Modern Microbiology

Wayne W. Umbreit

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MODERN MICROBIOLOGY

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Preface

A PREFACE is a place where an author can thank those who helped him, for no book is written alone. Sometimes he may want to explain why he wrote the book and set forth its purpose and plan. Perhaps, for example, I should explain why I put quotations at the head of each chapter. But I think that all these things will become evident as one reads the book.

However, there are some persons whose contributions are not elsewhere evident and without whose assistance this would have been impossible. One is Mrs. Virginia Force Merry, who again translated a manuscript into typescript and who kept the various threads of work from becoming completely tangled. My former colleague, Dr. Evelyn Oginsky, gave the manuscript a thorough and critical reading—but she is not responsible, of course, for whatever errors in concept or in fact I may have made. My immediate colleagues, Dr. Bernard Koft and Dr. Morris Solotorovsky, also have given much valuable advice. Students, too, have contributed; sometimes by providing, through their questions, a new idea, a new concept, or even an insight into what aspect of the science has been poorly explained to them. Aside from professional contributions, much else goes into a book. There are attitudes, and opportunities; there is time and quiet needed. There must be a place where one can think and work, and be protected from interruption. For this, and for much else besides, I sincerely thank my wife, Doris, and my children, Dorayne, Jay and Thomas. To them I dedicate this book.

I wish to thank the Williams and Wilkins Company, Baltimore, Md., for permission to reproduce 91 photographs from the *Journal of Bacteriology* and one photograph from the *American Journal of Tropical Medicine*. I wish to thank the Cambridge University Press, American Branch, New York City, for permission to reproduce 75 photographs from the *Journal of General Microbiology*. To Academic Press, New York, I am grateful for permission to reproduce 21 photographs from three separate books. The Rockefeller Institute Press has granted permission to use 4 photographs from the *Journal of Biophysical and Biochemical Cytology* and two from the *Journal of Experimental Medicine*. The American Association for the Advancement of Science gave permission to publish a photograph from

The Scientific Monthly, and the American Physiological Society permitted use of three from *Federation Proceedings*. E. Munksgaard and Co. granted permission for reproduction of one photograph from *Acta Pathologica et Microbiologica Scandinavica*, as did Masson et Cie for the *Annales of the Institute of Pasteur*. In all cases I have referred to the journal or book involved in connection with the illustration shown. Both authors and publishers have cooperated magnificently in making pertinent and valuable illustrations readily available.

May 1962 WAYNE W. UMBREIT

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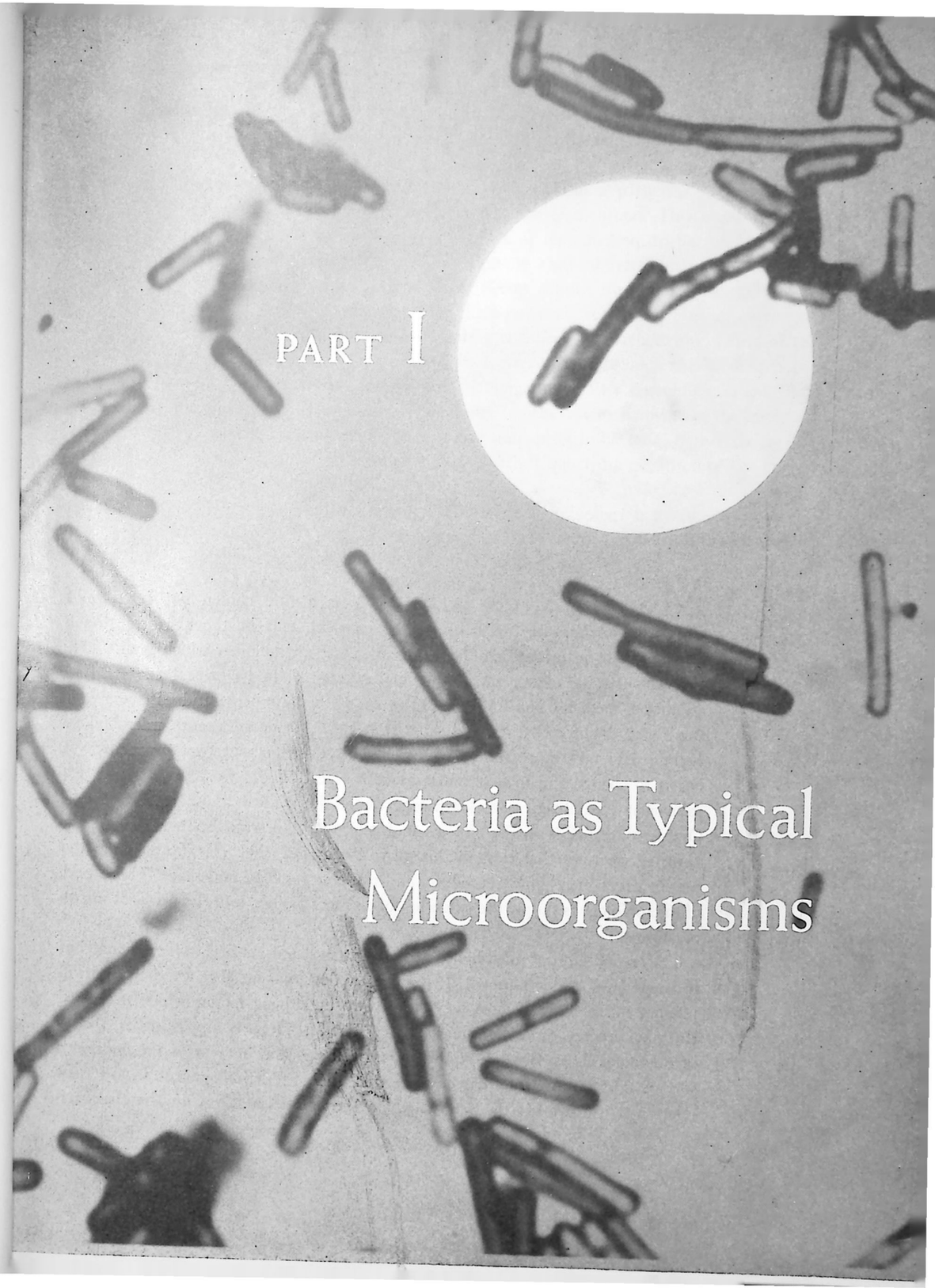
Facing page, *Lactobacillus helveticus*. [From Sundman, Bjorksten, and Gyllenberg, *J. Gen. Microbiol.*, **21**, 371 (1959). Courtesy V. Sundman.]



PART I

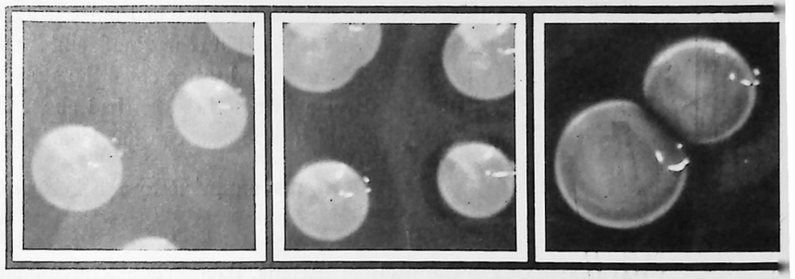
Bacteria as Typical Microorganisms

MODERN MICROBIOLOGY is a title chosen not because of alliteration or because this book, at the moment, may be the newest textbook available, but in order to emphasize an aspect of microbiology which is distinctly modern—that is, of our time. Microbiology started, in its present sense, as an adjunct of medicine and for decades this was its main impetus. Indeed, there were those who doubted that it was a separate science. For decades more, microbiology expanded to take in a variety of other disciplines, but about twenty years ago it began to change and to take on its modern aspect. Today there actually are two somewhat separate areas of knowledge under the general term, "microbiology." One is concerned with the biology of small (micro) organisms. It deals with the kinds of creatures that exist in this micro dimension; their classification and the way in which they grow, reproduce, and compete for food and space. This aspect of microbiology is simply the extension of classical biology into the world of microorganisms.

The second kind of microbiology is not just an extension of an older science but is a new kind of development. It is this new development that we choose to call modern microbiology. There seem to be two underlying factors which permitted the rapid progress of modern microbiology. The first factor is the distinctive quality of microorganisms. Because of their small cell size and because of the enormous surface thus exposed to their environment, their cellular organization and their relation to the world—the way in which they go about their life—is characteristically distinct from the higher cells. Microorganisms are more than, or at least different from, just small editions of larger organisms. Because of their small size, microorganisms bear a closer relation to one another than to higher cells. It is therefore pertinent to learn all that we can about a particular small group of microorganisms and then extend this knowledge to others. This means that we inquire into the morphology or the mode of reproduction, to be sure; but since there are widely similar appearances among vastly different microorganisms, we must also inquire into their mode of energy supply, their metabolic capabilities, and their genetic constitution. For these inquiries we use the tools of chemistry. Thus modern microbiology represents a chemical approach to the nature of the living cells of microorganisms. In Part I, we concentrate upon the bacteria as the typical and universal prototype of microorganisms. Part I could be called "Bacteriology." Part II expands this knowledge to other microorganisms of the microbial world, based upon their differences and similarities to bacteria. It thus covers in a brief fashion that part of microbiology which is merely an extension of the biology of higher cells to the microscale.

The second factor that underlies modern microbiology is a rather curious one and causes some lack of understanding between various microbiologists. The capacities of microorganisms are such that they impinge in an infinite variety of ways upon the life of man. Controlling their development, by both inhibiting and encouraging their activities, can be of great use to man. How to apply microbiology to the needs of man is so intimately bound with the development of the science that it is an important part of it. You cannot be an adequate microbiologist if you know only the theory. You must also know its applications. The science as applied to the daily life of man is discussed in Part III. The science of microbiology is peculiarly related to the diseases of man, animals, and plants; this direct application to health is discussed in Part IV.

Chapter 1



INTRODUCTION TO BACTERIOLOGY

"Having made sundry efforts, from time to time, to discover, if 'twere possible, the cause of the hotness or power whereby pepper affects the tongue (more especially because we find that even though pepper hath lain a whole year in vinegar, it yet retaineth its pungency); I did now place anew about ⅓ ounce of whole pepper in water, and set it in my closet, with no other design than to soften the pepper, that I could the better study it. This pepper having lain about three weeks in the water, and on two several occasions snow-water having been added thereto, because the water had evaporated away; by chance observing this water on the 24th of April, 1676, I saw therein, with great wonder, incredibly many very little animalcules, of divers sorts; and among others, some that were 3 or 4 times as long as broad, though their whole thickness was not, in my judgment, much thicker than one of the hairs wherewith the body of a louse is beset. . . . The second sort of animalcules consisted of a perfect oval. They had no less nimble a motion than the animalcules first described, but they were in much greater numbers. And there was also a third sort, which exceeded both the former sorts in number. These were little animals with tails, like those that I've said were in rain-water.

"The fourth sort of little animals, which drifted among the three sorts aforesaid, were incredibly small; nay, so small, in my sight, that I judged that even if 100

of these very wee animals lay stretched out one against another, they could not reach to the length of a grain of coarse sand; and if this be true, then ten thousand of these living creatures could scarce equal the bulk of a coarse sand-grain.

"I discovered yet a fifth sort, which had about the thickness of the last-said animalcules, but which were near twice as long."

DOBELL, *Antony van Leeuwenhoek and his Little Animals*

BACTERIOLOGY is the study of bacteria. Bacteria are microscopic, unicellular plants, containing no chlorophyll, which reproduce by binary fission. It is worth our while to examine each portion of this definition more closely. You will note that it consists of five parts:

1. microscopic
2. unicellular
3. plants
4. no chlorophyll
5. reproduce by fission

Let us consider each part separately. First **microscopic** means that the individual bacterium (a single organism is a bacter*ium*; a group are bacter*ia*) can be seen only when one uses a microscope that magnifies by 500 to 1000 diameters. Bacteria are generally not visible as individuals under the low-power but require the high-dry or oil-immersion lenses of the modern compound microscope. (See pp. 13–16 for a discussion of microscopes.) It is true, of course, that even an organism as large as a tree or an elephant is composed of individual cells, each one of which is microscopic. But these cells do not exist apart from the organism itself. The bacterium, a single cell, *is* an organism by itself. One may note here that bacteria are partly defined by size. If, on a larger scale, we chose to call everything between one and three inches long by a given name, we would tend to include several different kinds of creatures, even the caterpillar of *Alice in Wonderland*, for example. Hence, if we define all creatures that are microscopic (and have the other four characteristics) as bacteria, we will find several diverse kinds of creatures in this group. Some may well be like plants, some like animals. Hence, in addition to size, other characteristics are necessary.

The second characteristic is that the organism must be **unicellular;** that is, it must have only one cell. Within this single cell it can carry out its life. The single cell reproduces, by forming two "daughter" cells; in short, it lives and grows and has its being as one cell. Of course, the single cell can only grow to a certain size, for if it becomes larger than microscopic it will no longer be a bacterium. Being unicellular does not mean that two cells or millions of cells may not grow close to each other or that chains of cells may not form. A mass of such cells can grow at a particular spot and contain so many organisms that the entire mass is visible to the naked eye. Such a mass is called a **colony** of bacteria. It is composed of individual cells—just as a leaf is composed of individual cells—and certainly these cells in a bacterial colony exert an influence upon one another. But the cells in a leaf are interrelated and interorganized. If we crush the leaf and distribute the cells, what was a leaf is gone and can not be reconstructed from the individual cells, even though we are able to grow them individually. But if we disperse a bacterial colony, each cell in it is capable of growing up into billions of cells and these billion descendants are each capable of forming another colony entirely comparable to the first. This is the meaning of unicellular.

The third characteristic of bacteria is that

they are **plants.** This is perhaps the most vague and controversial point about their character. Anyone can tell the difference between a dogwood and a dog, and even between a cattail, a catnip, a cat, and a caterpillar. But suppose that one has only single cells; how does one tell whether or not they are a plant or an animal? It is not as easy as one would suppose. Some earlier biologist—Haeckel, for example—felt that one could not distinguish, and therefore created "kingdoms" of animal, vegetable, mineral, and "chaos" (or "protista"). Further, bacteria are first defined by size, and one might expect to have a mixture of sufficiently small plants and sufficiently small animals. Of course, if these small creatures have chlorophyll (certainly a characteristic of plants), one would consider them as plants, but we have excluded organisms containing chlorophyll from the bacteria. What other criteria could be used to distinguish plants from animals?

Animals, in general, move of their own will; plants generally do not. But some bacteria move and some don't—so by this criterion some are plants and some animals. Animals tend to use the polysaccharide glycogen as their cellular reserve food; plants tend to use starch. Some bacteria use a kind of glycogen, but most use polysaccharides only remotely related to glycogen or starch. Animals tend to have no cellulose in or about their cells; plants are composed largely of cellulose. Bacteria usually have no cellulose; some few do. All of these things are cited to show how difficult it is to decide whether bacteria should be called plants or animals. And indeed it is difficult even to define "plant" and "animal" at the unicellular microscopic level. But, before we go on, let us point out that in spite of certain difficulties it is an important question to be settled. For if bacteria are plants, they then should "partake of the nature of plants," as Aristotle would have said. We should then be able to attribute a wide variety of "plantlike" characteristics to them. But if they are animals, then we should expect a variety of "animallike" characteristics. And, since the organisms called bacteria are lumped together because of their small size, even if they should be a mixture of plants and animals, it would be important to know whether this is true or whether they are creatures really different from what we usually call plants or animals. These are not academic problems (why are unimportant problems always labeled *academic?*) but really are the heart of the matter, and are indeed of great practical concern.

The definition of bacteria classifies them as plants. Why? There seem to be several reasons, not necessarily sound. One reason is that the first people to study a wide variety of different kinds of bacteria were botanists. These new creatures were "obviously" plants. One can almost hear them say, "Why else would a botanist study them?" This was not then, nor is it now, a valid reason for considering bacteria as plants. But these botanists pointed out that bacteria (*all* of them) have a rigid cell wall. Plants have a rigid cell wall; therefore, bacteria are plants. But in plants, this cell wall is largely cellulose; in bacteria it is not. It has not been possible, however, to decide whether the rigidity or the composition is the more important. The whole debate reached such an impasse that there has been a sort of "gentlemen's agreement": in order to avoid further arguments, all of them inconclusive, we will all agree that since the botanists first called them plants, and since they have a rigid cell wall, we will all call them plants. But perhaps we should admit that they are not plants, nor animals either. Perhaps we should set them apart as a "separate kingdom" (for which there is already a name, the "protista"). Certainly the modern trend, and the modern information, is that bacteria are very much different from plants and animals—differences great enough so bacteria could indeed occupy a kingdom of their own. Some

bacteriologists do indeed do this. They classify all living things into animals, plants, and protista. By this scheme, the third item in the definition of bacteria would be protista, not plants. But, for our purpose, since the older definitions had plants, we will retain this word. Yet these are creatures respectable enough to stand in a world of their own and are so very much different from other living things that they bear careful study.

Bacteria are devoid of **chlorophyll,** i.e., they do not possess the green pigment characteristics of most plants. Single-celled plants which possess chlorophyll are called **algae.** Other plants which do not possess chlorophyll are called **fungi;** those fungi which are single-celled, microscopic, and divide by fission (the "fission fungi") are called bacteria.

Bacteria reproduce by **fission**—or, as it is sometimes called, binary fission. Bacteriologists used to say, "They just divide." As an overall description of the process, this is all right, since one cell does become two. But the process is, of course, much more complicated than this. What is meant here is that one does not find the fusion of cells or the complex division patterns seen in most higher cells. The process of binary fission is somewhat more complex than just splitting in two, and we shall examine it in more detail later (Chapter 3), but for the moment this will suffice to characterize the process.

These then are the bacteria, the microscopic, unicellular plants containing no chlorophyll and reproducing by binary fission. Such creatures possess the spark of life and, small though they are, they are not beneath our notice, for they may both help and hinder us. Indeed, some may kill us.

Bacteriology and Microbiology

Bacteriology is the study of bacteria. But in the course of time bacteriologists began also to study related creatures which were not strictly bacteria. The term microbiology came into use when it became common for bacteriologists to be familiar with organisms other than bacteria. After all, there is a variety of organisms capable of study by the methods of bacteriology and which the practicing bacteriologist continually encounters (molds, yeasts, streptomyces, for example). Since their properties were in a great part related to bacteria, it was only sensible that they should be studied by bacteriologists. However, some people felt that a bacteriologist should stick to bacteria, and so the word "microbiologist" was employed to designate this wider area. While the term microbiology could have meant any biology employing microscopic organisms, in actual practice it does mean bacteriology. A person doing diagnostic work in a hospital is likely to be called a bacteriologist. A person working with bacterial cultures in an industrial plant is likely to be called a microbiologist. But both may deal with creatures other than bacteria. If they work mainly with bacteria, but on occasion study other microscopic creatures, they may be called bacteriologists. If they work mainly with creatures other than bacteria, they may be called microbiologists. Microbiologist is the broader term.

Discovery of Bacteria

Bacteria were discovered by a Dutchman, Antony van Leeuwenhoek, in Delft, Holland, in 1675. A year later he sent a letter to the Royal Society of London, describing his discoveries. It is of interest that it was just 200 years before Koch's demonstration (1870) that bacteria cause disease.

Leeuwenhoek discovered a great many things: spermatozoa, blood corpuscles, yeasts, protozoa. He was considered almost uneducated by the standards of his day; he was, in fact, a cloth merchant, and in his spare time had the hobby of fashioning lenses, but this was only a hobby. In a letter to the Royal Society he says, "These observations concerning living creatures in the liquors spoken of, were indeed deserving of closer attention and description; but for that, there had been need too of a whole man, which my circumstances did not allow of; for I have only employed my spare time upon them."

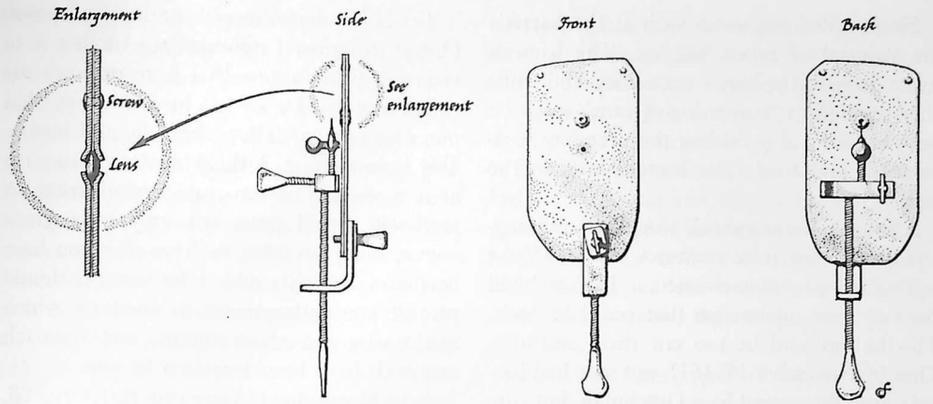


FIG. 1-1. *The construction of the Leeuwenhoek microscope.*

Of course, no one could discover organisms that could only be seen with a microscope—until he *had* a microscope. Along about 1600 the compound microscope had been invented and it became something of a new fad. Microscopes were built and used mostly by physicists who were interested in optics, but they really couldn't see much through them because of color aberrations.

The kind of microscope Leeuwenhoek used, however, was not a compound microscope but rather simple magnifying glasses. Basically these were spheres or bulbs of glass, sometimes filled with water. The modern "reading glass" is composed of opposite sides of such a sphere. The smaller the bulb, the greater the magnification, but even with a true sphere (and these small "beads" were hard to make as perfect spheres) the image is only clear in the center, and the best of them magnify only 2–300 diameters. This is not large enough to see bacteria; certainly not in the detail that Leeuwenhoek described them. How he did see them is still something of a mystery. He would not let anyone, even the Royal Society, have one of his microscopes. He told one visitor, "How I wish that I could show you my best lens, with my special way of observing, but I keep that only for myself and do not show it to anyone, not even to my own family."

We do know something about how the microscopes were made (Fig. 1-1): the little bead of a lens was placed between two metal plates. Focusing was so difficult that once the object was focused it was generally left under the lens, and a new microscope was built for another object. At any rate, with this device Leeuwenhoek probed about looking at everything he could think of—blood, water, milk, and other liquids.

As Leeuwenhoek says, one day he got to wondering why pepper tastes sharp. Did it have little sharp points on the grains which stick in the tongue? He started to look at the pepper, but since it doesn't wet well with water, he couldn't mount it for observation. So he let it soak for a few days, and in this mixture, which had actually fermented and decomposed during this time, he saw bacteria.

Single-celled organisms such as the bacteria are stripped of excess baggage. The bacteria make do with the barest necessities, and while they respond to "tender loving care," it is care only in terms of providing the necessary environment and food. The bacteria will do the rest.

Although Leeuwenhoek would not tell anyone how he saw these creatures, Robert Hooke in England was commissioned, in 1677, to build the very best microscope that could be built. This he did, and he too saw these creatures. Thus by November 15, 1677, not only had bacteria been discovered by a Dutchman, but confirmed by an independent method by an Englishman. It was 200 years before further real progress was made.

The Tools of Microbiology

Microbiology is concerned with study of microorganisms, those creatures on the borderline of life but a true part of it. You are now beginning the study of these creatures, and to proceed successfully, you will need certain tools. They will be described in some detail in each chapter as knowledge of them is required, which seems a better method than to confine them to the appendix or to a laboratory manual. Some of the tools are technical, some mechanical, some conceptual, some mathematical, but all are useful.

On the Purpose and Use of Textbooks

This is a textbook of microbiology. As such, its function is to enlighten, not to entertain. If my own experience is any guide, I do not suppose that anyone ever took the time to tell you what a textbook is supposed to do and how to use one. We will therefore do so. After all, this book is one of the tools you will use to learn microbiology.

Textbooks have several primary purposes. One is to transmit information. Another is to transmit organization—that is, to organize the varied aspects of a subject in such a way that one development follows logically and reasonably from another. A third function is to serve as a repository of retrievable information. A textbook should serve not only in a given course, but years later, perhaps after you have been away from its subject for years, it should provide you with answers to questions which might arise and whose solution and approach may well have been forgotten by you.

As such, you should know your textbook well, so that you know where things are. You should also use the book to record your own reactions; if your instructor tells you that some statement made by the text is no longer true, make a note of it, right in the book.

A textbook can, then, serve a useful function for you for years if you will learn how to use it. That is the purpose of these paragraphs. One never reads a textbook through at one time; it is to be taken chapter by chapter. It will help a great deal if you orient each chapter with what you have gleaned from the lecture on this same subject. This is frequently useful later when you wish to recall the matter. Again one rarely learns bacteriology or any other subject from a textbook alone, although it can be done. You will have lectures which will cover the same subject as the text, though sometimes in a different order. It is easier if your instructor follows the same organization as the textbook, but if he doesn't it should still be easy to see how his organization relates to the text. After all, it generally doesn't matter if Chapter 5 is studied before Chapter 3, and this textbook is written with this possible contingency in mind. There is more than one way of logical and reasonable organization, and different people prefer different orders. For example, the early

bacteriologists were generally botanists and were usually familiar with yeasts and molds. Hence early courses in bacteriology usually started with yeasts and molds and then took up bacteria later, saying, in effect, that bacteria are somewhat like the yeasts and molds you are familiar with. Many courses in bacteriology, and many good textbooks in it, are therefore organized in this fashion. But we prefer to organize it differently, since we think today's student is not familiar with yeasts and molds. We will start out telling you about bacteria and, near the middle of the book, write about yeasts and molds, saying, in effect, they are in certain ways like the bacteria with which you are now familiar. It is better if the lectures follow the order and organization of the textbook, but it is not really critical that they do so.

While we are at it, why lectures? This curious custom doubtless arose in the days before textbooks were widely available, when transmission by word of mouth was essentially the only way of communication. But it has been a persistent custom, and by and large a good one. As a tool for the transmission of information, attitudes, and feeling, the lecture does what no other method can do. In general, one should attend the lecture on the subject first, then find the portion of the textbook covering the same subject; a good lecturer will tell you *where* to find it. Then, in the quiet of your room, read what the text has to say, annotating it where reasonable. By thus reinforcing your audible impressions with the written impressions, even if they say the same thing, you will fix the matter in your mind, where it needs to be. After all, it does you no good to study bacteriology if the knowledge is not put into your mind. It is already in books, and in the mind of the lecturer, but unless it gets into your mind the whole effort is wasted.

A textbook is written to be as complete as possible because an author never knows whom he may be addressing. You may have had years of experience with a microscope, and so a section on the microscope is "old stuff" to you. In this case, skip it! There is no need for you to study further what you already know. But the textbook must have this section in it, since your neighbor may never have seen a microscope in his life and has no idea how one works. To use a textbook properly, there is no need to read all of it.

