonfilament.
Fragility of erythrocytes, minimal resistance of 0.42 per cent NaCl solution; maximal resistance, 0.32 per cent NaCl solution.
Galactose tolerance (liver function), less than 3 Gm. galactose excreted in five hours.
Glucose tolerance (Standard), blood sugar at end of half hour not more than 180 mg. per 100 c.c.; normal blood sugar at end of two hours; no sugar in urine.
(Exton), Same as above, except blood sugar at end of one-half hour not to exceed the first by more than 50 mg. per 100 c.c., and one hour sugar not to exceed the one-half hour sugar by more than 30 mg. per 100 c.c.
Hematocrit, 40 to 48 per cent cells.
Hemoglobin, men, 14 to 17 Gm. per 100 c.c. of blood; women, 13 to 16 Gm. per 100 c.c. of blood.
Hippuric acid, excretion of more than 2 Gm. hippuric acid.
Iron, blood, 46 to 55 mg. per 100 c.c.

Lecithin, plasma, 200 to 250 mg. per 100 c.c.
Lipoids (total), plasma, 500 to 550 mg. per 100 c.c.
Liver function, serum, bromsulfalein, no dye retention; galactose tolerance (see under galactose).
Nonprotein nitrogen, blood, 15 to 40 mg. per 100 c.c.
Oxygen capacity, blood, 18 to 24 c.c. per 100 c.c.
Phenolsulfonephthalein, urine (renal function), 50 per cent or more excreted in two hours.
Phosphorus, serum (inorganic phosphates), 3 to 4 mg. per 100 c.c.
Phosphatase, serum, 5 units per 100 c.c. (Bodansky): 14 units (King and Armstrong).
Potassium, serum, 16 to 22 mg. per 100 c.c.
Platelets, blood, 125,000 to 300,000 (or more) per cu. mm.
Proteins (total), serum, 6 to 8 Gm. per 100 c.c.
"Prothrombin time," plasma, less than twenty minutes.
Quick method 12 to 14 seconds.
Reticulocyte count, blood, 0.5 to 2.5 per cent.
Sedimentation rate (Wintrobe method), men, 0 to 9 mm. in one hour; women, 0 to 20 mm. in one hour. (Westergren's method), less than 20 mm. in one hour.
Sodium, serum, 315 to 330 mg. per 100 c.c.
Sugar (glucose), blood, 80 to 110 mg. per 100 c.c.
Sulfates, blood (inorganic), 3 to 5 mg. per 100 c.c.
Urea, blood or saliva, 10 to 40 mg. per 100 c.c.
Urea clearance, 40 c.c. or more blood cleared per minute if urine volume is less than 2.1 c.c. per minute; 60 c.c. or more blood cleared if urine volume is more than 2 c.c. per minute.
Uric acid, blood, 2 to 4.5 mg. per 100 c.c.
Uric acid, urine, 0.75 to 1 Gm. in twenty-four hours.
Urinary diastase, less than 300 units.
Viscosity, blood, 1:4 to 1:6.
Volume, blood, 70 to 100 c.c. per kilogram of body weight; 2800 to 3800 c.c. per square meter of body surface.
Volume index, erythrocytes, 0.9 to 1.1.

XI. MILLIEQUIVALENTS

Many biochemists prefer to express quantitative findings in milliequivalents per liter, rather than in milligrams per 100 c.c. The milliequivalents are the same as millimols per liter in all cases in which the valence is 1. If it is 2, the milliequivalents are twice the millimol values. A millimol is one thousandth part of a gram-molecule. The millimols per liter are calculated by dividing the milligrams per liter by the atomic weight of the element and the milliequivalents per liter are given according to the valence. For example, the normal ranges of certain human blood constituents are as follows:

	Mg. per 100 c.c. in serum	Milli-equivalents per liter of serum	Millimols per liter of serum
Sodium	320–350	139–152	139–152
Potassium	16–22	4.1–5.6	4.1–5.6
Chloride	350–390	99–110	99–110
Calcium	9–11	4.5–5.5	2.25–2.75
Magnesium	2–3	1.6–2.5	0.8–1.25
Carbon dioxide, volume per cent	50–60	44–52	22–26

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