In any new subject, part of the learning consists of learning the words associated with this subject. In this text until this point, the following words which may be new to many were introduced and most of them defined: bacterium, bacteria, "daughter" cells, colony of bacteria, "chaos," protista, polysaccharide, glycogen, rigid cell wall, molds, algae, fungi, fission fungi, fusion of cells, microbiology. This does not count microscopic, unicellular plants, chlorophyll, starch, cellulose, with which presumably everyone is familiar. At the end of each chapter, therefore, there is a question, "Define or identify," which contains the important new words that one might be expected to know.

There are references listed at the end of most chapters. These do not give you the key to the current publications; your instructor may do that if he wishes. Rather, the references tell you where you can find more extensive discussions of the subjects taken up in the chapter in question. They refer further to good discussions.

The Compound Microscope

A moment's digression to tell you something about how a microscope works will be useful. You do not need to know how a microscope works to be a bacteriologist, any more than

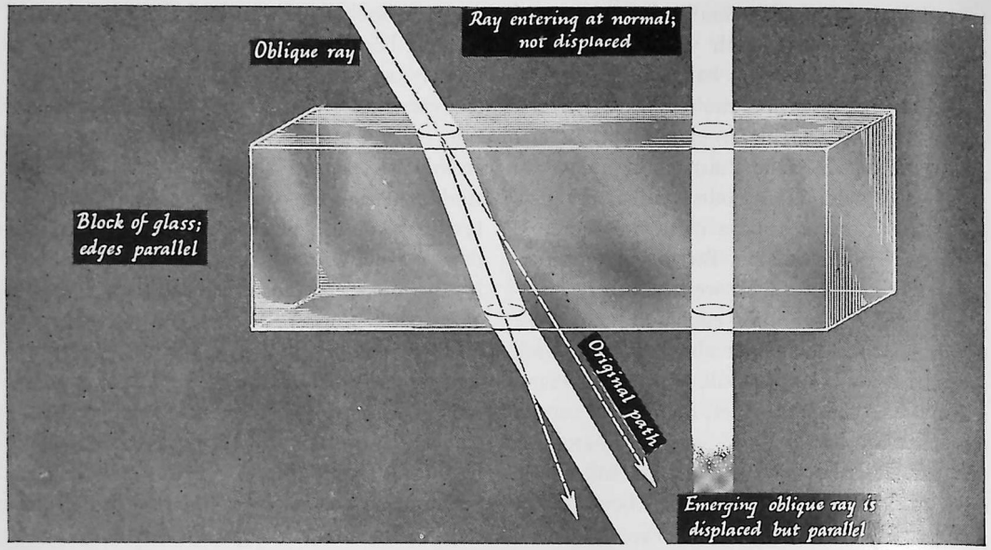
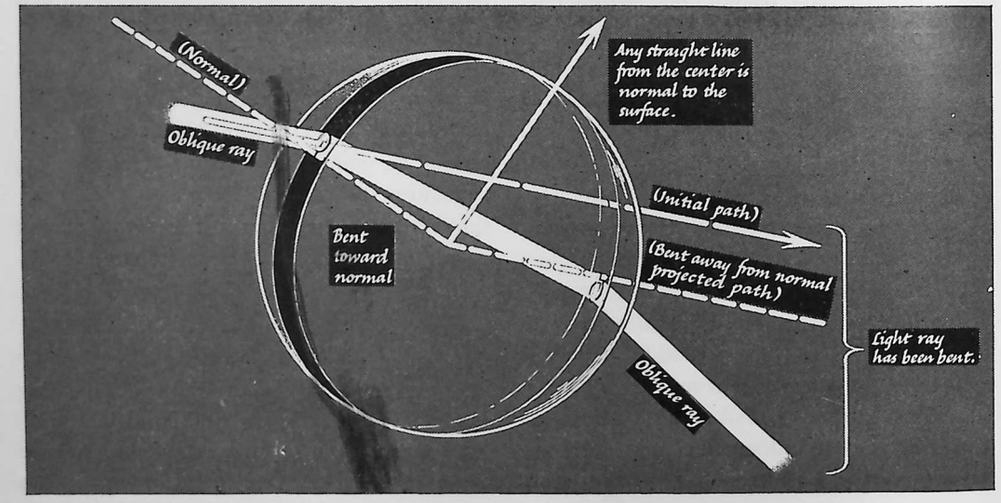


FIG. 1-2. *The first three optical laws. 1. When an oblique ray passes into a more dense substance it is bent toward the normal. 2. When passing through a denser material having parallel sides, the light ray is displaced latterally. 3. A ray of light entering normal to the surface is not bent.*

FIG. 1-3. *A ray entering and leaving a denser sphere is bent.*



you need to know how a television set works to see a picture on it, but frequently one is curious about the basic instrument one uses. An uncomplicated discussion of the laws of optics and how they may be used to develop microscopes may be helpful. (If you know all about this subject, pass on to p. 17.) You need to use a microscope to see bacteria and you will have to use it at the limit of its power, and not merely in a casual fashion.

The optical laws with which we are concerned are the following:

1. When an oblique ray of light passes from a lens dense to a more dense medium (say from air to glass) it is bent *toward* the normal, the normal being an imaginary line perpendicular to the surface (Fig. 1-2).

2. When an oblique ray of light is refracted (bent) through a denser medium bounded by parallel sides, it suffers no change in direction but is displaced laterally (Fig. 1-2).

3. When a ray of light enters at the normal it is not refracted.

The operation of these three laws is illustrated in Fig. 1-2.

4. With a curved surface (a sphere) an oblique ray is bent (see Fig. 1-3).

5. A **convex lens** is formed by the intersection (overlapping) of two spheres.

6. When parallel rays of light go through a convex lens, they cross after emerging (Fig. 1-4). The point at which they cross is called the focal point.

7. One may trace rays of light through a convex lens by using the following rules.

(a) Any ray going through the optical center emerges without bending since it enters and emerges on the normal (Fig. 1-4).

(b) A ray parallel to the lens (i.e., parallel to the axis of the lens) passes through the focal point (Fig. 1-4).

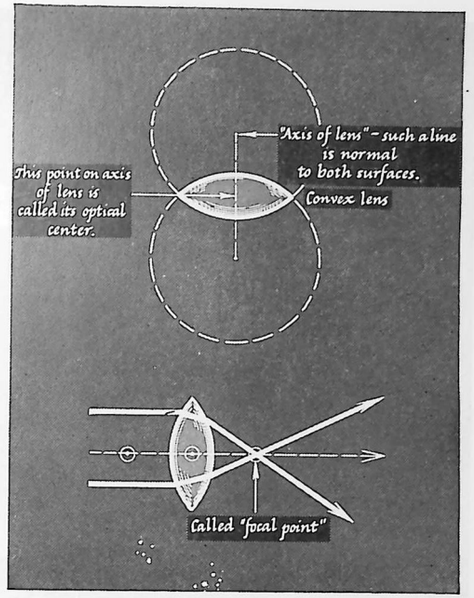


FIG. 1-4. *Parallel rays through a convex lens cross at the focal point.*

These rules are used in subsequent figures.

8. When an object is outside of the focal point of the lens, the image formed (where the rays cross) is real (it is the same light that entered) and inverted (Fig. 1-5).

9. When an object is inside the focal point of the lens, the image is virtual and erect (Fig. 1-5).

10. These principles are combined in a compound microscope by putting the object outside the focal point of the first lens (called the

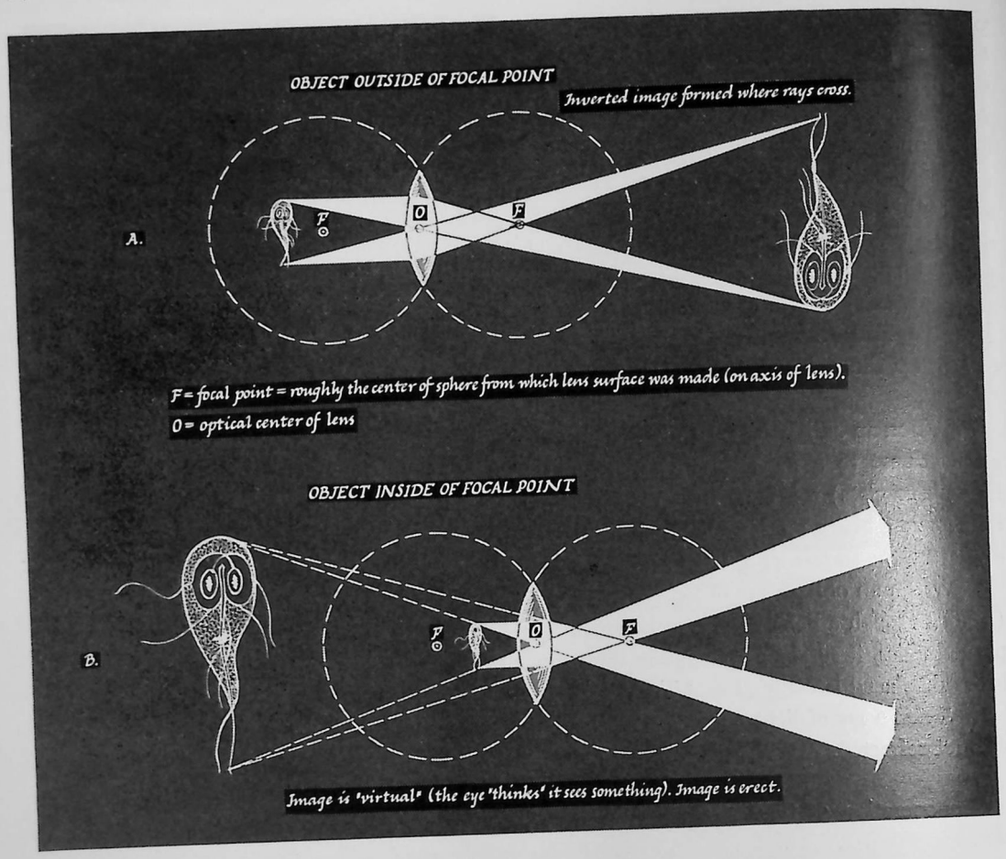


FIG. 1-5. *Real and virtual images.*

"objective" lens) and placing this image (real, inverted) inside the focal point of the second lens ("ocular" or eyepiece). Notice how this will magnify (Fig. 1-6).

11. The magnifying power of a lens depends upon

(a) the distance of the object from the focal point—the closer the object to the focal point, the greater the magnification;

(b) the curvature of the lens—the smaller the sphere from which it was derived, the greater the magnification.

These optical principles have been combined in your compound microscope. The instrument used in bacteriology has three objective lenses: low-power, high-dry, and oil-immersion. The ocular is usually 10X; X means the power of magnification. The properties of the objective lenses are listed in Table 1-1.

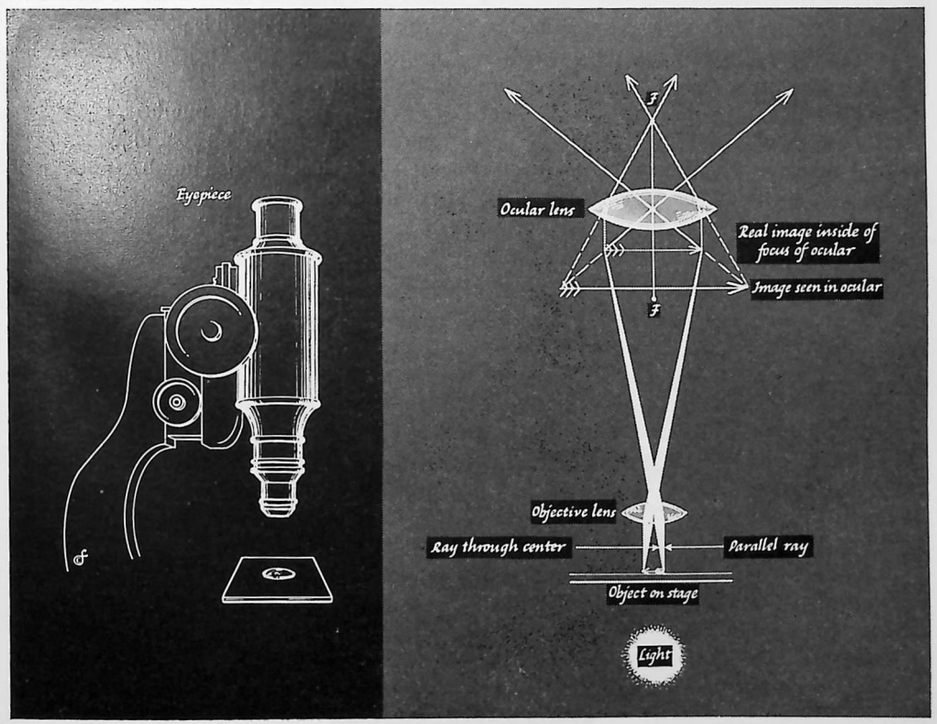
The oil-immersion lens is such a small lens that little light gets through it. When light emerges from a more dense medium (such as a glass slide bearing the object on the microscope stage), it will tend to bend away from the normal (Fig. 1-7); hence even less light enters the lens. To prevent this, oil is placed between the lens and the object. The oil usually has the same optical density (= refractive index) as glass. The light, therefore, does not move from a more dense to less dense medium, so it does not bend, but enters the lens. Since the smaller the lens, the greater the curvature of its surface and the greater its magnification, one would suppose that one could obtain any desired magnification by increasing the curvature. However, as the lens becomes smaller,

TABLE 1-1. *The Lenses of a Modern Compound Microscope*

	Diameter of Sphere from which Lens Is Derived	Focal Point (mm)	Magnification of Lens Itself	Magnification with 10X Ocular	Diameter of Field Seen	
					(mm)	(μ)*
Low-power	16	7	10	100	1.55	1,550
High-dry	4	0.6	43	430	0.31	310
Oil-immersion	1.8	0.15	95	950	0.16	160

* The Greek letter μ (mu) stands for micron (plural, micra), a unit of length which equals one thousandth of a millimeter.

FIG. 1-6. *The compound microscope. This instrument forms a real image inside the focal point of the occular lens. One therefore sees a virtual image of the real image of the object.*



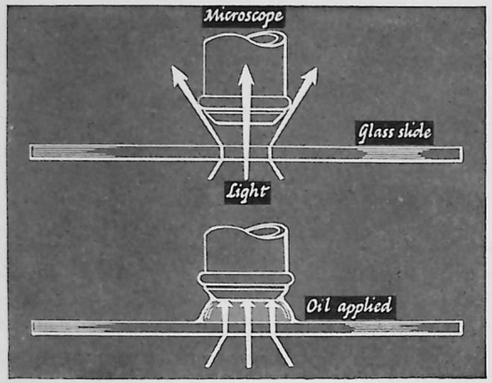


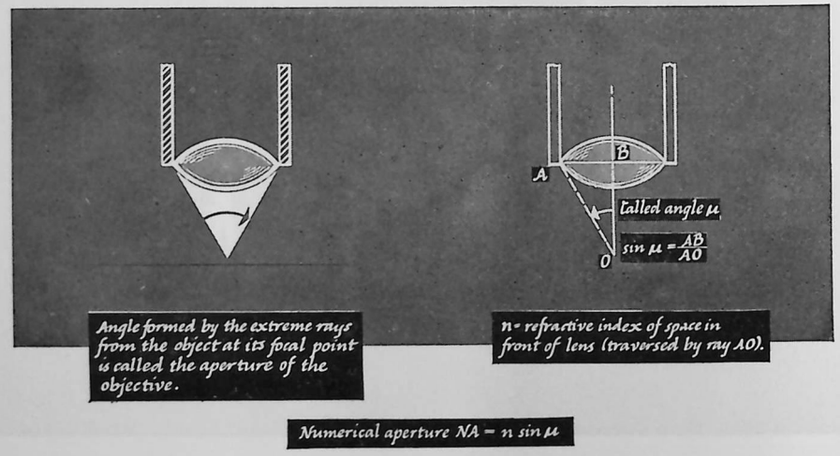
FIG. 1-7. *The function of oil in an oil-immersion lens.*

after a certain limit one cannot obtain enough light through it to be seen. The modern microscope uses transmitted light, reflected from a mirror or an actual intense lamp source, and an Abbe condenser, which is a lens system to focus the light rays on the object. Even with oil immersion 1000 diameters magnification is about the limit obtainable.

On your microscope lenses you may see some further markings: one of these may well be N.A. This is an abbreviation of numerical aperture, which is explained by Fig. 1-8. The N.A. is used in the following way. It happens that the wavelengths of light, divided by twice the N.A., measures the finest detail that can be revealed by the microscope. White light with a bluish tinge having a wavelength of 5000 Å and a N.A. of 1.0 will be able to reveal objects which are 5000 Å/2 = 2, 5000 Å = 250 mμ = 0.25 μ. (Note: Å stands for Ångstrom unit, which equals 10^{-8} cm.) That is, if two dots are less than 0.25 μ apart, they will look like a single dot in the microscope. If they are farther than 0.25 μ apart, they will appear as two dots. Since bacteria are generally about 0.5 to 1 μ wide (they may be several micra long) one can understand that perhaps not much detail about bacterial structure can be revealed by the oil-immersion lens. But this is what we must work with and sufficient detail is possible so that the compound microscope is extremely useful when used carefully.

The remainder of the numbers, etc., which you may find engraved on your objective lenses generally are identification numbers and usually have no optical significance.

FIG. 1-8. *The meaning of numerical aperture.*



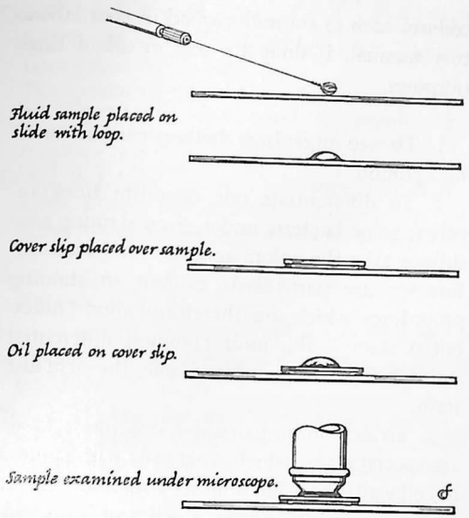


FIG. 1-9. *The wet mount.*

Methods of Studying Bacteria with a Compound Microscope

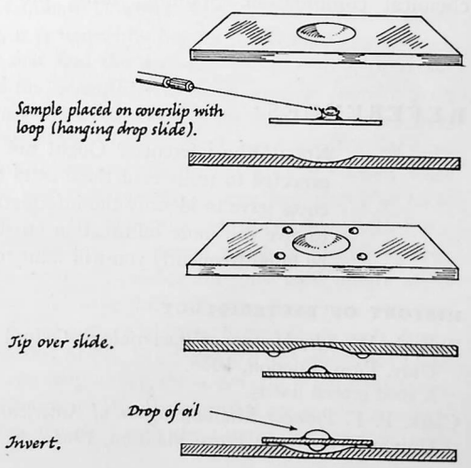
With the oil immersion lens, the compound microscope will give you a magnification of about a 1000 diameters. With this you will be able to see clearly the shape of the bacterial cells, but not very much else. There are some other kinds of microscopes which will be discussed later, but mostly these are specialized research tools and are not available to a beginner. Hence, you need to know how to get the most out of the 1000X magnification available to you.

You will need to examine bacteria, or materials suspected of containing them, in the **wet** condition and in a dried and **fixed** condition. The wet condition, generally fresh or live preparations, is necessary for the determination of motility, for once one has dried and fixed bacteria they are no longer motile. Bacteria are generally mounted on a glass (or sometimes plastic) slide and for wet preparations are covered with a coverslip, because oil will not stay on a water surface. Since the working distance of an oil immersion lens is 0.15 mm, the coverslip must be very thin, and, as you will find out in the laboratory, such glass must be handled carefully or it will break. Recently plastic cover slips which do not break readily have come into use.

There are two primary methods for examining bacteria in the wet condition. The one most widely used is the wet mount. In this case, one simply puts a drop of the fluid to be examined on a slide and drops a coverslip on it. The fluid will spread out (between slide and coverslip) to form a thin layer. Oil is put on top of the coverslip and the contents examined (Fig. 1-9).

The other method is the **hanging drop,** made as shown in Fig. 1-10. Both of these methods permit one to examine wet specimens for possible motility. The organisms are also in their natural shape, not distorted by drying or fixing. They may be examined in the living state or, of course, the specimen may be killed and still examined. The bacteria, however, are generally not too different in color or refractive index from the fluid, and at times they are hard to see. It is possible to color them (with dyes or

FIG. 1-10. *The hanging-drop method.*



stains) and then they are somewhat easier to see. However, wet mounts or hanging drops are generally used for determination of motility and for measurements of size and shape without distortion due to drying, and hence one usually does not want the cells treated with stains.

Bacteria may also be examined in the dry state, with or without staining. To stain bacteria, the suspension or material to be examined is thinly spread on a glass slide and allowed to dry. It is then "fixed" by passing it through a flame, with the side containing the dried preparation away from the flame. This fixing process prevents many of the cells from washing off the slide in subsequent operations. However, even the drying and the fixing do not necessarily kill all the cells. If one puts on ten million cells, easily contained in a small loop of a broth culture of bacteria, dries them, and heat-fixes them, one may have 10–1,000 cells left alive, i.e., capable of subsequent growth. If one is dealing with pathogenic bacteria, one must be most careful of these residual cells; otherwise it doesn't matter much.

Such a dried and fixed preparation may be examined as such (it rarely is) or it may be stained, i.e., colored with some dye or dye-chemical combination. Staining, using procedures such as those described in your laboratory manual, is done for any or all of three purposes.

1. To see organisms better; contrast with background.

2. To differentiate one organism from another: some bacteria under given staining conditions take the color; some do not. Such differences are particularly evident in staining procedures which are therefore called "differential stains," the most common differential stains being the gram stain and the acid-fast stain.

3. To determine particular structures: there are special stains which react only with spores, or cell wall, or nuclei, or other structures; hence an organism stained with a cell wall stain will show the presence or absence of a cell wall.

Such dried and fixed preparations, before or after staining, are usually examined with the oil placed directly on the specimen, rather than with a coverslip. These methods are the ones you will use in the laboratory and upon which much of our knowledge of bacteria is based. This is why we mention them separately here. The actual chemical mechanisms involved in staining are not precisely known.

REFERENCES

Note: Why references? Ought not the discussion in a textbook be complete? Is one expected to really read these extra things? Why references? In a scientific paper references serve to identify the information given, but in a textbook references are included to give you more information on subjects discussed. Many times our discussions must be brief; frequently you will want to learn more.

HISTORY OF BACTERIOLOGY

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A good general history.

Clark, P. F. *Pioneer Microbiologists of America*. Univ. of Wisconsin Press, Madison, 1961.

De Kruif, Paul. *Microbe Hunters*. Harcourt, Brace, New York, 1926. Reprinted in paper-bound form.
This is the story of some of the early bacteriologists, written by a man who knew some of them and who could write thrillingly about them.

Dobell, Clifford. *Antony Von Leeuwenhoek and his "Little Animals."* 1932. Reprinted, Dover Books, New York, 1960.
This contains not only the history but Leeuwenhoek's own letters to the Royal Society. The paper-bound reprint is a bargain; the original edition is now a collector's item.

Dubos, R. J. *Louis Pasteur, Free Lance of Science*. Little, Brown, Boston, 1950.
A well-written account written by a literate and experienced bacteriologist.

———. "Microbiology in fable and art." *Bact. Reviews*, **16**, 145 (1952).

——— and Dubos, J. P. *White Plague: Tuberculosis, Man and Society*. Little, Brown, Boston, 1952.

Nichol, H. *Microbes by the Millions*. Penguin Books, London, 1940.
A lively account of the activities of microorganisms in the soil.

Vallery-Radot, René. *The Life of Pasteur* (translated by R. L. Devonshire).
Written by his son-in-law. A little slow at times, but a fascinating study of development—human and scientific.

van Niel, C. B. "The 'Delft school' and the rise of general microbiology." *Bact. Reviews*, **13**, 161 (1949).

Winslow, C. E. A. "Some leaders and landmarks in the history of microbiology." *Bact. Reviews*, **14**, 99 (1950).

Zinsser, Hans. *Rats, Lice and History*. Little, Brown, Boston, 1935.
This is a literate work (what damning words these are) which, in a pleasant, witty style, propounds the thesis that epidemics have more to do with history than armies or generals. By one of the great bacteriologists, who knew what he was talking about.

COMPOUND MICROSCOPE AND OPTICS

I know of no really good and useful book on this subject which is both simple enough to be used by the beginner, yet detailed enough to be useful to the college student. Those which are sufficiently advanced to be useful, are so encrusted with technical language as to be difficult. However, Chapter 2 of the textbook by A. J. Salle has a good usable description of the microscope.

Salle, A. J. *Fundamental Principles of Bacteriology* (5th ed.). McGraw-Hill, New York. 1961.
This same textbook, in Chapter 3, has a good but chemically oriented discussion of staining, and the nature of stains.

BACKGROUND INFORMATION

In studying each chapter, you are dependent upon information derived from other sciences with which you should be familiar. However, because the students taking microbiology have varied backgrounds, it is frequently helpful if reference can be made to some convenient article where you may find the necessary background information. A good source of such information is the *Scientific American*, since it contains good authoritative articles written by scientists in one field to explain their work to scientists in other fields. The articles are clear expositions of experimental and scientific information, relatively free from the jargon usually associated with any area of science. They are, by and large, interestingly written, and they provide an excellent source of background information, occasionally even directly applicable to microbiology. Unfortunately, *Scientific American* uses a volume and page notation which is difficult to refer to: one must refer to the *year* and the *month* of publication since each month starts at page 1. Furthermore, not every library may have these volumes. Recently a selection of individual articles has been reprinted and are available, at twenty cents each, from: W. H. Freeman and Company, 660 Market Street, San Francisco 4, California. These reprints are supplied to bookstores, so you may obtain them directly if your bookstore has ordered them from the Freeman Company. We therefore list on p. 491 a variety of such articles relating to each chapter, together with the number assigned to each

particular reprint. There are other articles, not yet available in reprint form (and to which therefore no number can be assigned), although these may, in the future, be made available.

A typical citation is:

Wald, G. "The origin of life."
How did living matter first arise on the Earth? As natural scientists learn more about nature they are returning to a hypothesis their predecessors gave up almost a century ago—spontaneous generation.
Sci. Am., August, 1954, No. 47

QUESTIONS

Note: Many textbooks at the college level do not have questions. And many that do reflect a "busy work" attitude strongly reminiscent of the secondary schools. We include questions for two purposes: to set out new words which you may not know, and to stimulate you to new ideas which might otherwise never occur to you. Some of these questions may not be answered in the text. Perchance the answer is in your mind. After all, the purpose of education is not to reproduce what is in your textbook, but to provide you with sufficient information so that you may reason successfully and make progress in the field. What is in the books does you no good until it is in your mind.

1. Define or identify:

bacterium, bacteria
binary fission (cf. transverse and longitudinal fission)
fission fungi
mitotic pattern
microbiology
bacteriology
Hooke
optical normal refraction
fungi
focal point
Abbe condenser
N.A.
wet mount
Leeuwenhoek
bacterial colony
kingdom
protista
differential stain
hanging drop

We assume that you know: unicellular, chlorophyll, microscope, low-power, high-dry, oil-immersion, cellulose, cell wall, compound microscope, organism, polysaccharide, glycogen, starch, reproduction, convex lens, fixed preparations, slide, coverslip, working distance, motility, refractive index.

2. If you did not possess a microscope and could not ever see bacteria, do you think that you would know that they exist? What kinds of information would suggest that they exist?
3. What is the theory of spontaneous generation? What are the arguments both for and against it? Which are in error and why?
4. What methods could you think of to improve the use of the microscope?
5. Why does one stain bacteria?
6. What is the difference in principle between Leeuwenhoek's microscope and a compound microscope?
7. What is a real image? A virtual image? Which one is inverted?
8. List and explain the meaning of the five main characters which define bacteria.
9. How did lack of knowledge of microbiology retard and confuse early studies on spontaneous generation?
10. Give two methods by which the motility of bacterial cells may be determined.

Chapter 2

THE BASIC TOOLS OF MICROBIOLOGY

"Natural Science is a branch of knowledge by general consent not primarily based on the *a priori*. Natural Science observes and endeavors by observation to follow and trace the 'how' of what happens in Nature. It proceeds further to generalize about this 'how.' It tries to decipher something of it in the past and to forecast something of it in the future. . . . Not that Natural Science would admit, nor is it implied in the above, that Science's sole curiosity about Nature regards the material benefits it can extract thence. It would hasten to add, and with all sincerity, that at least in part its object is to learn the 'how' of Nature for the sake of that 'how' itself as being one aspect of 'truth.' "

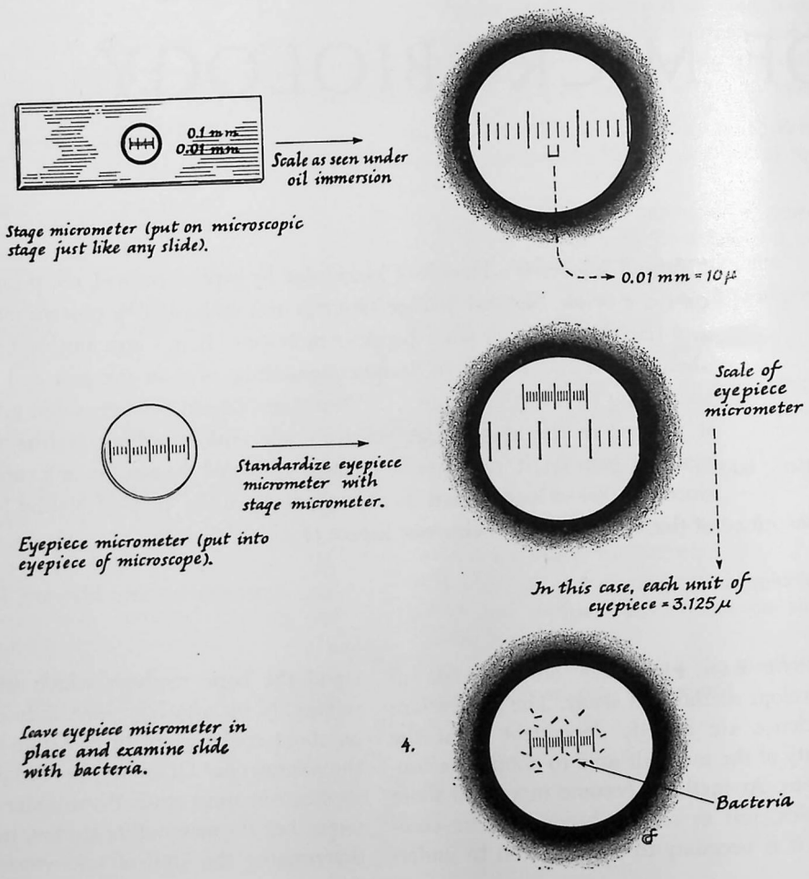
SHERRINGTON, *Man on His Nature*

SCIENCE CAN PROGRESS only as fast as it develops methods of study. The conclusions of science are directly dependent upon the validity of the methods used to obtain the conclusions. As methods become more and more complex, just as science becomes more complex, it is necessary to examine and to understand the basic methods which underlie the science. In microbiology, how, indeed, does one go about studying creatures visible only under the microscope? Of course, for certain types of studies, one must study them under the microscope, but for many other studies, methods for determining the kind of microorganisms, and

even their number, do not involve a microscope at all.

This chapter therefore outlines the basic methods upon which most of microbiology is based. It does this, further, recognizing two things. First, no one, at this stage, is going to provide you with an electron microscope or an automatic particle counter. The methods which you shall use are those capable of being conducted by no more fancy an apparatus than a compound microscope. It happens, however, that microbiology was founded with just such methods, and they remain the important part of microbiological study. The methods in this chapter are those which you are capable of doing in the ordinary microbiological laboratory, but it is better not to leave them solely to the laboratory manual. The actual technique of any particular method is not our real concern here, but rather the *principle* upon which it is based. For example, the microscope has certain limits, and hence man has devised more complex but also more effective ways of observing microorganisms. These, too, are described

FIG. 2-1. *Occular micrometers and stage micrometers.*



so that you may be familiar with the principles on which they operate, even though you may actually never see them in operation.

Second, we have described the methods in terms of bacteria, but it is to be understood that with slight modifications these methods are applicable to most microorganisms.

The basic methods are of several general types. First (beginning on p. 23), microscopic methods which tell one something about the nature of the cells. Second (beginning on p. 30), methods which are designed to obtain a single microorganism away from all others, and to let it grow "pure," i.e., with only its progeny. Such methods of isolation may be microscopic or not. Third (beginning on p. 43), methods designed to determine the numbers of microorganisms.

Microscopic Methods

We have earlier outlined the principles of the compound microscope, and mentioned briefly simple staining. In this section we need to answer certain questions. How does one measure the size of microorganisms under the microscope? What is the nature of other optical tools which may be employed? And what is the special staining method, the gram stain, which is universally employed in the study of microorganisms? The reasons for its use will become evident to you in our discussion.

Measurement of Microbial Size

Leeuwenhoek said of bacteria, "This kind of animal is a thousand times smaller than the eye of a large louse." This is hardly a very useful estimate. Curiously, the size of an eye of a louse is quite constant, so Leeuwenhoek had at least a constant standard of comparison. At that time, the cheese mite (a very small fruit fly) was "obviously" the smallest creature, so Leeuwenhoek said, "Bacteria are to a cheese-mite as a bee is to a horse." But after all, how does one measure a creature only half a micron across, when one's microscope cannot distinguish two dots a quarter of a micron apart?

The most obvious thing to do is to compare the size of bacteria with something whose size we know. If, for example, we were to mix a bacterial suspension with a suspension of particles of known size and then look at both under the same microscope, we could tell whether the bacteria were larger or smaller or the same size as the known particles. Today, we have grids in which the lines are a known number of micra apart (Fig. 2-1) and these can be used to standardize an eyepiece micrometer so that we can accurately measure bacterial size. But even without possessing such grids, called stage micrometer and eyepiece micrometer, you may readily standardize your microscope with simple things present in the laboratory. You may determine the width—and by $\pi d^2/4$, the area—of the low-power field by counting the number of lines of a piece of graph paper required to go across the field. For a particular microscope, a graph paper was used having 20 lines to the inch (= 1.22 mm between lines). It took 1⅓ lines to cross the field with the low-power lens, and hence the field diameter was 1.63 mm. A printed photograph from a magazine was examined under low power, and the dots which make up the printed photo were counted. It took 11 dots to cross the field; hence each dot from center to center was 1.63/11 = 1.5 mm = 155 μ. Two dots were required to span the high-dry field, whose diameter is thus 3.0 mm or 300 μ. The distance between the center of one dot to the center of another was just sufficient to span the oil-immersion field; the diameter is thus 1.5 mm or 150 μ. Thus even with very simple tools it is possible to obtain a reasonably accurate estimate of the size of objects seen in the microscope.

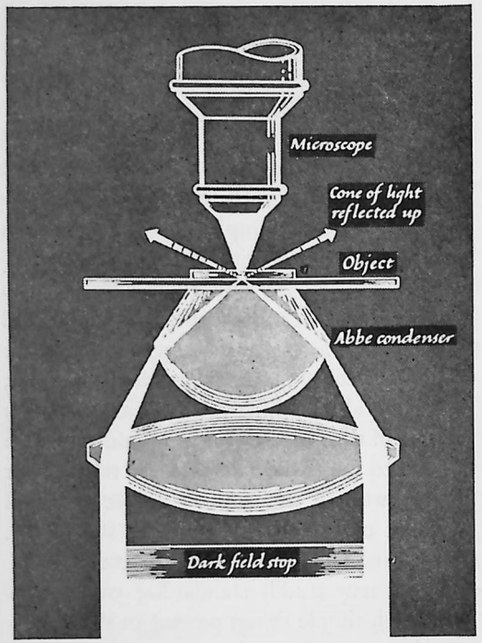


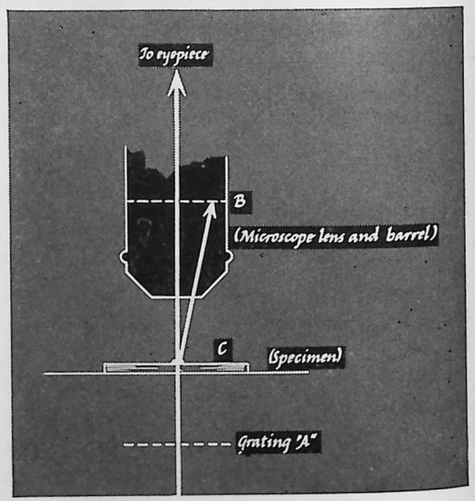
FIG. 2-2. *The dark-field system.*

Other Optical Tools

Three modifications of the compound microscope are in common use in bacteriological laboratories, although you may never have occasion to use them in general bacteriology. These are the dark-field system, the phase microscope, and the fluorescent microscope. Each of these will be discussed separately, but they all employ essentially the same compound microscope that you will use in the laboratory, with additional modifications.

The **dark field** system employs a dark field condenser with the result that the light is directed horizontally toward the specimen and not directly into the microscope lens (Fig. 2-2). Under these circumstances, when light strikes an object in its path, it is reflected into the lens of the microscope. It will be seen as a light spot in a dark field; hence the name "dark field." Now, light spreads in a spherical fashion and when reflected from a point source, as shown in Fig. 2-2, it will appear to the eye to be much larger than it really is. Thus, when this is magnified by the lens system of the microscope, a rather great magnification can be achieved—so great, indeed, that even colloidal particles can be "seen." With dark field, you can observe the light reflected from structures on the surface of bacteria which are too small to be seen otherwise—flagella, for example. Of course, you only see the light that they reflect and frequently all this tells you is that the object is there. It doesn't tell you much about what it looks like, just as sunlight reflecting from the surface of water tells you only that a reflecting surface is present and nothing about its nature or depth. Hence, dark field is not especially useful for determining details of structure but is mainly used to determine whether there are small structures present.

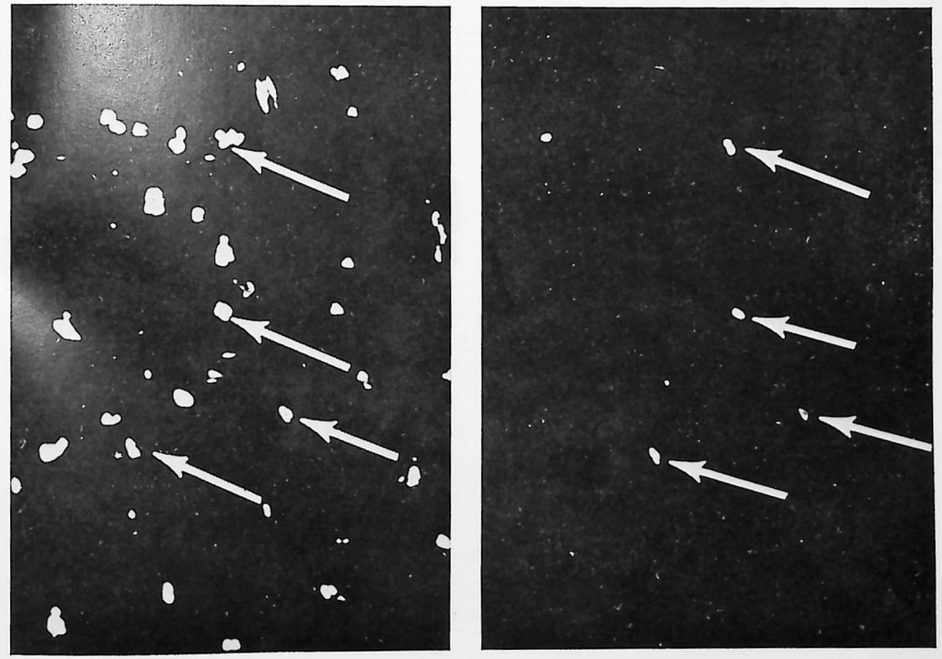
FIG. 2-3. *The principle of the phase microscope. Although the same result is achieved in a slightly different fashion, the principle is illustrated here. Light passes through a hole in grating A, which is lined up so the same ray passes through another hole in grating B. If the ray is bent, it will not reach the eyepiece or will reinforce the light coming through another hole in B.*



The **phase microscope** is a little more complicated to explain, but one may think of it as acting in the following fashion, as illustrated in Fig. 2-3. Light coming through grating (or screen) A continues in a straight line, on through grating B. But if in the region C it should pass through material of a different density, it would be bent and would not pass straight on through, but would either hit the upper grate B or reinforce another ray. Thus, wherever there was a change in density—a cell wall, a membrane, or a granule—one could see different light intensities in the eyepiece. In this way one can frequently see structures within living cells not otherwise visible. The gratings A and B may be put into an ordinary microscope to convert it into a phase microscope.

The **fluorescent microscope** depends upon the fact that certain substances give off visible light when placed in ultraviolet light. If the lenses of a microscope are glass, as most are, ultraviolet light will not penetrate well and what does may be removed by a glass "filter" placed in the eyepiece. If a specimen is illuminated with ultraviolet light, one will see noth-

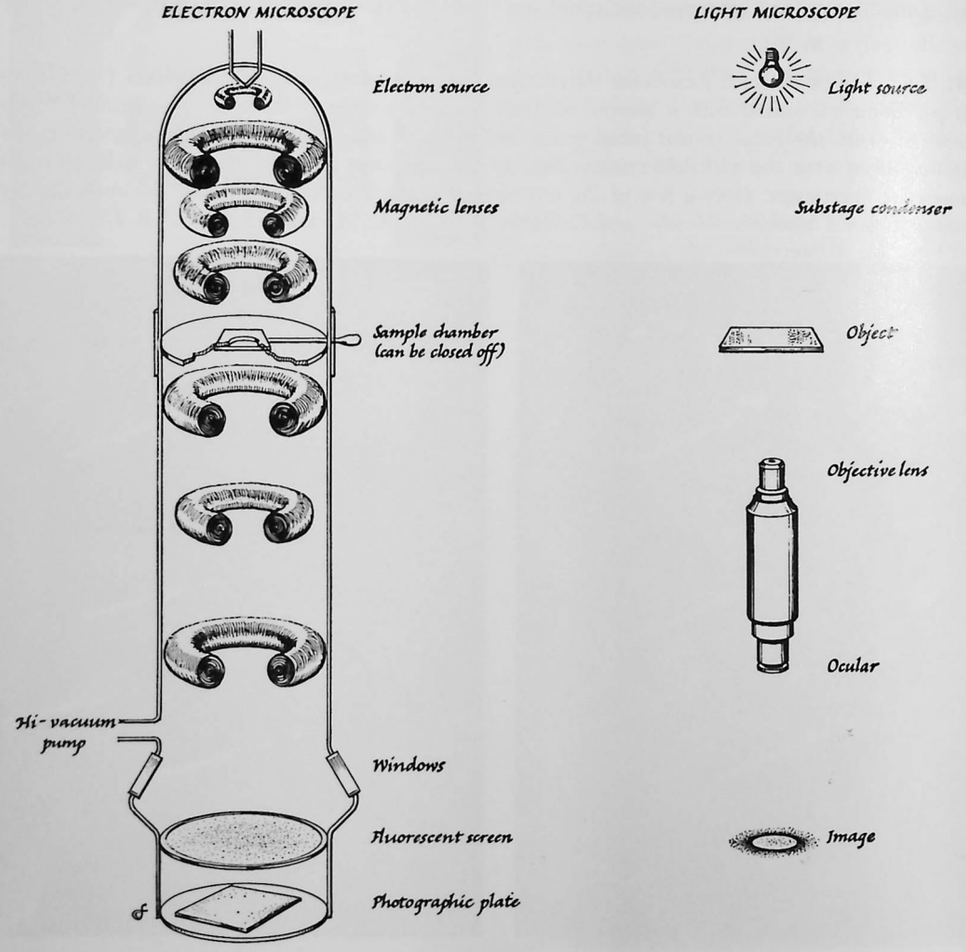
FIG. 2-4. *The use of a fluorescent microscope. This is a photograph of a bacterium* (Pseudomonas pseudomallei) *mixed with a specific antibody treated to make it fluoresce. The antibody combines only with the* Pseudomonas pseudomallei *and not with other organisms. The photograph on the left was taken with the dark-field system; that on the right was taken of the same slide with the fluorescent microscope. Only a few of the organisms are the* Pseudomonas*—these are indicated by arrows. [From Thomason, Moody, and Goldman,* J. Bacteriol., **72,** *362 (1956). S.A.B. LS-361.]*



ing in the microscope except where fluorescence has taken place and visible light, rather than ultraviolet light, is coming into the microscope. While relatively few natural substances are in high enough concentration to be seen by this method within cells, many fluorescent dyes may be used to reveal structures of bacterial cells (Fig. 2-4).

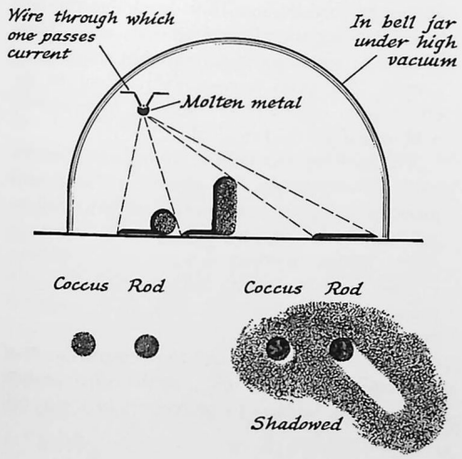
ELECTRONIC MAGNIFICATION. Two fundamental types of electronic magnification are possible. One of these is the electron microscope; the other, the television microscope. The latter is not used widely in bacteriology so we will mention it only briefly later, but the **electron microscope** is so widely used that it requires some understanding. This does not mean that one needs to know the electronic circuits required, but merely that one understand the principle. As illustrated in Fig. 2-5, a stream of electrons can be focused by magnetic lenses to pass through an object and then

FIG. 2-5. *Diagram of the electron microscope compared to a light microscope.*



refocused upon a fluorescent screen or photographic plate to form an image. With this arrangement, magnification of 200,000 diameters is possible, and resolution of some films is so high that further accurate enlargement of the photographs is possible. However, the electrons can only pass through a high vacuum and hence the entire system must be under high vacuum, specimens and all. This means that only dry materials may be examined. They must be mounted on very thin films of cellophane or collodion (usually supported on a wire screen). In such pictures a measure of the electron-scattering power of the object is obtained and the relation of this to the composition or function of the object seen is sometimes obscure. Occasionally "shadowing" may be used to obtain clearer estimates of size and shape. (See Fig. 2-6 for details.)

FIG. 2-6. *Shadowing for the electron microscope. The sample is exposed in high vacuum to metal vapors generated by a hot wire. These condense on the specimen. Depending on the angle of exposure one may obtain layers of differing thickness which permit one to judge the height or depth of a structure, and, in fact, sometimes reveal it when it is otherwise invisible.* [*Original sketch, courtesy of Dr. S. Weinreb.*]



A further development in electron microscopy has been observations of sectioned bacteria. They are imbedded in a plastic, which is then cut into exceedingly fine sections and examined in the electron microscope (Fig. 2-7).

The **television microscope** places a television camera at the eyepiece of a compound mircroscope and then projects the image on a wide screen. It may lose some detail, but it frequently enables easier viewing and some magnification. Its use is only beginning.

The Gram Stain

There is one stain known to and used by every bacteriologist. This is the **gram stain,** so named because it was first described in 1884 by a Danish bacteriologist named Gram. It has become so widely used that it no longer requires capitalization so Gram stain can be written gram stain. (The name has nothing to do with the weight unit, the gram.) It would seem that the story of how Gram happened to come across this unusual procedure would be of some interest, but apparently this has been lost to history. Gram describes what he did but not why he did it.

Gram's paper—published in *Fortschrifte der Medicine,* 2:185 (1884)—is, however, interesting enough to quote in part. Gram was working at this time in Friedlander's laboratory and Friedlander was editor of this journal. In the following direct translation from Gram's paper, the Koch-Ehrlich method referred to in the first sentence is the acid-fast stain. (I am indebted to Professor Solotorovsky for providing me with this historical information.)

> A good differential stain for the tubercle bacillus has been achieved with the Koch-Ehrlich method. The need of a comparable method for other bacteria may now be fulfilled by a differential staining method for pneumococci that I have developed in collaboration with Friedlander. The present report extends the method to other bacteria.

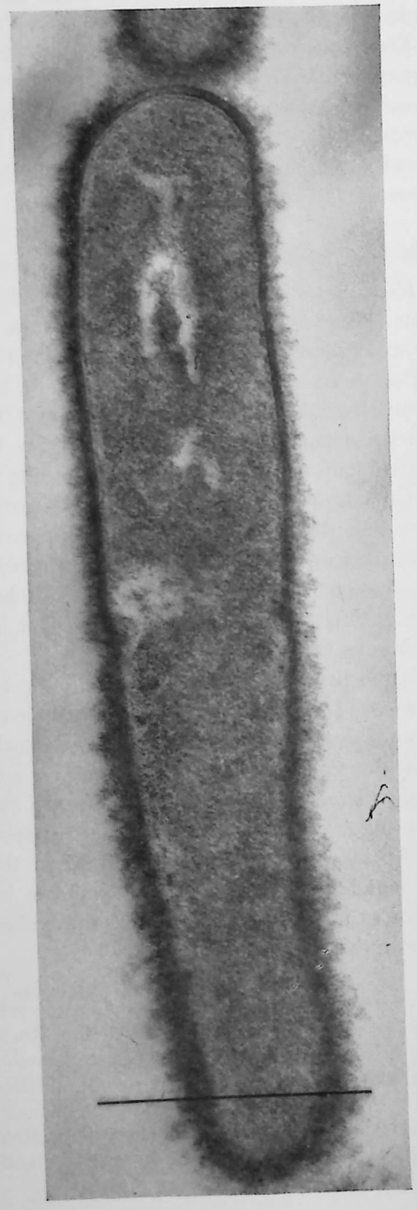


FIG. 2-7. *An electron microscope photograph of an ultrathin section of* Bacillus anthracis. *The organism, after suitable dehydration, has been embedded in epoxy resin; after solidification this was cut into ultrathin sections and photographed in the electron microscope. This is a section longitudinally through the center of the cell. Halfway down the cell there appears to be the beginning of cell division marked by a slight indentation of the cell. The line represents 1 μ.* [*From Roth, Lewis, and Williams,* J. Bacteriol., **80,** 772 (1960). *Courtesy of Dr. R. P. Williams.*]

As already described by Friedlander, the sections are treated with absolute alcohol and then immersed in Ehrlich's aniline gentian violet solution for 1–3 minutes. The stained slides are transferred without rinsing or rapid rinsing in alcohol to an iodine-potassium iodide solution (1 gram iodine, 2 grams potassium iodide, 300 grams water) for 1–3 minutes. The blue-violet color of the section changes to blackish purple-red. The sections are treated with absolute alcohol until completely decolorized and clarified in cedar oil. Bacteria are stained intensely blue and the background tissue is light yellow. The background tissue may be counterstained with Bismarck brown. Such preparations retain their stain for four months. The method is rapid, convenient and also applicable to smear preparations.

Other aniline stains such as fuchsin aniline, and aqueous gentian violet are not satisfactory. Tincture of iodine or aqueous potassium iodide cannot be used because they decolorize the bacteria. Rinsing the stained sections with dilute alcohol or water produces variable staining reactions. The stain is partially resistant to decolorization with 3% solutions of hydrochloric acid or sulfuric acid in alcohol.

The method was applied to organisms associated with croupous pneumonia, pyemia, suppurative nephritis, suppurative arthritis following scarlet fever, multiple brain abscesses, osteomyelitis, typhoid fever, liver abscesses, erysipelas, tuberculosis, anthrax and gas gangrene. [Here follow several pages of observations.]

In closing, I should like to re-emphasize that the aniline gentian violet, unlike other aniline dyes, stains bacteria in preference to tissue cell nuclei.

This method appears to be an advance in bacteriological technique.

[There is then a curious testimonial, as follows.] Addendum of the Editor (C. Friedlander): I should like to note that the Gram method is of outstanding, indeed in many cases, the best of available methods for staining bacteria.

The gram staining procedure is fundamentally the following. The details and concentrations of the materials are not critical and are given in laboratory manuals.

A heat-fixed smear is stained with crystal violet, preferably a solution buffered at a slightly alkaline *p*H. It is then washed in water and treated with a dilute solution of iodine. The next step is to decolorize with alcohol, or acetone-alcohol mixtures. Some kinds of bacteria are not decolorized and remain purple (the color of the crystal violet) and these are called gram-positive. Others lose all their color and, since they are difficult to see, they are usually stained with mild stain, easily distinguished in color from the violet; usually safranin, a red dye, is used. In this procedure, using safranin, gram-negative organisms are red.

It is not the color of the cells which is of any real importance. Rather, the reaction to the gram stain is related to deep-seated differences between organisms. Gram-positive organisms, for example, are inhibited by certain dyes, may form spores, may be inhibited by penicillin, by halogens, etc., and indeed have a cell wall of different composition and nature (see Chapter 3) than gram-negative cells. The gram-positive character is associated with certain bacteria, yeast, molds, streptomyces (described in Chapters 15 and 16); all other cells are gram-negative. The standard "norm" is gram-negative, and gram-positive cells may lose the gram-positive staining character and appear gram-negative. If, however, at any time during its life an organism stains gram-positive, it has a "gram-positive type of physiology." That is, some organisms stain gram-positive when they are young, but as the culture gets older they stain gram-negative; others are negative when young but positive when older. To be sure that a gram-negative organism one sees is not really a gram-positive which has lost its staining ability, one must examine it at various stages and under various conditions. This generally causes no difficulty for an experienced bacteriologist. Indeed, each bacteriologist, after some experience, sets up his own modified procedure for a gram stain; after a while he begins to know how organisms react and how far he may trust his own stains. You, too, will learn the same.

There are two basic facts about the gram stain which you should know. First, you must use crystal violet or gentian violet, which is an impure mixture containing crystal violet. This used to be called "methyl violet" and it was one of the first dyes made by Perkins, the man who discovered aniline dyes. It was (under the name "methyl violet") the first dye used by Ehrlich to color bacteria successfully. Today the term "methyl violet" refers to a different, but related, dye. Other dyes do not work; the first dye must be crystal violet.

Second, one must use iodine. A few other things will work, but so poorly as to be useless. For all practical purposes, the staining procedure must be crystal violet, then iodine. Nothing else will do. To decolorize, one may use alcohol, isopropanol, acetone, etc. The decolorizing agent is not critical and of course it is immaterial what dye one uses for the counterstain. It is only necessary that it be contrasting in color to the violet and not be too intense a stain.

While the real cause of the gram stain lies in the difference in composition of cell wall and protoplasm and in the differing "nature" of the cell between gram-positive and gram-negative cells, just how the stain works to show up this difference is not known for certain.

There are two major theories, with some evidence both for and against each.

1. Chemical theory. In the gram-positive organism the iodine combines with a chemical substance in the cell (or in the cell wall) and ties the crystal violet to this substance. The substance is thought to be an unsaturated fat, which could readily be iodinated, or ribonucleic acid.

2. Physical theory. In the gram-positive organisms the crystal violet enters, followed by the iodine, and inside the cell the two combine to form a molecule so large that it cannot move out again, even in alcohol, in which the dye-iodine compound is soluble. Gram-positive cells which are crushed become gram-negative.

Methods of Obtaining Pure Cultures of Microorganisms

The term **culture** is applied to masses of individual bacteria. A **pure culture** is one in which the cells are all of one kind. The early work in bacteriology was done with impure cultures, in which one kind of bacteria predominated but others were present. For example, souring of milk is caused largely by a gram-positive coccus (*Streptococcus lactis*), which is the predominant organism in a bottle of sour milk; but other organisms (gram-positive and gram-negative rods, for example) are also generally present in smaller numbers. Before the study of bacteriology could be put on a sound basis, it was necessary to have adequate means of preparing and maintaining pure cultures. After all, how could one be sure that the milk turned sour due to the coccus present? Maybe it was the gram-positive rod that was souring it.

The problem actually arose in another form. Koch, during the years 1873–1876, had demonstrated that anthrax could be caused by a large rod-shaped bacterium, which could form spores when it was exposed to air. Moreover, if an animal had a case of anthrax, this bacterium (or one that looked like it) could be found, but rarely, if ever, could this same bacterium be found in healthy persons or animals. But the cultures were not demonstrably pure. Koch couldn't be sure that some other organism, present in smaller amounts, was really causing the disease and this just happened to carry along with the large rod. A culture in which only one kind of organism was present had to be obtained to settle this problem. A pure culture was a vital necessity.

As a matter of fact, in the 1870's and for many decades thereafter, work with mixed cultures in other diseases led to confusion, so that it became important to try to define how one could go about establishing an organism as the causative agent of a disease. These approaches were expressed in what are called "Koch's postulates," as follows.

In order to relate a given organism as the cause of a given disease, one must prove certain points.

1. Find the organism present in all cases of the disease and not present in healthy individuals.

2. Isolate and cultivate the organism in pure culture, demonstrating that it alone is present.

3. Induce the disease in a healthy individual (an animal, if possible) with this pure culture.

4. Demonstrate that the organism is present in the induced disease, and that on reisolation in pure culture it is the same organism that was used to induce the disease.

When one can fulfill Koch's postulates, one *knows* that the organism involved caused the disease. If one can complete only part of the procedure, one knows something, of course, but not as much as if all four provisions could be demonstrated.

In essence, while organisms exist mixed in

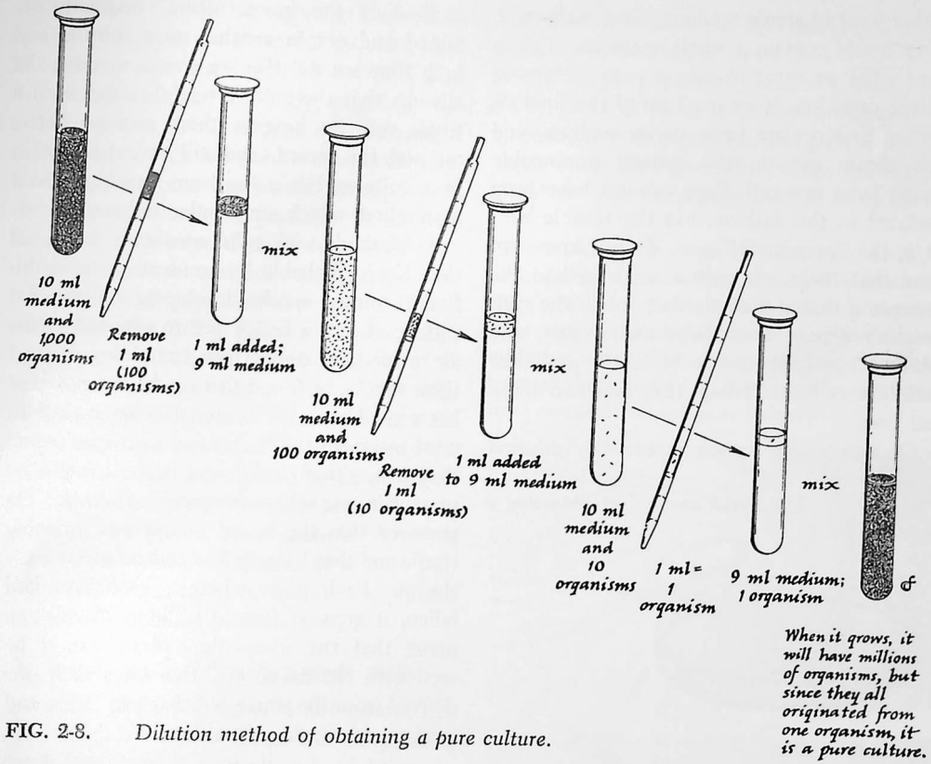


FIG. 2-8. *Dilution method of obtaining a pure culture.*

nature, when one wishes to study their properties one must separate them into pure cultures. The process of preparing pure cultures is based upon the principle that if all the organisms in a culture came from a single cell, they would all be of the same kind. Hence, the methods for obtaining pure cultures are based upon obtaining a single bacterial cell, and allowing it to grow to millions of cells without other organisms getting in.

Methods of Historical Interest

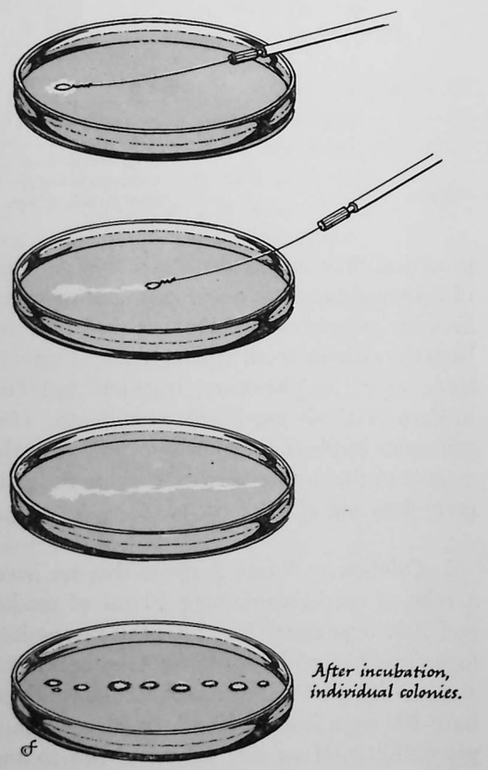
There are some basic methods of obtaining single cells and permitting them to grow uncontaminated, which are no longer in use, having been replaced by simpler methods. However, since most of the older methods are rather primitive, they can be done with a minimum of equipment and are therefore useful to know in case an emergency requires isolation of bacteria without modern equipment. Further, these were the pioneering methods and our modern methods are based upon them. The principles involved are sometimes more clearly evident in the simpler methods, where the gadgetry does not obscure them.

1. *Dilution methods.* Suppose that we have a tube of media containing 10 ml of media and 1000 organisms (Fig. 2-8); this means 100 organisms per milliliter. If we take out 1 ml and mix it with 9 ml of sterile media, we then have 100 organisms in 10 ml, or 10 organisms per milliliter. If we add 1.0 ml of this to an-

other 9 ml of sterile medium, then each milliliter would contain a single organism. This is just what we want to start a pure culture—a single organism. If we use 1 ml of this final dilution to inoculate some sterile medium, and we obtain growth, this growth presumably came from one cell. Pure cultures have been isolated in this fashion, but the trouble with it is the "presumably"—we do not know for sure that there was only a single cell in the inoculum that started the last tube. The cells might not have been distributed evenly, and perhaps two happened to be in the particular milliliter we took. Perhaps they were two different ones!

Of course, we could repeat the dilution method on the "pure culture" originally obtained and obtain another pure culture, and each time we did this we would increase the chances that the culture was obtained from a single cell. So long as there was no better method, this served (and still serves) to obtain pure cultures, but it has been largely replaced by methods which are simpler and surer.

FIG. 2-9. *The streak method of obtaining a pure culture.*



2. *Methods with solid media.* It is related that Koch, probably by accident, noticed different colored specks developing on the flat surface of half a boiled potato exposed to the air in his laboratory. Upon examining each of these specks, he found them to be composed of bacteria. They were bacterial colonies, but in most instances the bacteria in each speck were the same, although different bacteria might be present in one colony compared to another. He reasoned that the boiled potato was probably sterile and that bacteria had fallen onto it from the air. Each place where a bacterium had fallen, it grew; it formed millions of cells, so many that the mass—the colony—could be seen with the naked eye. But since each was derived from the single cell that had fallen and grown, each was a pure culture.

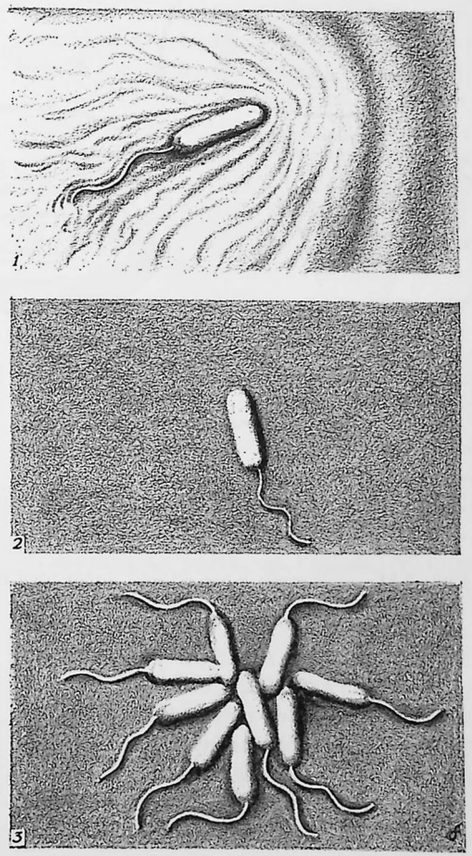
Now how to make this useful? One could, of course, spray a suspension of bacteria in the air and let it settle, but how could one tell if an organism came from the spray or from the air itself. In any event, one would not want to spray the air with disease-causing organisms. Two simpler methods were devised in Koch's laboratory by Loeffler and Gaffky, who later became famous bacteriologists. The first was to **streak** the potato or some other suitable *solid* medium with the organism. This, in fact, is a "dilution" method; Fig. 2-9 shows how it works. At the end of the streak isolated colonies would appear, derived from originally isolated single cells; thus pure cultures could be obtained. This procedure is the direct ancestor of our "streak plate" methods of today.

The second method developed in Koch's laboratory was based on a somewhat different idea. If bacteria were distributed in a liquid, and the liquid could be suddenly solidified, the solid condition would prevent the remixing of the bacteria which went on in the liquid state. Each separate organism would be trapped. If there was food and the possibility of growth, then each such trapped single cell would grow up to be a colony, and essentially each a pure colony, since all organisms were derived from a single cell (Fig. 2-10). For this purpose, Koch used gelatin, which was liquid at above 15°C but would solidify below this point. This method worked quite well except that the materials had to be incubated in the cold and the gelatin was itself decomposed by many organisms. However, this is the clear and direct ancestor of our present **pour plate** methods. It required only the development of a more suitable solidifying agent to be *the* most important method of obtaining pure cultures.

Digression on Solidifying Agents: Agar

Today's solidifying agent is not gelatin but agar, which is the basis of much of our present-day bacteriology. It controls and conditions to a very large extent what can and what cannot be done. It is related that in 1882, Frau Hesse, the wife of a graduate student in Koch's laboratory, suggested agar-agar, as it was called in those days. This was a material derived from seaweed and was used in jellies and other foodstuffs as a thickener, particularly in the Dutch East Indies, where Frau Hesse had lived. A 1–1.5% solution of it has most unusual properties. It melts at 100°C but it does not solidify until it is cooled to below 42–45°C. The exact temperature of both melting and freezing (= solidifying) depends to some extent upon the concentration of the agar, concentration of salts, etc. It further happens that a temperature of 42–45°C can be tolerated by most organisms, at least for a few moments. The procedure, then, is to place the agar media in a boiling water bath—that is, raise it to 100°C—and melt the agar. One may then cool it to just about 42–45°C where it still remains liquid—inoculate it with organisms, and pour it into a plate, where the temperature drops quite rapidly. Very soon the temperature of the plate is

FIG. 2-10. *The principle of a plate method for obtaining a pure culture. An organism, isolated from others in a fluid media is suddenly trapped when that medium solidifies and the organism can no longer move freely. It can, however, grow, but since all the organisms at this particular spot came from one cell, this is a pure culture.*



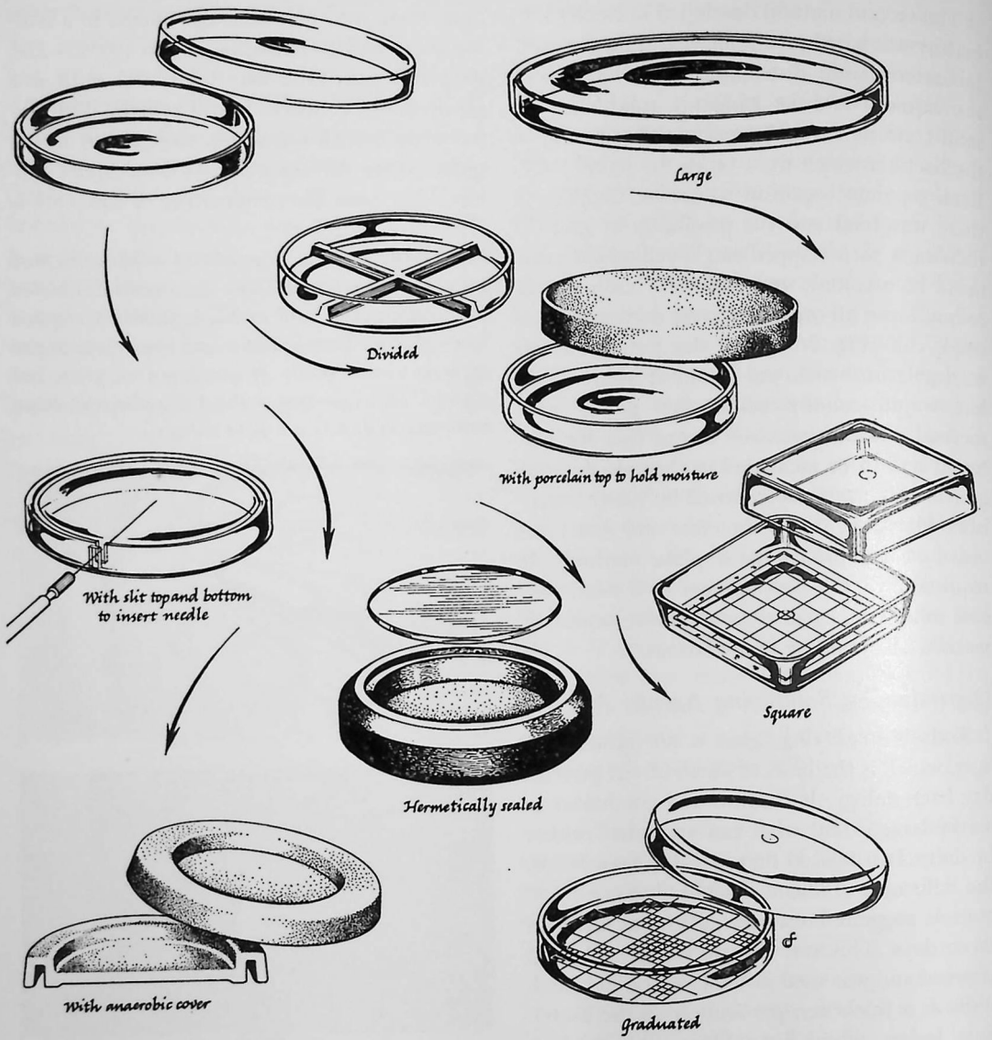


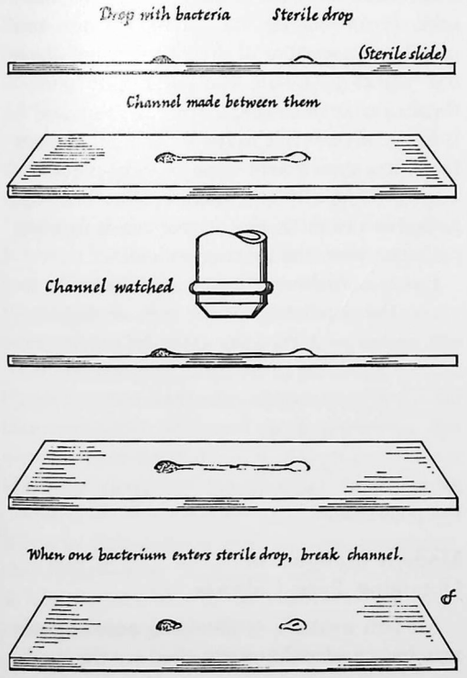
FIG. 2-11. *Various types of petri dishes used in microbiology.*

considerably below 45°C, and the agar solidifies and traps the organisms which are suspended. Such a plate can then be incubated at any temperature one desires, even as high as 80 or 90°C without the agar remelting. This use of agar has become so widespread that it is the basis of virtually all the plate and streak methods used in modern bacteriology.

The petri dish (Fig. 2-11) was invented in 1887 by another German bacteriologist, named Petri. Up to this time Koch had used photographic slides or any other flat piece of glass, which were subject to considerable contamination. Petri devised the covered glass dish, which is more or less standard in today's laboratories.

The agar itself which is used in microbiological media is relatively inert and is attacked by very few organisms. Its primary purpose in virtually all media is for solidification. A variety of other solidification agents have been tried—silica gel and many other types of polysaccharide materials—but none has been as convenient or as widespread as agar. It is, of course, possible that we are still missing some organisms which exist in nature but which are unable to isolate or cultivate because they cannot stand the temperature of agar at 42–45°C. This seems unlikely, however, since we can prepare streaked plates without subjecting the organism to these temperatures. With the development of modern plastic materials and modern polymers, it is probably time for some further developments in the field of solidifying agents, but since agar was put to this use in 1882, it has been and will continue to be for a long time the mainstay of solid bacteriological media.

FIG. 2-12. *Spallanzani's method of obtaining a single cell.*



The petri dish, as we have mentioned, is a convenient method of preparing solid media and in a condition where one may observe the growth that appears on it. For many years, petri dishes were made of glass and virtually all were sterilized by heating in a dry oven rather than sterilizing in the autoclave (see p. 225). The reason for this was primarily that the earlier petri dishes were made of softer glass; in the course of autoclaving two or three times, the glass became cloudy and one was unable to see through it. Sterilizing in a dry, hotter oven prevented this, presumably because the outer surface of the petri dish tended to melt very slightly and to keep the glass clear. Today it is possible to obtain plastic petri dishes which are prepared aseptically; that is, they are sterile and sometimes they even contain the media that one may wish to use. These are, so far, not autoclavable, but are simply discarded. Petri dishes, in general, are incubated upside down; this is to prevent condensed water from dropping on the medium and causing the organisms to smear together.

Single-Cell Methods

If one could somehow obtain directly a single cell, pick it up with a fine—a *really* fine—tongs, and then put it into a medium in which it could develop, one would have a pure culture. Today there are such tongs. Back in 1790 there were none, yet in that year Spallanzani obtained a bacterial cell all by itself and "grew" it; at least he watched it divide. The method, like most good methods, was incredibly simple. As illustrated in Fig. 2-12, a drop containing the bacteria was placed on a flamed, and thus sterilized, slide. Close by, a drop of sterile media was placed and a narrow channel made to connect the two drops. Spallanzani focused his micro-

scope on this channel, and watched and waited until a bacterium, a single bacterium, entered the channel and went into the sterile drop. He then brushed away the connecting channel, and there was a single bacterium all by itself. It seems incredible, indeed, that such single cells were obtained so long ago, when even a hundred years later the direct single-cell methods were so difficult.

ENRICHMENT METHODS; MEANING AND IMPORTANCE. One would have a better chance of obtaining a pure culture and of isolating a given bacterium if it is greatly predominant in the population. It would be almost impossible, by any methods, historical or modern, to isolate an organism which comprised perhaps one in a thousand or even one in a hundred. But if one could change the relative proportion of this organism to the rest of the population, if one could increase or enrich this population with the organism one desired, one would improve one's chances of isolation enormously.

Enrichment could be achieved by permitting growth under conditions favoring the organism you wish, so it grows faster than others; thus there would be more of it in proportion to the others. If one had a medium on which only one organism would grow, and others would not, then one could obtain a pure culture without further isolation; but this situation is so rare as to be negligible. Even under the most extreme conditions, the only *sure* way of obtaining a pure culture is actually to obtain a single cell.

Enrichment methods, therefore, have as their objective the alteration of the mixed population to favor the organism one wishes to isolate; this permits the use of methods for obtaining single cells which could not otherwise be applied. A few examples will suffice to illustrate the principles involved.

Suppose one wished to isolate a sulfur-oxidizing organism from nature; or, for sulfur, substitute phenol, gasoline, or corn cobs, etc. Now soil, or water, or other places in nature may contain one organism oxidizing sulfur (or phenol, etc.) for every 100,000 organisms doing other things. If, however, we inoculate a medium containing sulfur (or phenol, etc.) as the sole or principal source of energy, only the organisms capable of oxidizing sulfur (or phenol, etc.) can grow; hence they will become more predominant. One can use several enrichment steps to build up the population one desires.

There are in nature certain organisms which grow at 60°C. They are called **thermophiles.** (See p. 126.) One might enrich for these by carrying out the incubation at 60°C. Since spores are somewhat resistant to heat, one might enrich for spore-formers by giving successive inocula a heat treatment. Organisms living in the intestinal tract, where bile lowers the surface tension, are capable of growing in solutions of low surface tension, whereas many other organisms are not. In many cases one may have a mixture of organisms from which one wishes to isolate the pathogenic ones—those capable of causing disease. In this case it is frequently possible to use the animal or plant to achieve enrichment. One inoculates the animal or plant with the mixture; since only the pathogen can grow, and so one can isolate the pathogen from the diseased animal.

Briefly, enrichment serves as a device to increase the population of the type of organism one wishes so it becomes easier to isolate. Enrichment consists of inoculating a suitable medium and so controlling the chemical and physical environment to favor the organism one wishes, and repeating this process until the organism one desires is a predominant part of the population.

Modern Methods of Obtaining Pure Cultures

Modern methods of obtaining pure cultures may be considered in parts. First are the methods in common everyday use by the majority of bacteriologists; these are based on the use of solid media, generally containing agar. The others are methods used for special purposes. We shall separate this discussion on this basis into "common methods" and "special methods."

Common Methods

These methods are based upon the principle that a solid medium is capable of separating, and keeping separate, individual cells of bacteria, thus permitting pure colonies or pure culture development from single cells. Agar has the unique property that a 1.5% solution melts at 100°C, but does not solidify until 42–45°C. This property is utilized in two ways: (1) the streak plate and (2) the pour plate.

1. The **streak plate.** A petri plate or other suitable device containing a suitable solid medium—generally an agar medium which has been allowed to solidify—is "streaked" in any of a variety of fashions (illustrated in Fig. 2-13). This is, in essence, a dilution, and eventually individual cells are isolated (as earlier shown in Fig. 2-9) from which pure colonies develop. The pure culture may be preserved by "picking" it or "fishing." This consists of transferring a bit of the colony with a sterile needle to a tube of sterile medium in which it is capable of growth (Fig. 2-14). This method has the advantages that it is relatively simple, quick, takes little apparatus or time, and works effectively in most instances. It has the disadvantage that one is really not quite sure that each isolated colony came from a single cell; certainly the chances of having two cells, perhaps different, form a single colony is higher than in the pour plate method. Still, one may overcome this objection by "replating" the pure culture as often as one likes so the chances of a mixed colony become negligible.

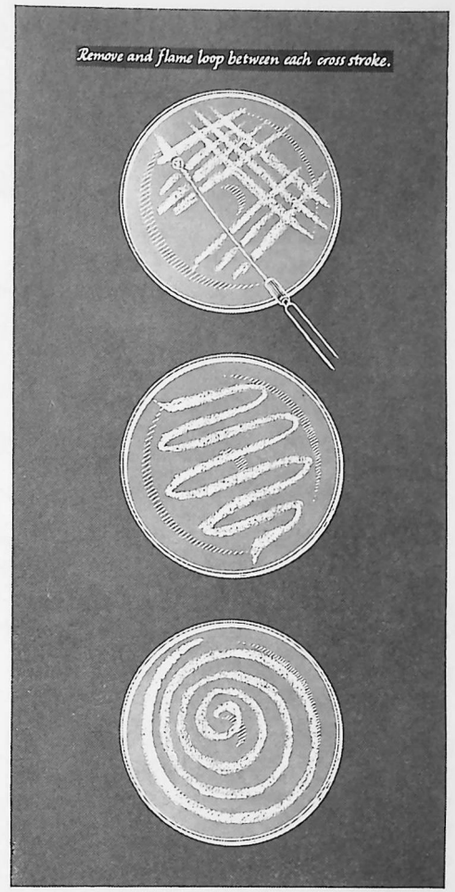


FIG. 2-13. *Three of several methods of streaking plates.*

2. The **pour plate** method. Because of agar's properties, one can place a mixture of bacteria into liquid agar at 42–45°C, disperse them throughout, and quickly cool to solidify the medium. After solidification the plate may be incubated at any temperature one chooses, although most bacteria do not grow above 75°C —in fact, few grow above 45°C. The single-cell organism swimming in the fluid medium is suddenly trapped and held in position when the medium solidifies. When the single cell

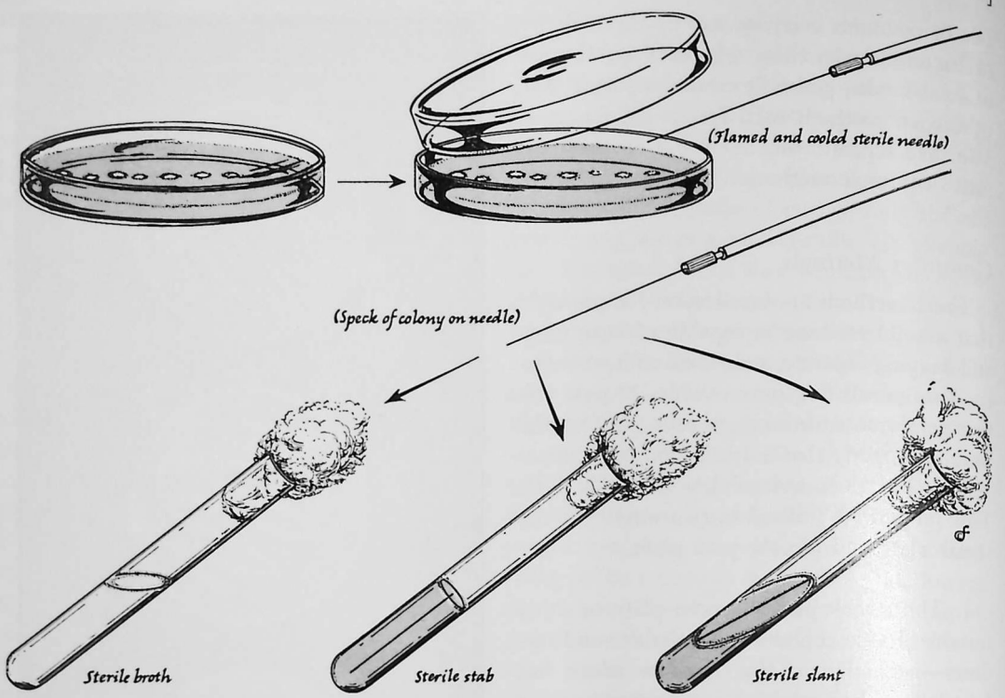


FIG. 2-14. *Picking a colony.*

grows, it produces a colony which can be picked off to other media as a pure culture. The agar, while it prevents the free movement of bacteria from one spot in the medium to another, is soft enough that it does not prevent growth. As the cells divide and the colony expands they just push the agar away.

In order to obtain a pure culture under practical conditions, the colonies must be far enough apart so that they do not touch each other during growth. Otherwise organisms derived from two cells would mix and one could not be sure that one had a pure culture. Hence one needs isolated colonies, each one a good distance away from the other, for pure culture isolation. This dilution is achieved either by "loop dilution plates" (Fig. 2-15) or by "dilution" plates. The purpose of these dilutions is to obtain few enough organisms on the plates to ensure separated colonies.

It is always possible that a given colony arose, not from a single cell, but from two or more cells—a **clump**—which happened to be close together when the medium solidified. A culture derived from such a clump would not be pure, but one can eliminate this possibility by **replating** the pure culture. On serial replating, it becomes less and less likely that the particular colony picked always arose from two or more cells. The main advantage of the pour plate method over the streak plate is that one is relatively surer that the colony came from a single cell. Further, it is easy to put this method on a quantitative basis. By knowing precisely how much one has diluted the cells, and by counting the number of colonies produced, one can quantitatively determine the number of

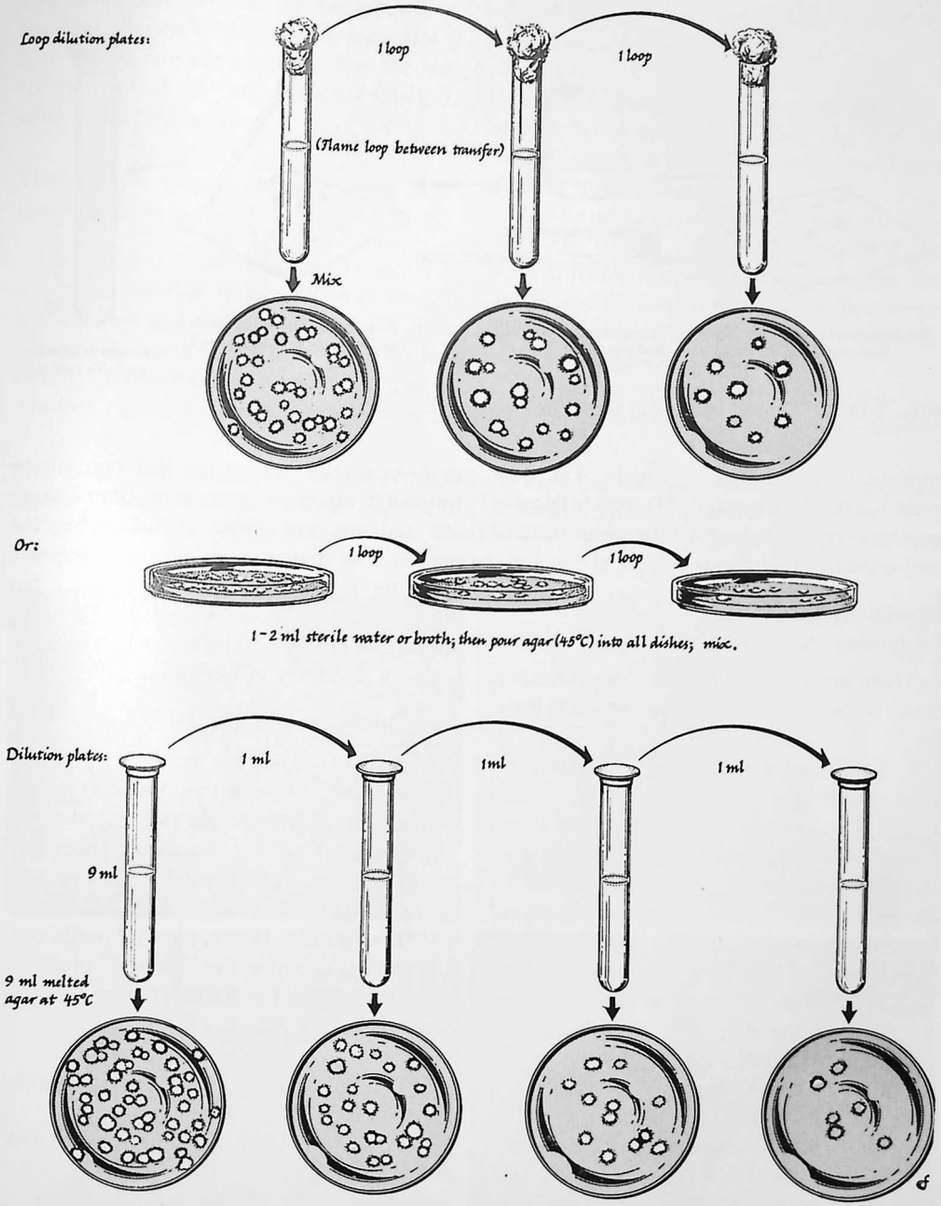


FIG. 2-15. *Pour plates; loop dilution and dilution in water.*

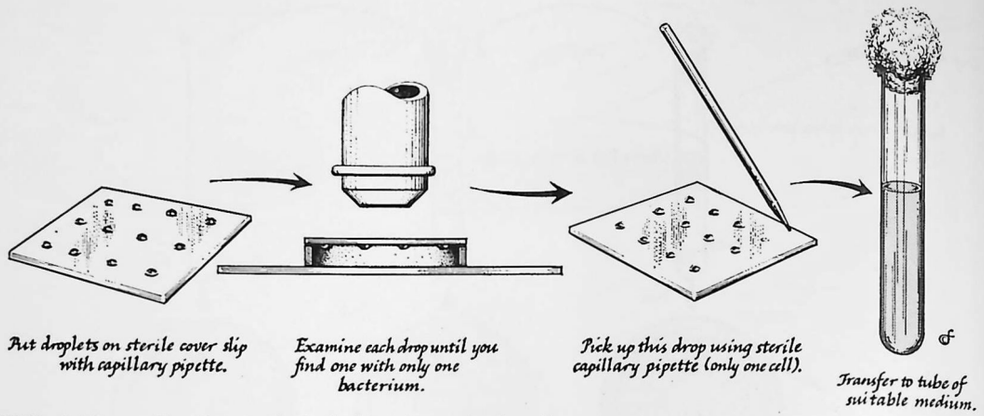


FIG. 2-16. *Droplet method of obtaining a single cell.*

organisms in the original sample. The pour plate has the disadvantage of being a little more cumbersome and taking a little more material and apparatus than the streak plate.

Special Methods for Obtaining Pure Cultures

These are all single-cell methods. One separates a single cell and then permits it to grow.

1. The **droplet method** requires relatively little apparatus. With a sterile pipette drawn to a capillary, put several small drops of a suitably diluted culture medium on a sterile glass coverslip, as shown in Fig. 2-16. Sometimes India ink or a dye is added (being first rendered free from living bacteria) in order to see the bacteria better. One then examines each drop until one finds one containing only one organism. Remove this drop with a sterile capillary pipette to fresh medium and so start a culture with a single cell. Obviously, this method is a little bit tricky and tedious, and the possibility of contamination is also relatively great.

2. Micromanipulators (Fig. 2-17) have been built which permit one to pick out a single cell and transfer it to sterile media. The details of operation are evident from the figure. The advantages of the method are that one can be reasonably sure the cultures come from a single cell and one can obtain strains within the species. The disadvantages are that extensive apparatus is required—not to mention a fair degree of technical skill. Both types of single-cell methods are relatively slow and tedious and have the possibility of contamination.

Proof of Purity of Cultures

Assuming that one has isolated a pure culture, how does one know that it is pure? By definition, a pure culture is one in which the cells are all of one kind, but it does not necessarily mean that they are all identical; some may be young, some old, for example. But it does mean that they are alike. Hence, proof of purity consists of demonstrating the "likeness" of organisms in a culture, and likeness is based on certain criteria.

1. The organisms look alike microscopically and stain in the same fashion.
2. When plated, the colonies formed all look alike.
3. Streaks, stabs, etc. are uniform.
4. Several isolated colonies do the same thing, i.e., ferment the same sugars, and so on.

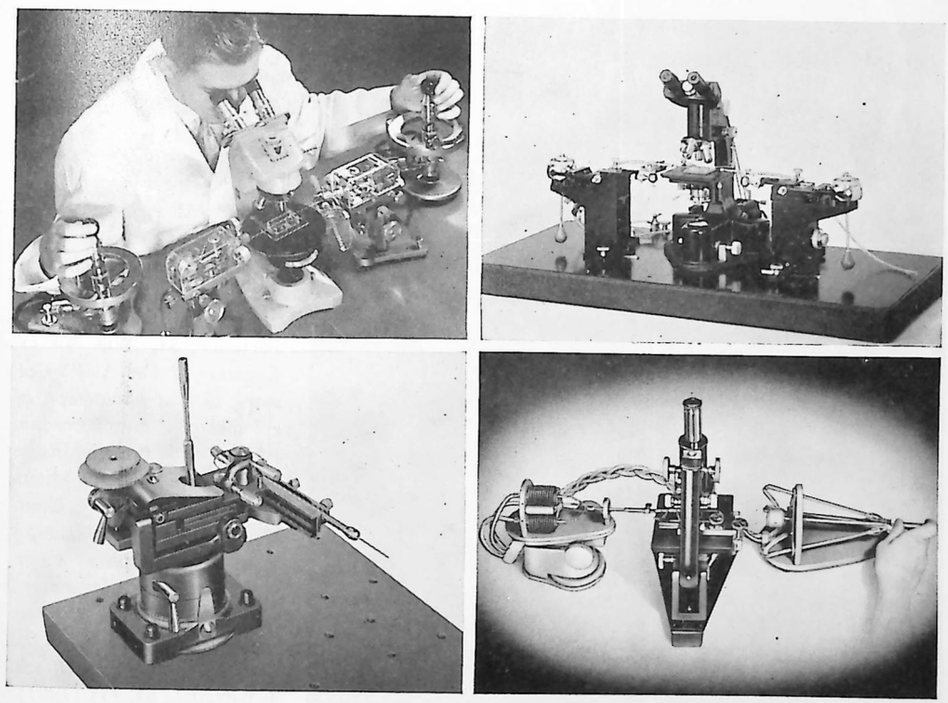
One problem faced by the bacteriologist in assessing likeness is that all the organisms in pure culture are not identical. But the basic criterion is that they are capable of doing the same things.

Methods for Characterizing Pure Cultures

Later discussion of taxonomy (Chapter 7) will point out that various different *kinds* of bacteria exist and that one of the functions of a bacteriolgist is to be able to tell one type from another. To do this, the bacteriologist first determines certain simple things which permit him to tell, at least roughly, what kind of an organism he is dealing with.

1. The nature of the colony on agar—primarily the surface colony. (See Fig. 2-18 for several colony types.) Very smooth colonies usually indicate capsules.
2. The gram stain, usually made on cells from the surface colony. In addition to the gram reaction, this stain will show the size, the shape, and the general character of the individual cell. Gram stains will frequently give indications of spores and capsules.
3. The nature of growth in broth, i.e., whether the organism grows diffuse, as a **pellicle**

FIG. 2-17. *Micromanipulators. Upper left, two de Fonbrune micromanipulators in use. (Courtesy of Aloe Scientific Company.) Upper right, a Leitz micromanipulator. (Courtesy of E. Leitz, Inc.) Lower left, an Emerson micromanipulator. (Courtesy of A. H. Thomas Co.) Lower right, the Cailloux micromanipulator. (Courtesy of C. H. Stoelting Company.)*



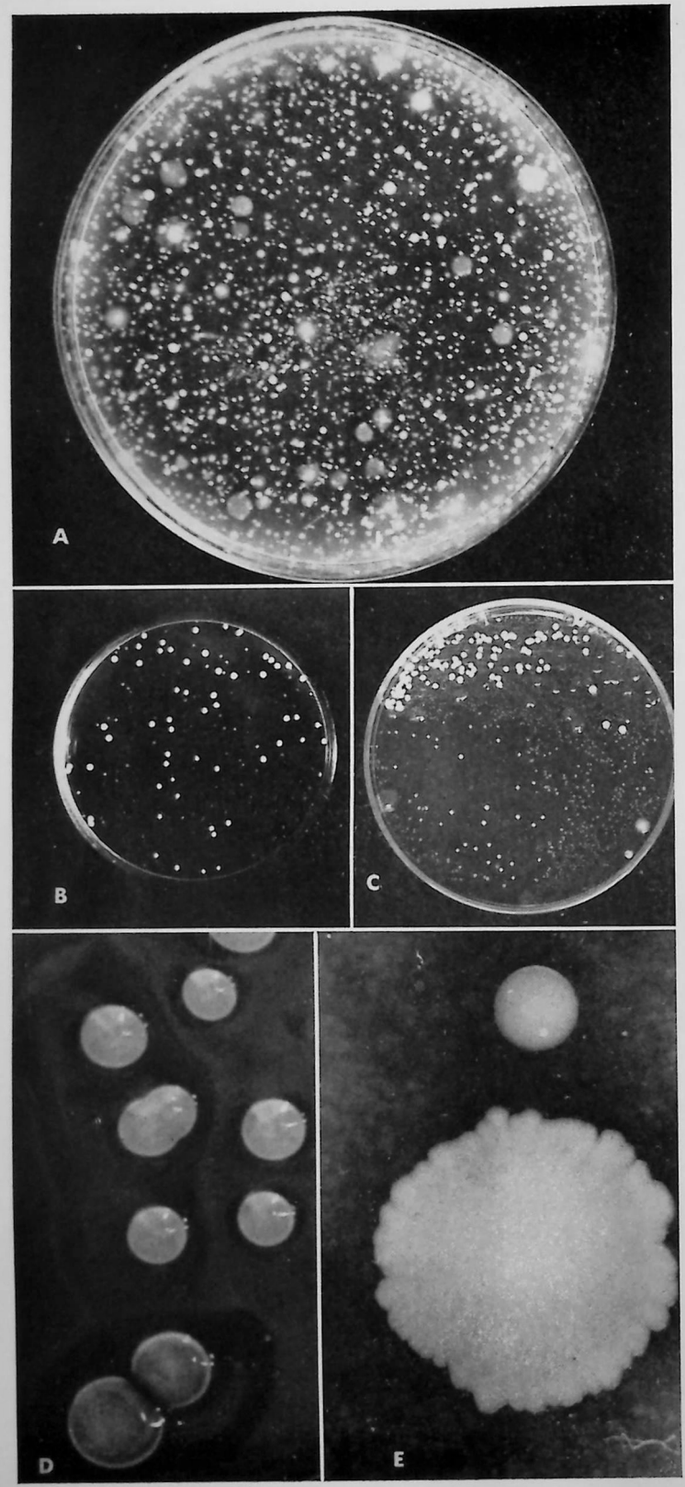


FIG. 2-18. *Colony types. A is a photograph of a plate exposed to a violent sneeze. Note the various types of colonies developed.* [*From Jennison, ASM, LS18.*] *B is a surface culture of* Aerobacter (*large colonies*) *and* Shigella. *Note that the colonies are easily distinguished. In subsurface colonies the two organisms have an identical appearance.* [*From Hentges and Fulton, J. Bacteriol.,* **79,** *457 (1960). Courtesy of Dr. D. J. Hentges.*] *C is a plate of 10% sucrose agar showing varied types of colonies. All except the large, gummy ones can be eliminated from such a plate by the addition of sodium azide.* [*From Mayeux and Colmer, J. Bacteriol.,* **81,** *1009 (1961). Courtesy of Dr. A. R. Colmer.*] *D is a photograph of colonies of a myxobacterium which attacks the agar.* [*From Veldkamp, J. Gen. Microbiol.,* **26,** *331 (1961). Courtesy of Dr. H. Veldkamp.*] *E is a photograph of a normal colony (the large one) of* E. coli *and a small colony variant, both growing on the same medium.* [*From Hirsch, J. Bacteriol.,* **81,** *448 (1961). Courtesy of Dr. H. M. Hirsch.*]

(floats on top), or as only a sediment. A wet mount from broth is used to determine motility.

4. A **slant culture.** The growth of a streak on a slant culture varies considerably in apperance from one bacterium to another.

5. A **stab or shake culture** (Fig. 2-19) indicates something of the requirements for air and oxygen.

These five simple determinations tell one a great deal about the nature of the pure culture one has isolated and permits one to make a fairly accurate estimate as to its nature, which supplementary criteria can later confirm or adjust.

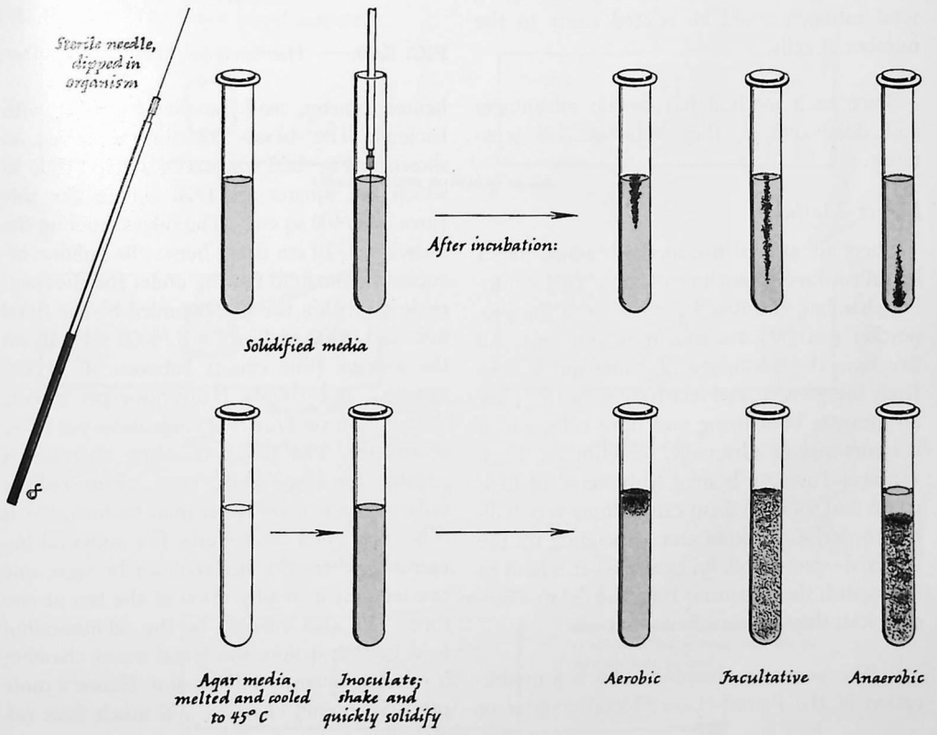
Maintenance of Cultures

Once a pure culture is obtained, one generally wishes to keep it, sometimes for years. Methods for doing this are discussed in Chapter 4 (pp. 112–120). For the present, let us merely say that a culture may be transferred every few days from one slant to another or from one stab to another.

Methods for Determining the Numbers of Microorganisms

There is an array of methods for determining the number of microorganisms in a given sample and these generally apply to either deter-

FIG. 2-19. *The value of stab and shake cultures in determining the oxygen requirements.*



mining the total numbers of individual cells, or the numbers of different kinds of organisms present. These methods are based on several general principles.

1. *Direct methods.* One counts visually, with suitable magnification, the numbers and kinds of organisms present in a known (but small) sample of material being studied.

2. *Cultural methods.* After suitable dilution, one permits the organisms to grow. Since each colony came from a single cell, the number of colonies growing is a measure of the numbers of cells in the sample.

3. *Indirect methods.* Instead of measuring each cell, one measures a result of the action of many cells. For example, one might measure the amount of microbial nitrogen. Since each cell has a given quantity of nitrogen in it, the total nitrogen could be related easily to the number of cells.

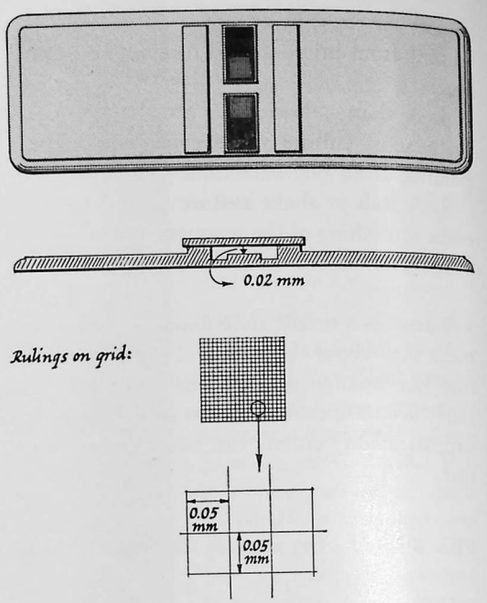


FIG. 2-20. *The bacterial counting chamber.*

Since each method has certain advantages and disadvantages, they are discussed separately.

Direct Methods

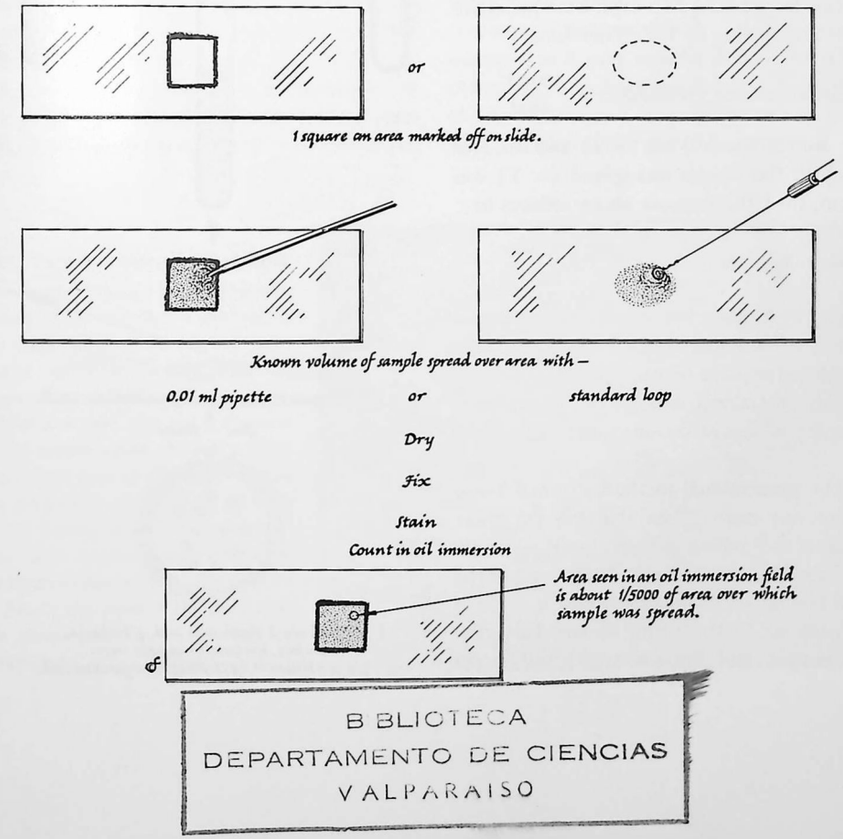
There are at least five methods which might be called direct, but three of them (the counting chamber, the Breed method, and the proportion method) are similar in principle. All five have the advantage of being quick, relatively inexpensive, and relatively accurate. They all measure both living and dead cells, which is sometimes an advantage, sometimes a disadvantage. They are limited to material of high count and some of them can become very tedious to perform. Sometimes, depending on the material—pus or soil, for example—it is hard to distinguish the organisms from the debris. Nevertheless, they have an important use.

1. The **counting chamber.** This is a modification of the Petroff-Hauser blood counter or hemacytometer, made smaller for using with bacteria. The blood counting chamber, as shown in Fig. 2-20 consists of a ruled slide in which the squares are 1/20 cm on the side (area = 1/400 sq cm). The ridges, holding the slide, are 1/10 cm deep; hence the volume occupied by the fluid flowing under the slide and enclosed within the box bounded by the ruled lines is 1/4000 of 1 cc (= 1/4000 ml). If, on the average (one counts between 50 to 100 squares), one counts 1 organism per square, the solution contains 4000 organisms per cubic centimeter. The blood counting chamber is suitable for blood cells, yeast, tissue culture cells, etc., which are larger than bacteria, but it is not very good for bacteria. For under oil immersion, where the bacteria can be seen, one can see only a small portion of the box at one time. The area covered by the oil-immersion field is 0.02 sq mm; the blood count chamber is ruled in squares of 25 sq mm. Hence a more precisely ground chamber and much finer ruling is required for bacterial counting, but the principle is the same. The bacterial counter has squares of an area of 0.0025 sq mm and a depth of .01 mm; therefore each box represents 2.5×10^{-7} ml, and an average of 1 organism per field means a count of 2.5×10^{7} per milliliter. The mechanical precision of preparing such slides is not the only difficulty, however. Bacteria are hard to see unstained; hence, frequently they are suspended in fluid containing stains. They may be motile and run from one square to another when one is trying to count them. However, the counting chamber is useful and is capable of considerable precision.

2. The **Breed method.** In this method, named after the man who worked it out, the sample is spread evenly over a known area, usually 1 sq cm (Fig. 2-21). A sample of the order of 0.01 ml is taken. This may be measured out in a 0.01 ml pipette but a simpler way to do it is to use a standardized loop. If a loop is made, and it is used with the same fluid, it will generally deliver almost the same amount of fluid each time. The exact amount of any loop can be determined by transferring about 50 loopfuls to a weighed dish and weighing the added liquid. A loopful, containing a known amount of fluid (say 0.01 ml), is then spread over the known area (say 1 sq cm) and spread evenly.

The smear thus prepared is allowed to dry, and then is fixed, stained, and counted under oil immersion. The diameter of most oil-immersion fields is 0.16 mm. You can determine the diameter of the field of your own microscope by the simple method given earlier or by use of a standard ruled slide. The *area* seen in

FIG. 2-21. *The Breed count method.*



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the oil-immersion field, πr^2 is thus $3.14 \times 0.08 \times 0.08 = 0.02$ sq mm. One square centimeter (the area over which the sample was spread) is 100 sq mm. Thus there would be $100/0.02 = 5000$ microscopic fields in the 1 sq cm. Each organism we see per field thus represents 5000 in the 1 sq cm, and since we used 0.01 cc of sample, this will represent 5000 per 0.01 cc or 500,000 per milliliter. Thus, each organism seen per field represents 500,000 per milliliter in the original sample.

If the diameter of the microscope was d mm, then the area seen in the field of the microscope is $\pi(d/2)^2$ sq mm. The sample, Z ml, is spread over a field of Y sq cm. The total area occupied by the sample is thus 100Y sq mm. There are $100Y/\pi(d/2)^2$ microscopic fields in the area occupied by the sample. For each organism N seen in a field, there are then $N[100Y/\pi(d/2)^2]$ in the area over which the sample was spread. This can be converted to organisms per milliliter by multiplying by $1/Z$. Therefore

$$\text{microorganisms per milliliter} = \frac{400NY}{Z\pi d^2}.$$

If the sample was 0.01 ml ($= Z$) and the area over which the sample was spread ($= Y$) was 1 sq cm, then the formula above reduces to

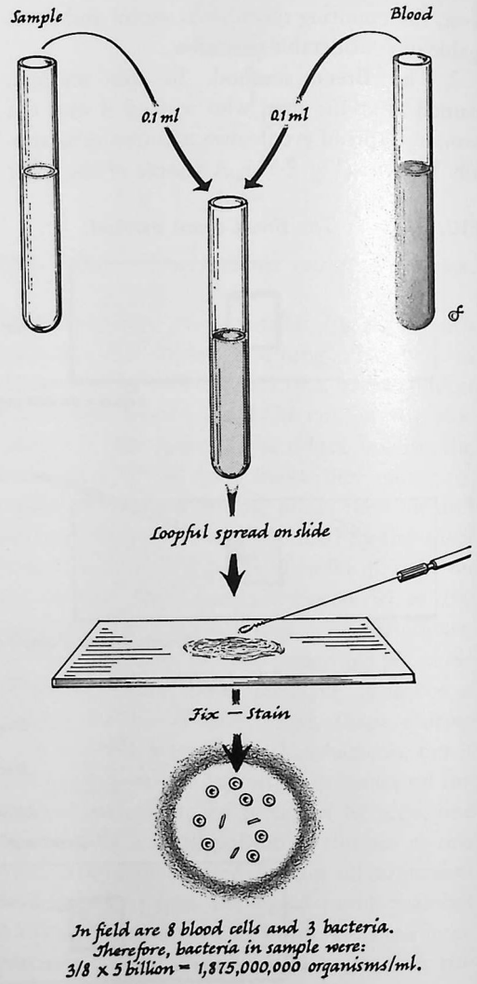
$$\text{organisms per milliliter} = N\frac{4 \times 10^4}{\pi d^2},$$

which is the formula one usually sees in books.

The Breed method is quick and reasonably accurate and requires no complicated apparatus or highly specialized equipment. It is widely used and you should become quite facile in its use.

3. The **proportional method.** Normal blood contains very nearly 5 million cells per cubic millimeter or 5 billion red blood cells per milliliter. This, of course, is easily determined in the Petroff-Hauser or chamber counter if there is any doubt as to the count of any particular blood sample. But the count of 5 billion per milliliter is so common that if one lacks other standards, the count may be readily used. One may, for example, mix 0.1 ml of blood plus 0.1 ml of the suspension of microorganism, or simply equal amounts of blood and the material to be counted, spread them out on a slide, and count the number of microbial cells and the number of blood cells for each field. (See Fig. 2-22.) The microbial count per field divided by

FIG. 2-22. *The proportional method, with blood as a standard.*



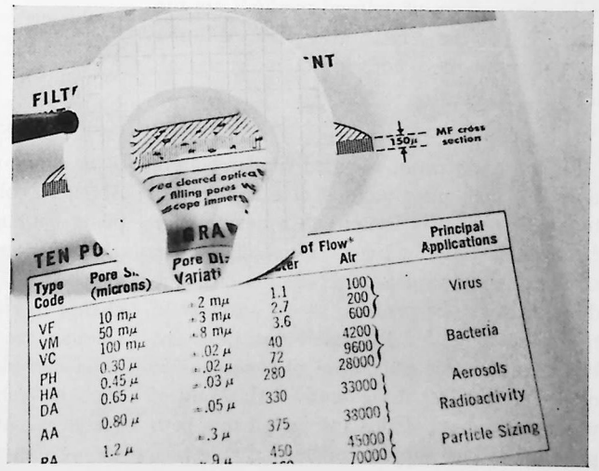
the blood count per field times the number of blood cells per milliliter gives one the number of microorganisms per milliliter. Instead of blood, one may use a standardized suspension whose contents one knows, or any other particles; standardized suspensions of plastic beads, of carbon particles, etc. have been used for this purpose. Under such circumstances, one does not need to measure the amount of sample that one uses. All one needs to do is to have the same amount of one's standard suspension and the bacterial suspension mixed together, and to count an adequate number of fields.

4. **Filtration method.** It is possible to prepare or to purchase thin, cellulose filters with uniform pores in them so that they are capable of "screening out" bacterial particles; see p. 53. We may, for example, filter 10 ml of water through such a filter. The filter, while it is opaque, can be made completely transparent when treated with immersion oil, and then, it is possible to count in the microcope the number of microorganisms found (Fig. 2-23).

5. The Coulter **counter** is an electronic device (Fig. 2-24) which counts individual particles, whatever their nature. Fluid containing the particles passes through an exceedingly small orifice (10–100 μ in diameter). The electrical resistance across this orifice is measured. When a particle comes through, the electrical resistance changes, compared to the suspending fluid without a particle, and this change in resistance is counted electronically. Until very recently the method was most useful for counting red blood cells or yeast, but it has now been increased in sensitivity so that it can handle bacterial suspensions as well.

The advantages of all of these direct methods are that they are relatively quick, are inexpensive, and, with adequate numbers of counts are relatively accurate. The disadvantages are that they are limited to material of high count; that they measure both dead and living cells; in the microscopic methods, if one is counting several hundred fields and has a great many counts to do, they can become excruciatingly tedious. Finally, under some circumstances it may well be difficult to tell the microorganisms from the debris if one is trying to count bacteria in suspensions of pus, or soil, or something of this sort.

FIG. 2-23. *Bacterial filter. The disc at the top of the photograph, scored to permit easy counting, is the filter. A drop of oil has been placed on it so that it becomes transparent and through the transparent area one may see a diagram of the construction of such a filter. The lower part of the figure shows the properties of a variety of such filters having different pore sizes. The data hidden under the nontransparent part of the filter is "rate of flow"; the smaller the pore size, the slower the flow. (Courtesy of Millipore Corp., Bedford, Mass.)*



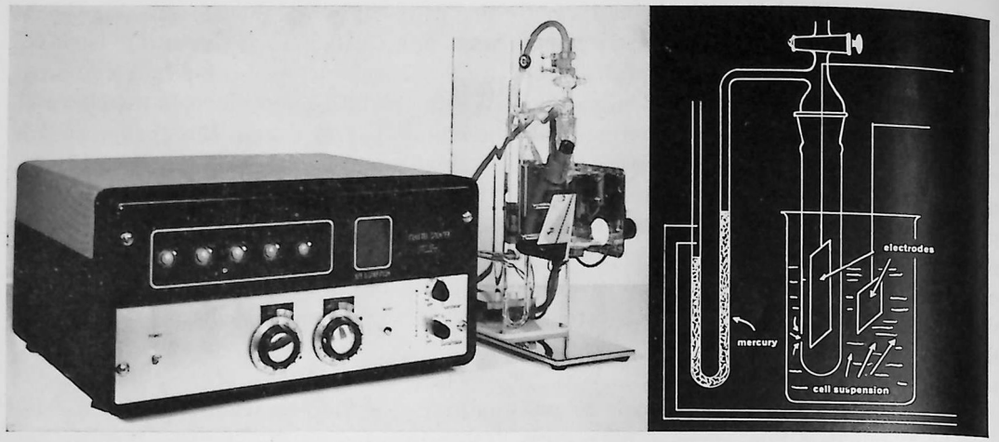


FIG. 2-24. *The Coulter electronic particle counter. This instrument, when fitted with a special 30 μ aperture tube and a 50 μl sampling manometer, can count bacteria. The principle is illustrated in the diagram. A drop in the mercury in the manometer pulls a cell suspension through the aperture. The microscopic lens in the photograph is focused on the aperture so one can see it is not clogged. The mercury also activates the switch turning on the counter and turns it off when the constant amount of fluid has passed through the aperture. An electric current flows through the aperture between the electrodes on either side of it. When an individual cell passes through the orifice, it displaces some of the fluid and changes the electrical resistance of the orifice contents. This resistance increase produces a voltage pulse which is amplified, displayed on the screen, and counted. The size of voltage pulse is proportional to the size of the cell. (Photo courtesy of Coulter Electronics, Inc., 590 W. 20th Street, Hialeah, Fla.)*

Cultural Methods

The basic types of cultural methods are the plate count, the Frost little plate, dilution methods, and the membrane or molecular filter.

1. The **plate count** is based fundamentally upon the pour plate method of isolating bacteria, but it isolates bacteria in a quantitative fashion. As shown by Fig. 2-25, one dilutes the sample through various stages of dilutions. The dilution might be made in sterile water or in the media itself, but it is made quantitatively. For example, one puts 1 ml of a suspension of bacteria, shakes it vigorously with 99 ml of water, takes 1 ml of this and puts it in a petri plate, and then supplies melted agar (cooled of course to 45°) mixes, and allows it to solidify. Then each colony that one finds growing on this plate after a suitable incubation would represent 100 organisms in one's original sample—and so on, down through the continued dilutions.

For counting individual colonies, choose a plate containing between 30 to 300 colonies. About 50 colonies are usually preferable, since plates containing more colonies than this are not only too difficult to count, but are so crowded that all organisms do not have a chance to grow; plates containing less than 10 colonies are subject to too great a variation from one plate to another. There are various tools or techniques available—illumination of plates, magnifying glasses, electrical counters, etc.—to assist in counting the plates, but they

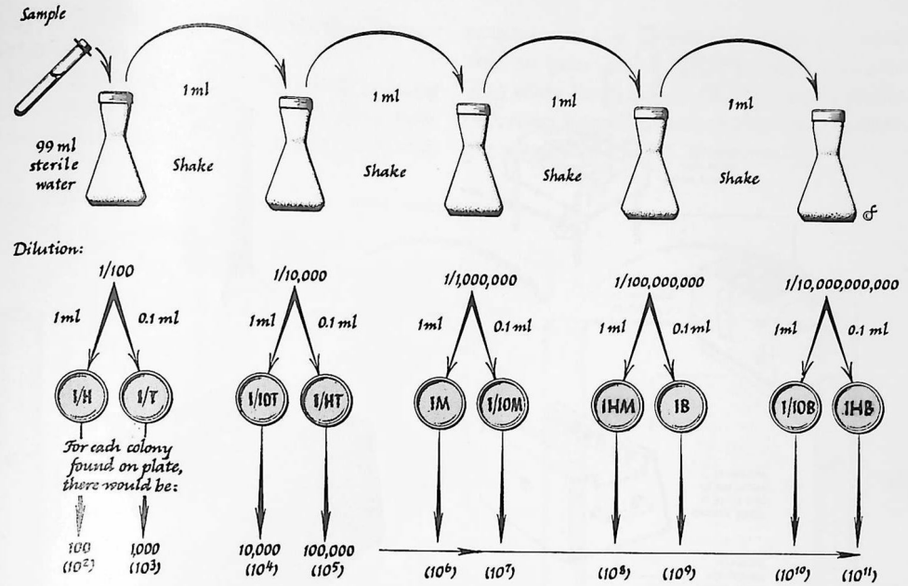
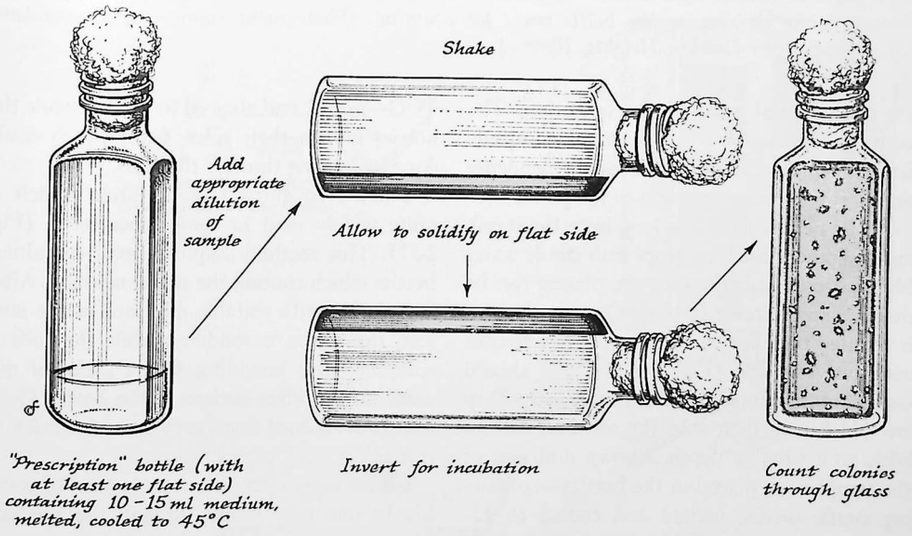


FIG. 2-25. *Dilutions used in making a quantitative plate count.*

FIG. 2-26. *Plate counts can be made in prescription bottles.*



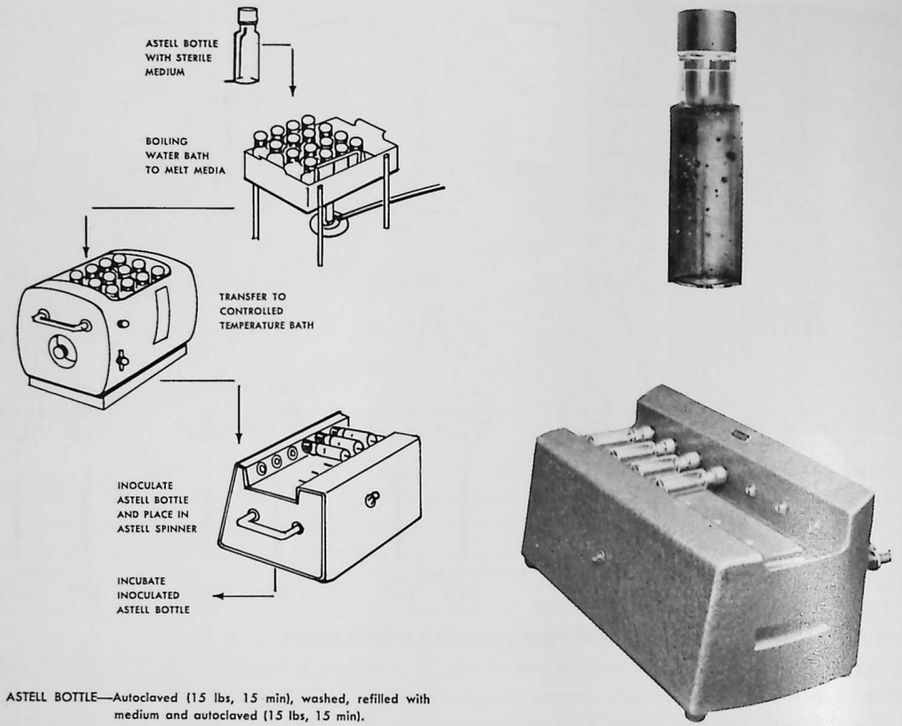


FIG. 2-27. *The Astell counting method. The diagram on the left illustrates the procedure. The photograph at bottom right shows the spinner used to cool and solidify the agar. The upper right shows colonies growing in the bottle ready for counting.* [*Photographs courtesy of Consolidated Laboratories, Inc., Chicago Heights, Illinois.*]

are not essential parts of the operation. The petri plates, as we've said, are incubated upside down to prevent water which might condense on the cover from dripping into the plate.

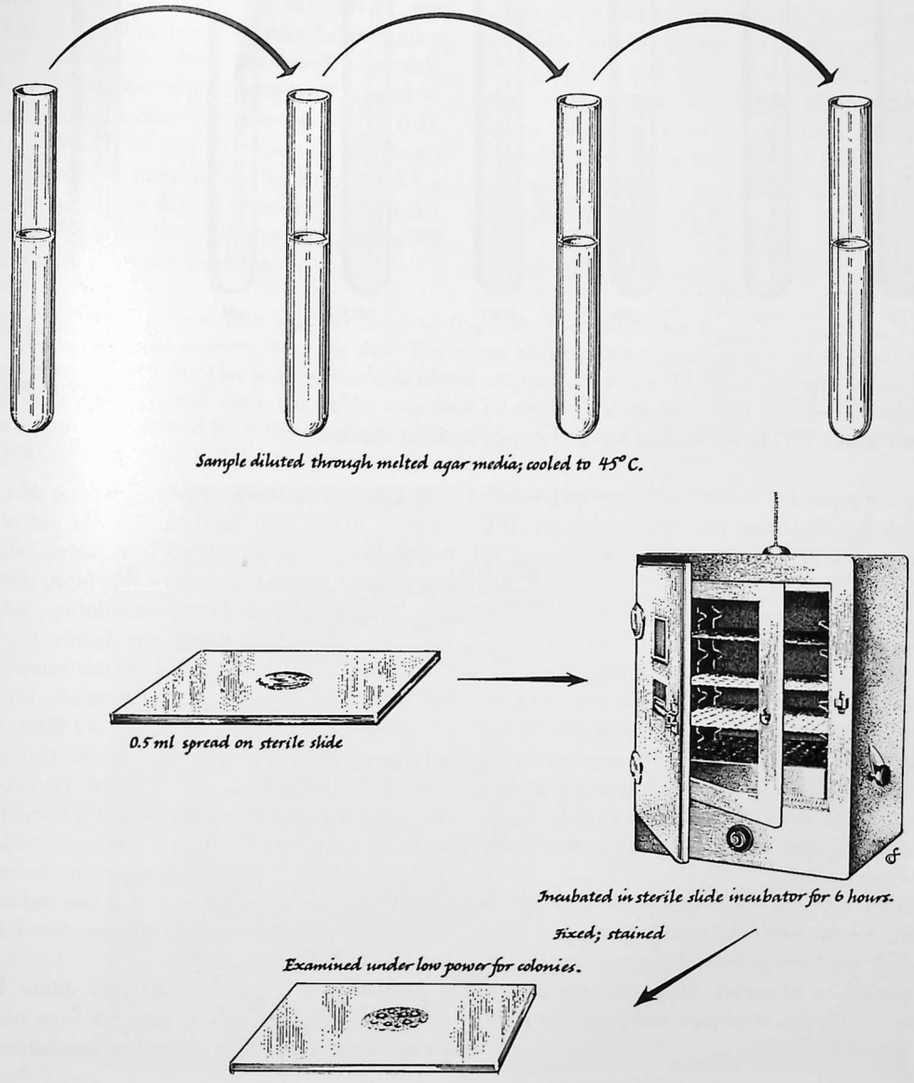
While petri dishes have long been the standard equipment used, together with sterile water blanks, sterile pipettes, etc., the plating can be done in any suitable fashion. One simple way is to use "prescription" bottles, or any bottles with one flat side (Fig. 2-26). These should each contain enough medium that, when they are laid flat on their side, the medium will solidify to a suitable depth. Known dilutions of the samples are placed in the bottles—containing sterile media, melted and cooled to 42–45°C—mixed, and allowed to solidify while the bottles are on their sides. Counting is easily done by looking through the glass.

There is a further modification which is quite widely used in some laboratories (Fig. 2-27). This method employs small cylindrical bottles which contain the sterile medium. After inoculation with suitable dilutions of the samples, the media is solidified while the tube is spinning, thus providing an even film of medium on the inner surface of the bottle. Colonies growing in it may be examined visually or through the microscope.

Under some circumstances, instead of water blanks one may dilute through the medium itself. It is critical, however, that the dilution be quantitative.

Some limitations should be noted in using plate counts. One is that not all living cells which may be in a sample are necessarily able to grow; i.e., one counts only those organisms able to grow under the conditions provided. The other limitation is that the water blanks, etc., must be well mixed, so that the organisms are evenly distributed. If clumps of organisms

FIG. 2-28. *Procedures used in the Frost little plate.*



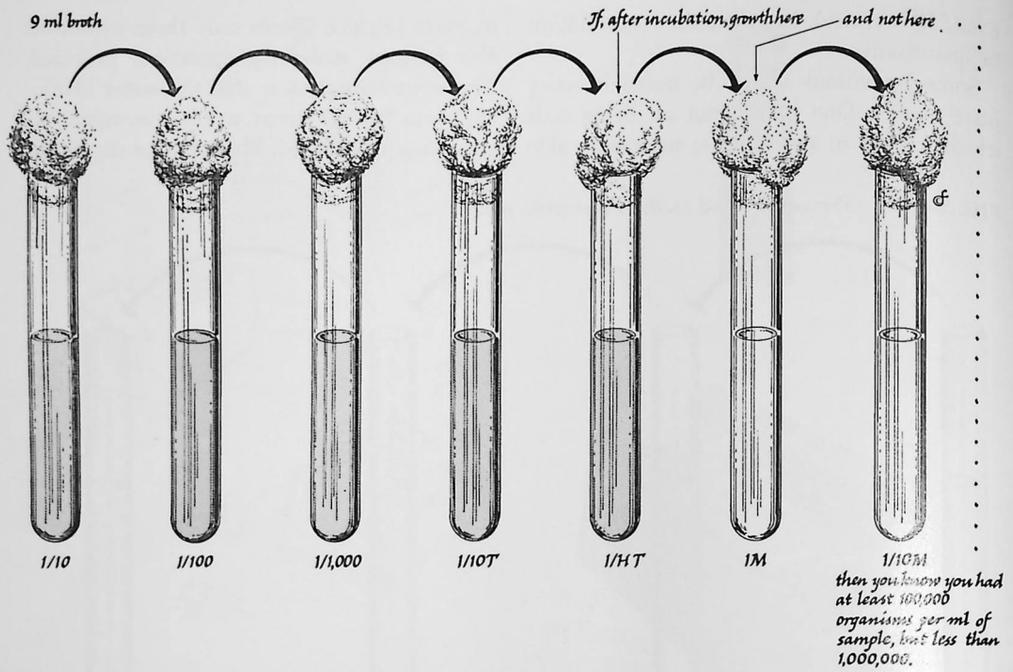


FIG. 2-29. *Dilution method for determining bacterial numbers.*

are not separated, several cells may give rise to a colony, rather than only one cell. Under such circumstances several cells may be counted as one cell.

2. **Frost little plate.** In an effort to produce a more rapid plate count, Professor Frost, many years ago, developed what is called the Frost "little plate" (Fig. 2-28). He diluted the sample through various dilutions of media and then placed 0.5 cc of the inoculated agar media on a sterile slide. He incubated this for 6 or 8 hours in a small incubator, heated to fix it, stained it, and counted the organisms under the microscope.

This method, while reasonable and precise and demonstrably adequate, has never been widely used—even for milk, where it would be supposed to be useful. Apparently the incubation time of the usual plate method is not really a serious limitation.

3. **Dilution methods.** If one is making dilutions, as in the plate method, they do not of course have to be inoculated into plates, but can serve to inoculate tubes. In this form, one may have growth at 1/100,000 dilution but none at 1/1,000,000; hence one knows that there were 100,000 organisms in the sample but not 1,000,000 (Fig. 2-29). Frequently this is all one really wants to know, i.e., not the exact number but the order of magnitude. If one finds 10,000 organisms, under many circumstances, this is 10,000 too many, and it doesn't matter if there were 10,560 or 20,000 or 50,000. So long as one is making dilutions in water blanks, one may as well plate, but if one makes the dilutions in the media itself, the dilution method is frequently simpler.

As shown in Fig. 2-29, one may dilute 1 cc of a sample through a series of 9 cc nutrient broth tubes, and after the incubation

determine the approximate numbers of cells.

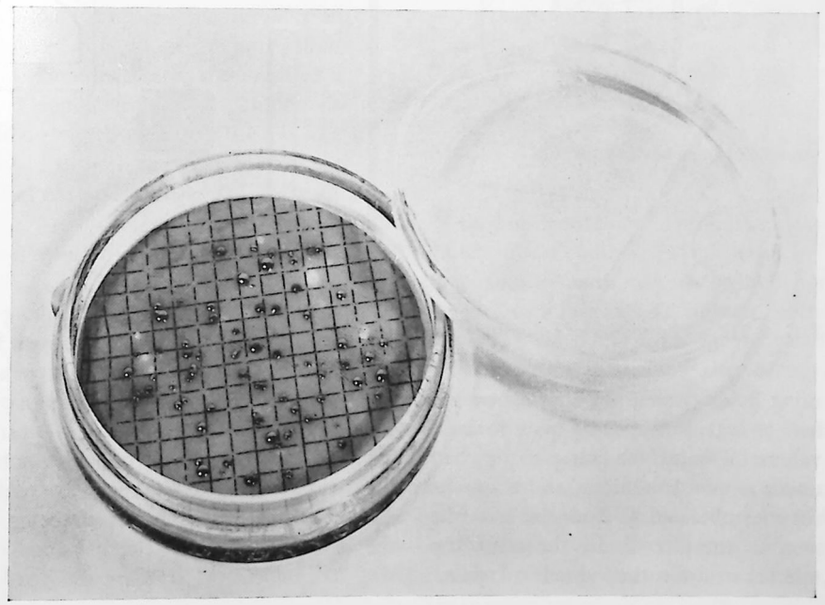
4. **Molecular filter** (*Millipore filter method*). Recently there has come into use a submicroscopic filter; the trade name of one brand is "Millipore filter," which is described in detail here. This is a fine cellulose membrane containing per square centimeter approximately fifty million capillary pores of uniform size (Fig. 2-30). The pore size may be controlled and one may purchase filters with pore sizes from 10 mμ, the approximate size of small viruses, to 5 μ. Filters with pore sizes of 0.3, 0.45, 0.65, 0.8 μ are all useful for bacteria. The larger the pore, the more rapid the flow; the 0.3 μ accommodates a flow of 40 ml/min/sq cm at 1 atmosphere, the 0.8 μ filter accommodates 330 ml under the same conditions.

Filters with pore sizes of about 0.5 μ are useful for retaining bacteria, i.e., they can not only be used to free a liquid from the bacteria that may be in it, and thus sterilize it, but one may also count the actual bacteria which have been removed, either by a direct method or by a cultural method.

Such filters can be printed with a counting grid. They become transparent in oil (see earlier Fig. 2-23), and hence the organisms per square centimeter can be counted. Or the filter may be placed on a nutrient medium (or on a selective medium to be described later), the organisms allowed to grow, and the colonies counted (Fig. 2-30).

These methods are especially useful for meas-

FIG. 2-30. *Millipore filter used for count of live cells. The sample has been filtered and the filter placed on sterile medium in a petri dish. This allows the live cells to develop into colonies which may then be counted. This is a water sample placed on Endo medium. The dark colonies indicate sewage pollution of the water; the lighter ones have no sanitary significance. This is a special or selective medium devised for a specific purpose, discussed in Chapter 17. (Courtesy of Millipore Filter Corp., Bedford, Mass.)*



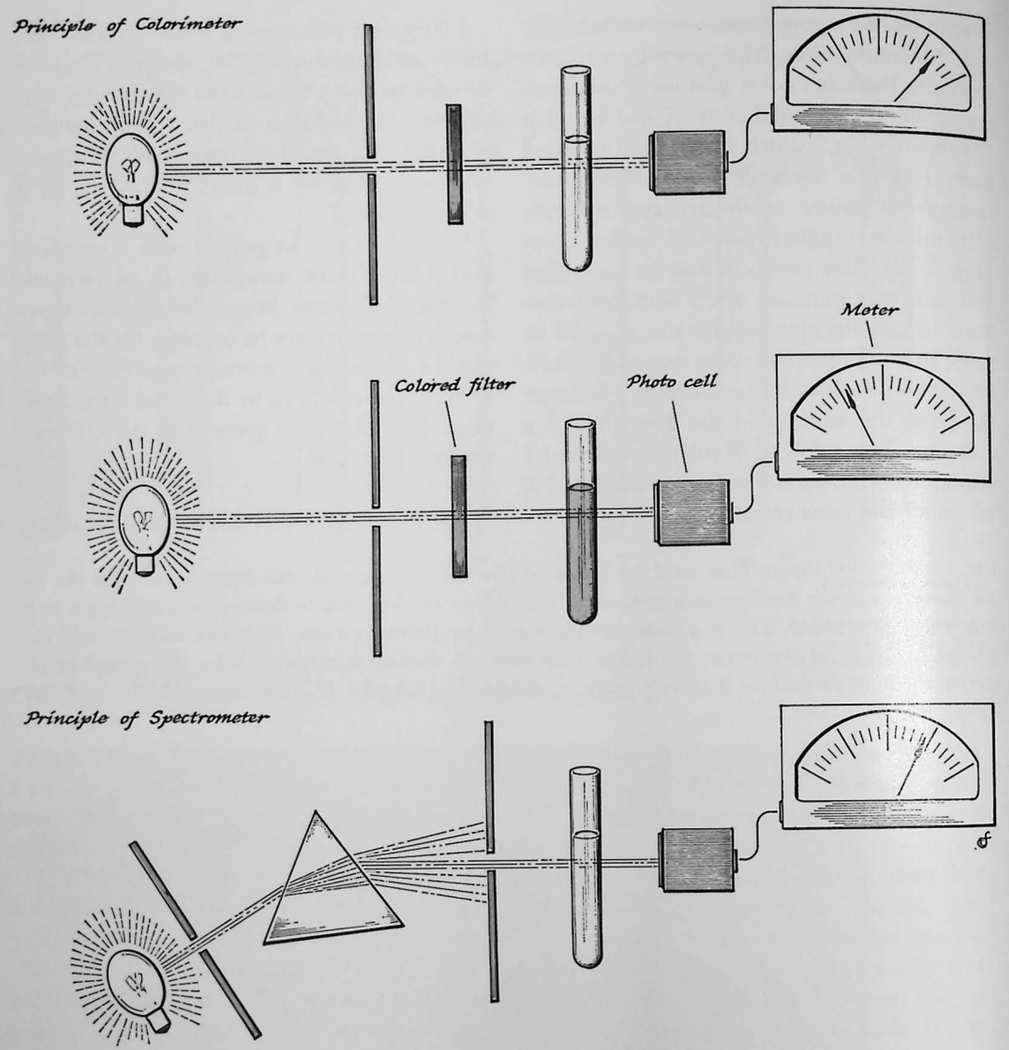


FIG. 2-31. *Principles of colorimeter and a spectrophotometer.*

uring fluids (water, air, etc.) where the numbers of organisms may be quite low. A known volume of water, for example, may be filtered under aseptic conditions, and thus—from the colonies obtained or from the individual cells seen on the filter under the microscope—the number in the entire volume is known.

Total Quantity Methods

In these methods one does not count individual bacteria but measures some characteristic property of masses of them. These methods generally have the advantage of speed and convenience. They have the disadvantage that

they are limited to comparative measurements under standardized conditions and for their use one must have large populations of cells. Some of them distinguish and some do not distinguish between living and dead cells.

Turbidity measurements are by far the most common estimates of bacterial numbers. These may be made quantitatively in a colorimeter, which we shall describe below. The first thing one does to determine whether bacteria have grown in liquid media, however, is to look for turbidity; one makes a rough estimate of growth by the degree of turbidity. Actually, this turbidity can be related readily to the number of organisms present. In a clear broth medium the organism *Escherichia coli* is just visibly turbid when it has reached a population of one million organisms per milliliter.

The early bacteriologists, who had no photoelectric colorimeters, measured turbidity quantitatively by two very ingenious methods. In one case they set up a series of tubes containing increasing quantities of the insoluble barium sulfate (thus producing increasing degrees of turbidity) and compared the turbidity of various cultures with these barium sulfate standards. In the other case, they pushed a standard-sized loop down through a culture until they could no longer see it from the top. The deeper they had to push the loop, the less the turbidity; a sort of standard scale was thus available.

Today, to measure turbidity, we have photocolorimeters (Fig. 2-31) and spectrophotometers, which provide light of any color we wish. All we need do is to determine how many cells equal one unit of turbidity. So long as we use the same conditions for measuring turbidity, we have a simple estimate of the number of organisms in a suspension. This kind of method does not distinguish between living and dead cells since each contributes to the turbidity. However, it can be used for measuring growth by measuring increased turbidity with time, and

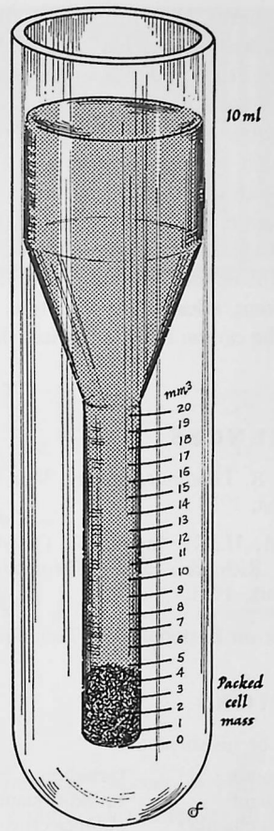


FIG. 2-32. *A centrifuge tube used for measuring cell volume.*

is one of the most convenient methods of measuring growth under controlled conditions.

Volume measurements may also be used. For example, as shown in Fig. 2-32, we may centrifuge down the microorganisms in graduated tubes and determine the volume they occupy. Or we may, having centrifuged them, determine their weight—either wet (called **wet weight**) or after drying (called **dry weight**). An easy method for molds is to filter off the mold pod and weigh it. Or, having separated the bacteria, we might determine something characteristic of them: their **nitrogen content,**

the **phosphorus**, the **carbon**, the **ribonucleic acid**, the **deoxyribonucleic acid** (DNA), the **protein**, etc., each of which, under standardized conditions, could give us a pretty accurate, although indirect, estimate of how many bacteria were present in a given sample. All of these measure both living and dead cells.

But, as an estimate of how many bacteria we had, we could also measure something that they do: the glucose used, the ammonia assimilated, the ammonia released from protein, the acid formed, the carbon dioxide formed, the oxygen taken up, and many other **metabolic activities**. Twice as many bacteria would be expected to do twice as much within a given time. These methods, of course, measure only those organisms that are doing something—which means, under most circumstances, those which are alive. These, then, are the basic methods of microbiology, widely applicable, generally used. As we go on we will describe other more detailed methods for specific purposes, but you have now in your possession the major tools with which you can work.

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QUESTIONS

1. Define or identify:

direct count	turbidity
Breed count	turbidity standards
counting chamber	photoelectric colorimeters
Petroff-Hauser	spectrophotometers
standard loop	volume measurements
proportional method	metabolic activity
cultural method	dilution method
plating in bottles	molecular filter
water blanks	millipore filter
Frost "little plate"	
total quantity method	

2. Which of the methods of determining the numbers of bacteria is most suitable for the following situations:
   (a) the numbers of bacteria in a sample of milk to determine whether it is likely to spoil soon?
   (b) the number of bacteria in a fermentation vat; 25 vats to be sampled every hour?
   (c) the number of bacteria in a sample of water, i.e., are there 100 or only 1 per milliliter?
   (d) to determine whether any bacteria at all are alive in a heat-killed suspension of bacteria, e.g., a vaccine?
   (e) to determine how many dead bacteria there are in a heat-killed suspension of bacteria, e.g., a vaccine?
3. How could you tell whether a bacterial cell was living or dead?
4. You are engaged in a foreign mission in an uncivilized part of the world and you have only a microscope available. You must determine the exact number of cells in a vaccine preparation which has just been flown to you, but which is unfortunately not labeled as to the cell number. How would you do it?
5. You find that a culture of a microorganism uses up 1 g of glucose per hour. You wish to know how much glucose each cell uses up. What method would you use to determine this?

Chapter 3

THE NATURE OF THE INDIVIDUAL CELL: BACTERIA

"Man must approach natural phenomena with the view, indeed, of obtaining information. But not, however, in the character of a pupil, who listens to all that his master chooses to tell him, but rather in the character of a judge who compels the witness to reply to those questions he himself thinks fit to propose. To this single idea must be ascribed the revolution by which natural science was at last conducted into the paths of certain progress after groping in the dark for so many centuries."

KANT, *Critique of Pure Reason*

The Cell Theory

The first chapter described the situation that occurred when Leeuwenhoek reported his finding of bacteria to the Royal Society of London (in 1675–1676) but refused to provide a microscope to see them. The Royal Society therefore commissioned one Robert Hooke to build the very best microscope he could, and through which he saw bacteria, in 1677. This same Robert Hooke had been working with microscopes before, but they were compound microscopes,

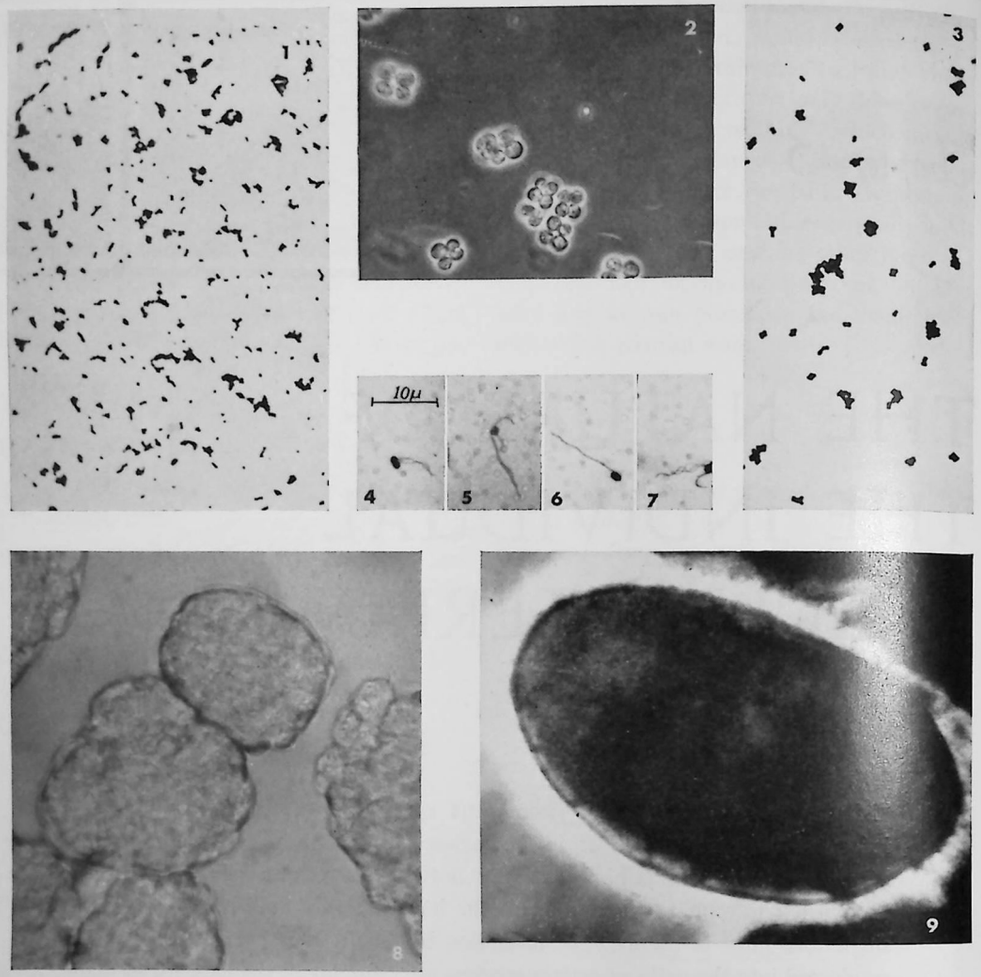


FIG. 3-1. *Cocci. 1 and 3 are typical cocci as usually seen under oil immersion. 2 is a* Sarcina, *i.e. cocci existing in packets of 8. 4, 5, 6, 7 is a series of unusual cocci which are motile (the organs of locomotion, the flagella, are stained); most cocci are not motile. 8 is a higher magnification of a packet of* Sarcina. *9 is an electron micrograph of a single* Sarcina *which is also unusual in that this particular organism produces cellulose, not in the cell wall, but outside the cell (the clear, white area in the photograph). [1 and 3 courtesy of Dr. C. F. Niven. 2, 8, and 9 from Canale-Parola, Borasky, and Wolfe, J. Bacteriol., 81, 311 (1961); courtesy of Dr. R. S. Wolfe. 4, 5, 6, 7 are from Langston, Gutierrez, and Bouma, J. Bacteriol., 80, 714 (1960); courtesy of Dr. C. W. Langston.]*

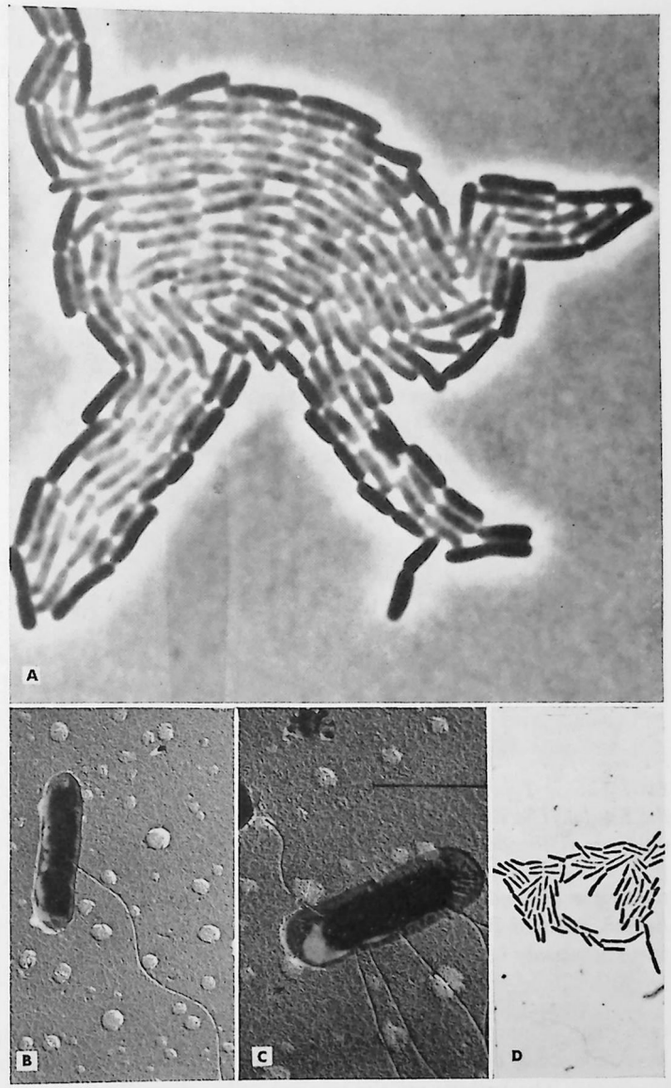


FIG. 3-2. *Types of cylindrical organisms. A and D, microcolonies of* E. coli. *[From Hoffman and Frank, J. Gen. Microbiol.,* **25,** *353 (1961).] B and C are electron micrographs of* Xanthomonas. *[From Hayward and Hodgkiss, J. Gen. Microbiol.,* **26,** *138 (1961). Courtesy of Dr. Hodgkiss.] Both of these organisms would be regarded as having somewhat rounded ends. Note the degree of variation in size found in completely pure cultures.*

not simple ones like Leeuwenhoek's. And Hooke, as early as 1665—some say in 1667—had reported on the structure of cork. He found that it was composed of tiny empty boxes, which he called cells. Shortly thereafter, on examining fresh plants he found these same boxes "filled with juices."

As time went on, and more came to be known about the nature of life, it became evident that cells were always found with life. Wherever life existed, there too was cellular organization. Destroy the organization, and one destroyed the life. Even the most complex creatures are based upon cellular organization—

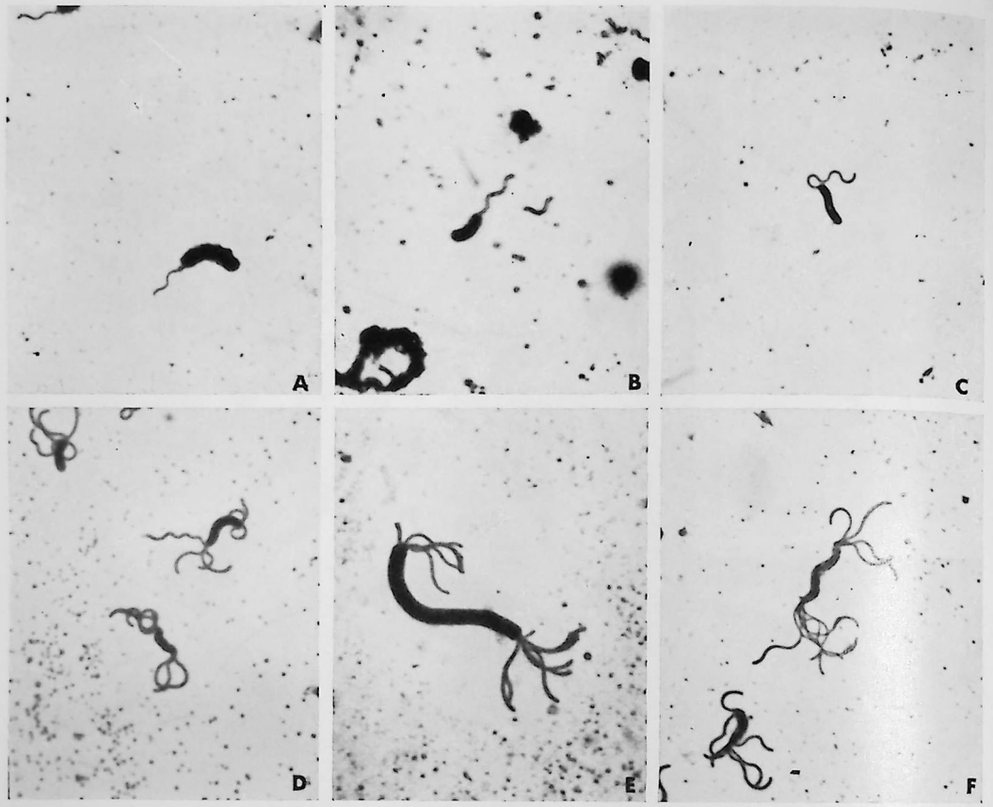


FIG. 3-3. *Types of curved rods. These are all stained to demonstrate the organs of locomotion, the flagella. The cell form ranges from a mere bend* (A) *to a coil* (F). *The organisms are* A, *an unknown vibrio species;* B, Vibrio cholera; C, Vibrio tyrogenus; D, Spirillum linum; E. Spirillum serpens; F, *a spirillum from water. Note how the distinction between* Vibrio *and* Spirillum *depends on the degree of bending of the cell and on the number of flagella* (*i.e., compare C and D*). [*From Leifson,* Atlas of Bacterial Flagellation, *Academic Press, 1960. Courtesy of Dr. E. Leifson.*] *Electron micrographs of* Spirillum *may be found in Fig. 3-19.*

even man. Complex, multicellular creatures have more cells, but the living unit is still the single cell. The bacteria are the smallest cells. Hence, if we wish to study bacteria, we must first look to the nature of their cells.

Nature of the Individual Bacterial Cell

Bacteria exist in the form of cells. Each cell is an individual bacterium, capable of carrying on all of the *essential* parts of the attributes of the bacterium—growth, reproduction, metabolism. Bacteria exist in three shapes:

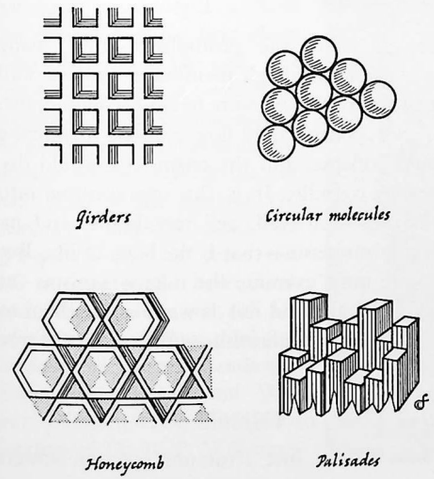
1. Spheres. These bacteria appear under the compound microscope as circles or dots, and are called cocci (the singular is coccus). "Coccus" comes from the Latin corruption of the Greek *Kokkos*, meaning a seed. See Fig. 3-1.

2. Cylinders. These appear under the microscope as rods and are sometimes called bacilli (singular, bacillus). The word comes from the Latin *bacillum*, meaning little rod, being the diminutive of *baculum*, meaning a staff. Cylinders may have rounded ends, or abrupt ends, etc., hence there are a wide variety of rod forms, as shown in Fig. 3-2.

3. Spirals. They appear under the microscope as definitely and regularly curved rods, ranging in length from a comma to a coiled spring, and ranging in the tightness of the coil from a spring to a wave (Fig. 3-3). The spiral shape is rather rare; most organisms are spheres or cylinders. Spirals are distinct from spirochetes, which are a form of higher bacteria discussed in Chapter 15.

The size of bacteria may vary considerably; after all, the definition specifies only very broad size limits, but the vast majority of bacteria are about 0.5 μ wide. Cocci, since they are round, are also 0.5 μ long, although many are not perfect spheres. Rod forms, while about 0.5 μ wide, are from one to several micra long. Generally, younger cells are wider and longer than older cells.

FIG. 3-4. *Present conceptions of possible cell wall structures.*



Actually, however, not much could be learned about the detailed structure of bacterial cells with the ordinary microscope. Methods for greater magnification had to be developed. The information derived from these methods, especially from the electron microscope, will be used when convenient, but we shall try, insofar as we can, to describe methods usable with the usual light microscope so you can make these same observations yourself. We shall also illustrate our discussion largely with one organism, *Bacillus megaterium*, which has several advantages for a beginner in bacteriology: it is a large, rod-shaped organism, easy to cultivate and easy to see. You will handle it in the laboratory and become familiar with it. Further, in most cases it is reasonably typical of bacteria in general.

An Imaginary Excursion into a Cell

It may be helpful at this point if we imagine a bacterial cell magnified so that it was the size of a modest house. If we were approaching it, what might we see? First, we would probably encounter a layer differing from the surrounding medium. This layer might be deep or shallow, it might be soupy or firm, but it could be penetrated some distance. While we might find the going distasteful, progress would not be impossible. This jelly-like area is not really cell—nor yet medium. It is called the capsule, or slime layer, the composition and depth of which will depend upon the bacterial species, the medium, and the environment on which it is grown.

When we have made our way through this slime layer, we come against something solid. This is the cell wall, built of girders we might suppose, but certainly of unusual design. Figure 3-4 shows some of the present-day notions about the detailed geometric structure of the cell wall. But it is rigid and unyielding and

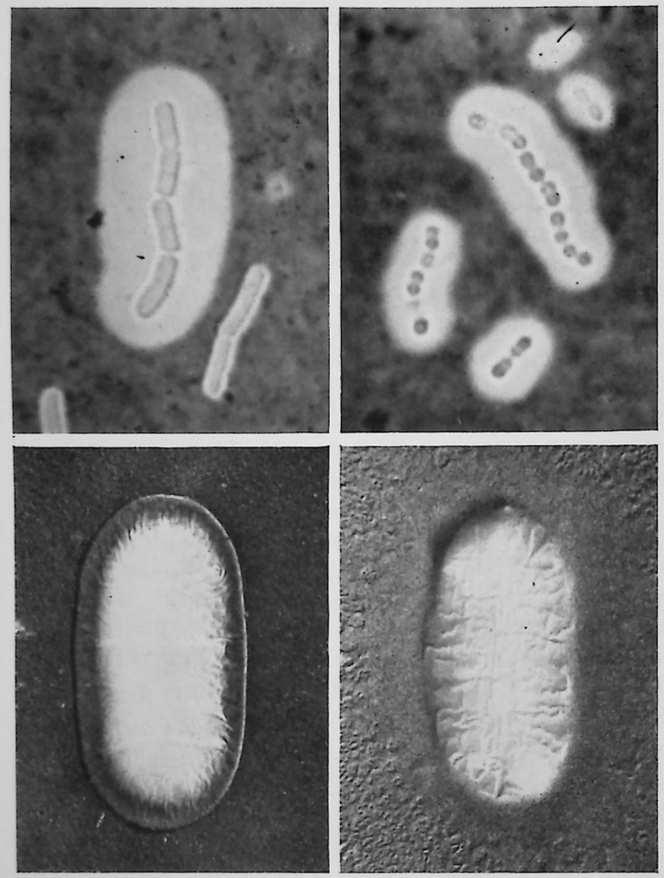


FIG. 3-5. *Capsules. At the top are capsules demonstrated by suspending the cells in India ink. Left is* Bacillus megaterium, *an organism in which the capsule is a polypeptide. Right is* Diplococcus pneumoniae, *in which the capsule is a polysaccharide.* [*Courtesy J. P. Duguid, magnification about* 3500 ×. *At the bottom are electron micrographs at about* 30,000 ×. *At left is an organism* (Shigella) *which does not have a capsule. The outline of the cell can be seen clearly. At right is a related organism* (Klebsiella) *which possesses a large capsule. While this capsule is not directly seen in these shadow casts, the outline of the cell is obscured by the presence of the capsule.* [*From Duguid,* J. Gen. Microbiol., **21**, 271 (1959).]

were we to kick it, it would not yield. But beneath it there is something else—something rigid, indeed—pressed out against the retaining cell wall, but having more the rigidity of a tent rather than that of a wall. This cellular membrane is not just a canvas wall but has depth to it, and while it is held together as a network, it has "holes," and pumps, and generators, and conveyor belts in it. So complex is it, indeed, that we hesitate to try to draw it, even in imagination, and so we have not. Sober scientific data on its nature is unusual enough.

The membrane we cannot penetrate. There is no hole large enough for us to enter, and no conveyor belt onto which we fit. Back of this membrane lies the protoplasm—the "living stuff" itself (although membrane and cell wall are living too). If we were to cut the membrane, the protoplasm would flow out, the membrane would collapse, and the entire cell would disintegrate rapidly. It is the organization into structures—cell wall, cell membrane, and internal protoplasm—that is the basis of life. But now we must examine the microorganisms "as they really are, and not as we imagine them to be"—which, as Paracelsus said, constitutes true science.

Slime Layer or Capsule

This is the first structure one encounters when approaching the bacterial cell from the outside. The **capsule** is commonly defined as a microscopically demonstrable, gelatinous or slimy layer covering the cell wall and having a definite external surface. However, in many bacteria there is not exactly a definite external surface, and there is a considerable variation in the thickness and the firmness of this outer slime layer (Fig. 3-5). The material exists outside the cell wall and in some cases forms a regularly firm and strong structure. At other times it almost diffuses out into the medium. Usually the layer is jellylike; at times it may be pulled out into long strings. It does not take up dyes within any degree of regularity. Chemically, there are two types of capsule or slime layer material. The first of these are **polysaccharides,** a material composed of dextrans, levans, etc., much like the plant gums. The other type of capsule is composed of **polypeptides.** Certain bacteria contain a peptide composed primarily of chains of glutamic acid molecules linked together.

There are several primary functions of the capsule. It serves to protect the cell from the environment. There are several instances where strains possessing such capsules are able to live in environments in which the noncapsulated strain is eliminated. Or, under some circumstances, the capsule may be used as a reserve food and may actually be redigested by the organism when other food is lacking. Or such

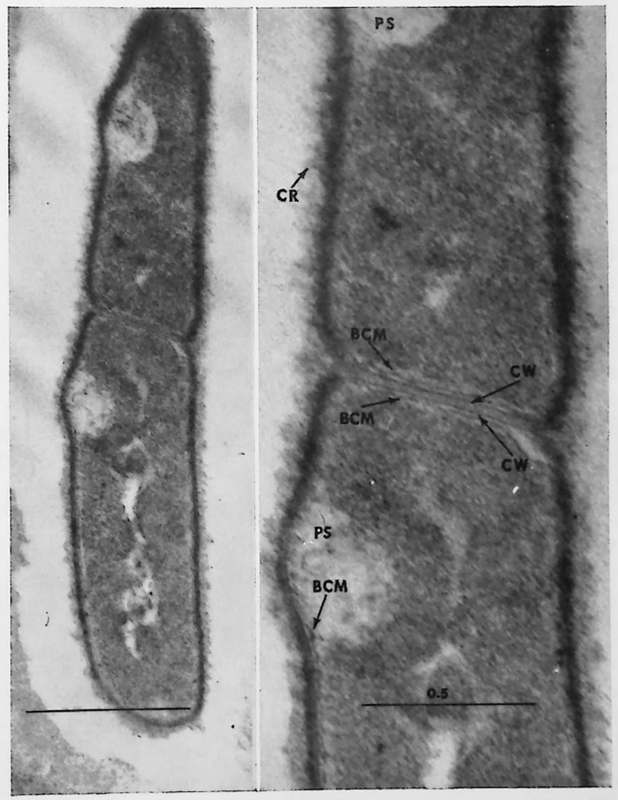


FIG. 3-6. *Ultrathin section of* Bacillus anthracis. *The line in the first figure represents 1 μ. Note that the cell wall has bent about a swelling within the cell. The right-hand figure is a twofold enlargement of the central part of the first figure. CR represents the capsular residue, CW is the cell wall, BCM is the bacterial cell membrane, and PS stands for "pre-spore"—that is, from this area a spore would have developed. Note how the cell wall has definitely bent and how thick it is compared to the cell membrane.* [*From Roth, Lewis, and Williams,* J. Bacteriol., **80**, 772 (1960). *Courtesy of Dr. R. P. Williams.*]

capsules may be merely waste products. For example, there are situations where an organism can attack sucrose, a sugar composed of glucose and fructose, but can only metabolize its glucose part. The fructose is then built into a capsular or slime layer material and excreted into the environment. All these functions have to do with the functions of the capsule for the organism itself. The capsule, however, does serve the purpose of sticking cells together; this is important in pasting soil particles together, for example, and thus plays a role in preventing soil erosion. Also, organisms may form large capsules in milk, converting it to ropy milk, an undesirable trait. As mentioned, capsules do not readily take up stain. Normally one uses a simple negative stain.

The negative stain. A heat-fixed smear (or on occasion an unfixed loopful of the specimen) is treated with a drop of aqueous nigrosine spread thinly over the surface. (Purified India ink may be used, but most India ink has organisms in it which will show up in the stain.) In this case, we may outline the cell, frequently detecting capsules, especially if we treat already stained cells in this fashion and compare the size of the conventionally stained cell with the negatively stained cells. Unfortunately, there are no good capsule stains as such, applicable to all organisms. The capsule generally does not react with dyes, so a negative stain is frequently the best we have.

The Cell Wall

A characteristic feature of the bacterial cell wall is that it is rigid. It is not absolutely rigid, and it can be bent (see Fig. 3-6, where a cell wall has been bent), but it has the rigidity of perhaps a lead or copper tube, and is, so far as we can judge, not elastic. When deformed it does not apparently spring back into place. But it is hard to bend. After all, the forces involved in fixing and staining bacteria are relatively enormous compared to the bacterial size, and the very fact that we see rod-shaped organisms under these circumstances is reasonable evidence that the cell wall is rigid. If we remove the cell wall (discussed below), all cells become spheres.

Plants also have a rigid cell wall, mostly composed of cellulose. In plants, this cell wall is the structural basis of the plant; the dead cell walls are what give rigidity to the wood. But bacterial cell walls are not made of cellulose and, of course, do not serve the structural function of supporting a higher organization, as in a plant. But it does serve a slightly different kind of structural function, as we shall see later.

The cell wall of *B. megaterium* can be broken by supersonic vibration. When this happens, the rest of the cell runs out, leaving the empty shell of the cell wall—still a rigid tube, looking something like a fragile test tube (Fig. 3-7). The cell wall may also be cut with a very fine microdissection needle, and the contents of the cell diffuse out, again leaving just this hollow empty tube—the cell wall.

Before we examine chemically what this cell wall may be, there is one experiment which gives a clue to both the composition and the function of the cell wall. There is an enzyme, **lysozyme,** which occurs in—of all places—tears; but it is more readily obtained from egg white. Lysozyme is capable of dissolving the cells of certain bacteria, acting to destroy the cell walls. When the walls are destroyed, the cells swell up and burst.

Live cells of *B. megaterium* were then treated with lysozyme in a solution of higher osmotic pressure—for example sucrose at above 0.6 *M*, KCl or NaCl at and above 0.8 *M*, Na_2HPO_4 at and above 0.5 *M*. In this treatment, it was discovered that the cell wall was dissolved, as before, but the cells did not burst (Fig. 3-8). Instead, they became spheres and kept right on living, getting along pretty well without their cell wall. Of course, they would

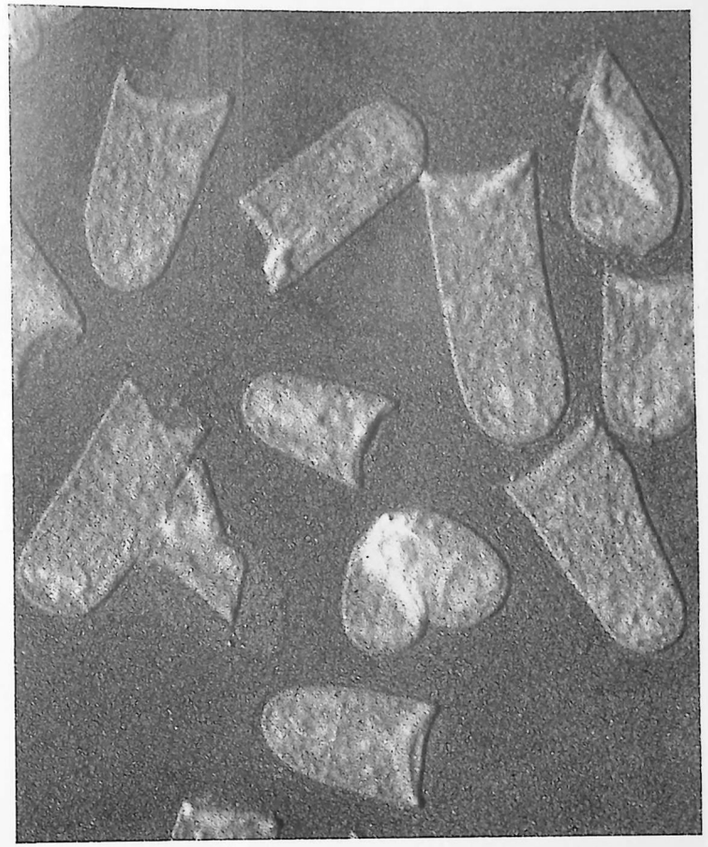
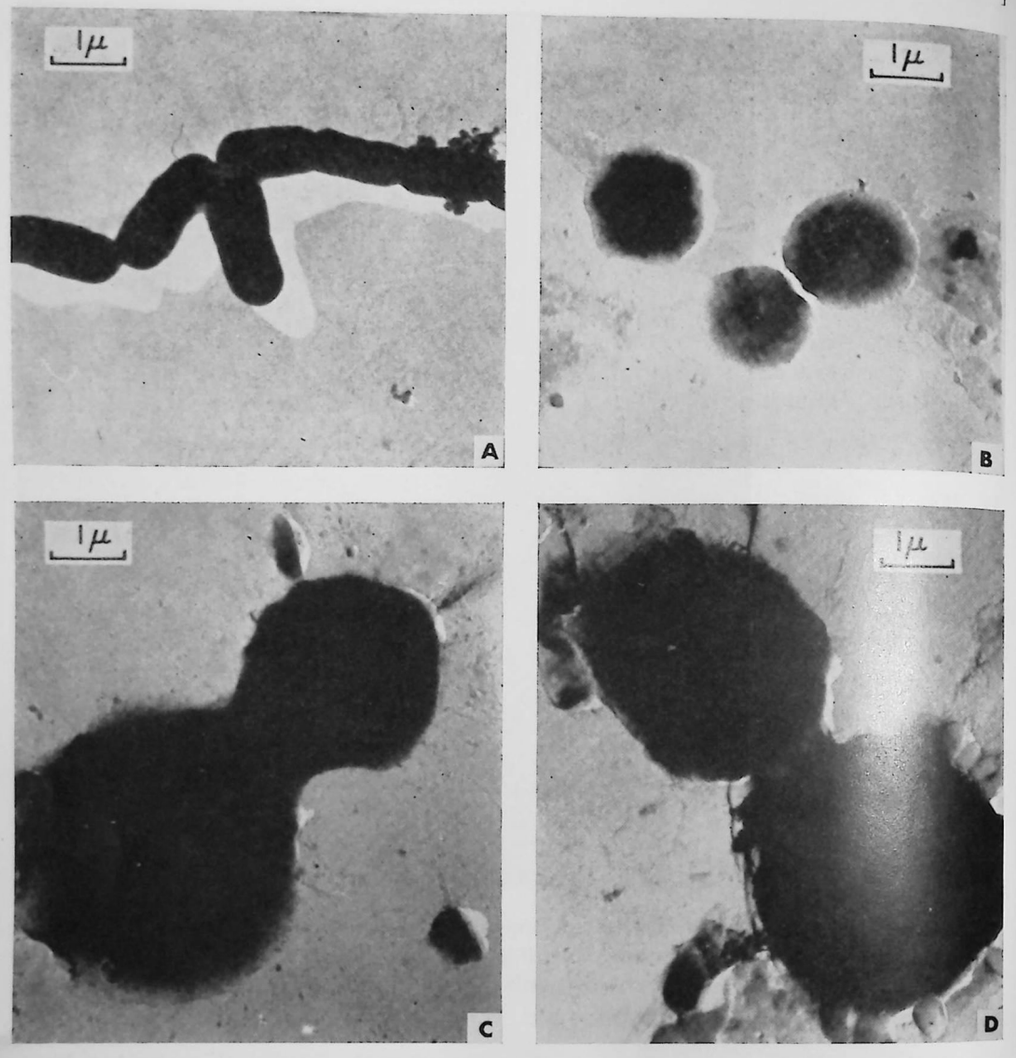


FIG. 3-7. *Isolated cell walls. These are pure cell walls of* Mycobacterium tuberculosis (*BCG*). *Note their rigidity and how they retain their original shape.* [*From Ribi, Brown, and Goode,* J. Bacteriol., **79,** *142 (1960). Courtesy of Dr. E. Ribi.*]

burst if the osmotic pressure was lowered—if, for example, the solution were merely diluted with water. These "round" structures, devoid of cell wall, are called **protoplasts.**

The osmotically fragile protoplasts can do many things that the intact cell, with its cell wall, can do, i.e., respire or take up oxygen from the air, increase in weight and size, synthesize protein and nucleic acids; they are even capable of developing spores. But as soon as we dilute the solution, the protoplasts burst, while the intact cell is unharmed.

In many organisms it was surprising that more protoplasts than cells were obtained; if one started with 100 cells, one might find 200, or 300, or even 400 protoplasts. It became apparent that many rod-shaped cells were, in fact, a series of protoplasts held together as a rod by a continuous cell wall (Fig. 3-9). Our ideas of rod-shaped organisms have undergone a



revision in the last few years and we no longer regard the rod as necessarily "one" cell.

However, such studies have shown us a most important function of the cell wall of bacteria. The cell wall prevents the cell from expanding too far in an environment in which it rapidly absorbs water. It thus acts as a sort of corset holding in the cell contents, which under most circumstances are at a higher osmotic pressure than the surrounding environment. Since water will move into regions of higher osmotic pressure, the cell would expand until it burst if the cell wall did not prevent it. When the cell wall is removed, as with lysozyme, the rest of the cell does just that—it absorbs water until it bursts and can only be held as a protoplast in solutions with enough osmotic pressure to prevent the entry of water.

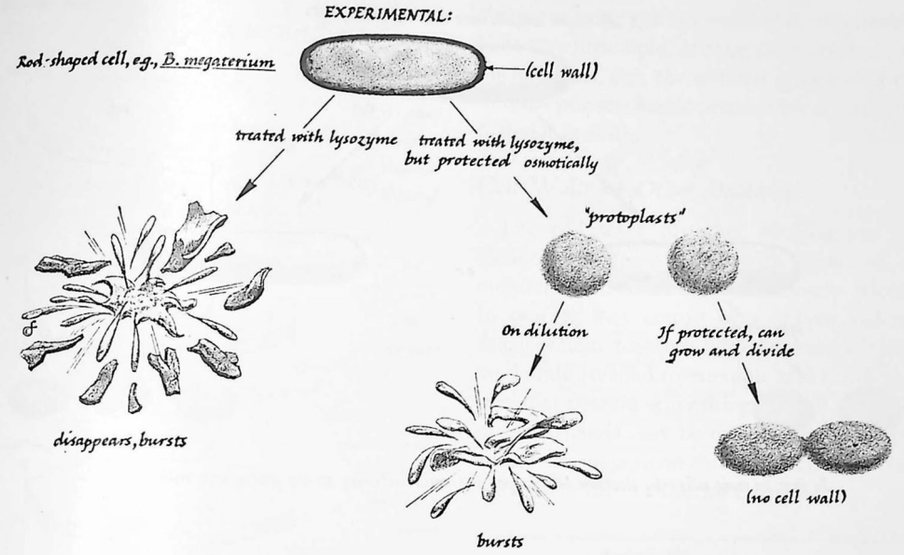
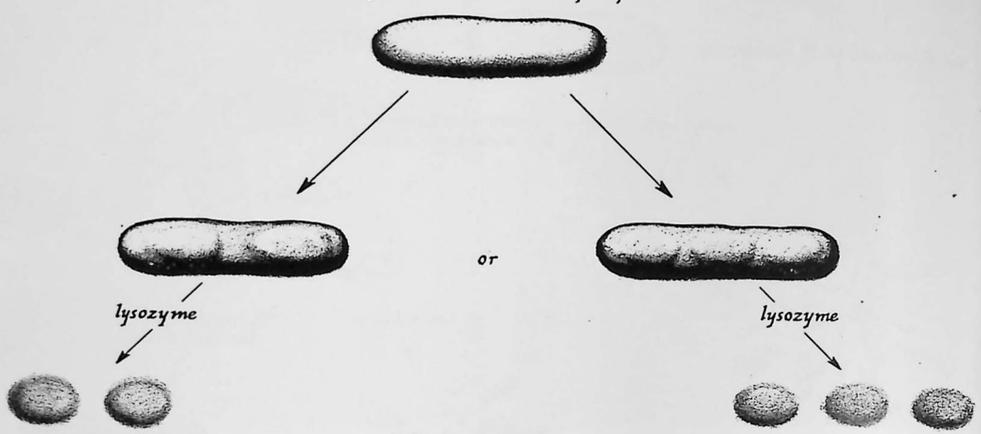


FIG. 3-8. *In photograph:* A, *Whole cells of* Bacillus megaterium; B, *protoplasts after treatment of the whole cells with lysozyme in an osmotically protected medium. Note that they are round and not cylindrical.* C *and* D, *such protoplasts after 9 hours' incubation. The protoplasts have grown and have apparently divided without reforming a cylindrical cell.* [*From Fig. 24 of McQuillen, "Bacterial Protoplasts" in Gunsalus, I. C. and Stanier, R. Y. (Ed.)* The Bacteria, *Volume I; Academic Press, N. Y., 1960. Courtesy of Dr. K. McQuillen.*] *The drawing above shows the process pictorially.*

The protoplast, of course, tells us little about the cell wall except that a cell can live, although precariously, without one. To study the cell wall itself, it is necessary to prepare the cell walls free from other materials. This has been done by breaking up the cells in a variety of ways—for example by violent shaking and then centrifuging out the cell wall fragments. Such fragments are examined under the electron microscope to demonstrate that they are free from other cell components and to study their morphology. The free cell walls from *B. megaterium* are completely dissolved by lysozyme, showing that what is isolated is the same material that is dissolved by this enzyme when acting on the intact cell.

Cell walls so isolated from a variety of bacteria are not all the same. Some seem to be quite smooth, others show more detailed organization. The cell wall is generally about 10–20 mμ thick and thus is invisible in the light microscope. The cell wall does not stain under ordinary conditions; what one sees in stained smears under the light microscope is the protoplasm below the cell wall.

However, what is perhaps surprising is that the cell wall accounts for about 20% of the weight of the cell, surely a very high proportion. As a specific example, cell wall isolations from *B. megaterium*, when dried, weigh from 20–25% of the dry weight of the whole cell. The cell wall therefore comprises from ¼ to ⅕ of the cell. Chemically, the cell wall of *B. megaterium* contains protein which, when hydrolyzed, yields 10 amino acids. It is, however, an unusual protein in that it lacks aromatic



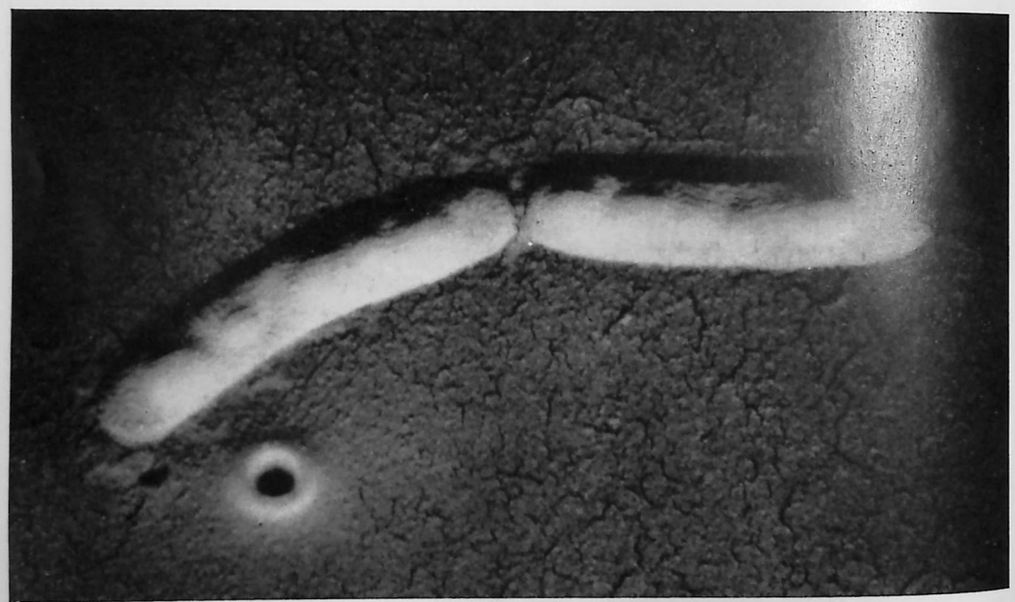


FIG. 3-9. *Multiple protoplasts within a single cell. The electron micrograph is of* Bacillus megaterium, *and was furnished by Dr. G. P. Vose of the Texas Woman's University, Denton, Texas.*

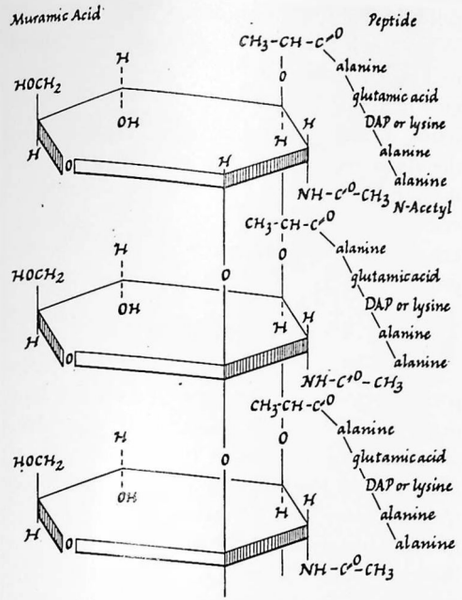


FIG. 3-10. *Chemical structure of muramic acid.*

amino acids (tyrosine, tryptophan, phenylalanine), the sulfur amino acids (cystine, cysteine, and methionine), and arginine and proline. But it does contain a new amino acid—diaminopimelic acid, structurally related to lysine—which occurs in nature only in the cell walls of certain bacteria. Thus the protein found in the cell wall is a most unusual protein.

In addition to this unusual protein, the cell wall of *B. megaterium* contains, after hydrolysis, about 45–48% of its dry weight as glucose (evidently existing in the unhydrolyzed cell wall as a complex polysaccharide) and 18% as **hexose amine.** This last compound proved to be a new kind of amino sugar, which has been given the trivial name of muramic acid. This material exists in the cell wall of *B. megaterium*, and of most bacteria, in the form of its N-acetyl derivative (Fig. 3-10). Thus, in addition to an unusual protein, the cell wall of *B. megaterium* contains an unusual polysaccharide as well. The cell walls of *B. megaterium* have very little lipid, at least after isolation, no nucleic acids, etc.; the unusual protein and the unusual polysaccharide account for most of the material present.

Cell Walls of Other Bacteria

The cell walls of other bacteria are not identical in composition with those of *B. megaterium* but are certainly closely related. In general, they consist of a unique and unusual protein together with an unusual polysaccharide (related to muramic acid), although they may contain other things as well. As noted earlier, bacteria may be divided into two large groups—gram-positive and gram-negative. Their cell walls differ in the following ways.

	Gram-Positive (as *B. megaterium*)	Gram-Negative
Amino acid composition	Unusual protein; aromatic amino acids lacking; frequently possess diaminopimelic acid.	Unusual protein; aromatic amino acids present; usually possess diaminopimelic acid.
Polysaccharide	Muramic acid; reducing sugar: 40–60%; hexose amine: 10–20%.	Muramic acid; reducing sugar: 20–40%; hexose amine: 3–5%.
Lipid content	Possibly 2–4% lipid.	15–20% lipid.

Studies on the nature of the cell wall of gram-negative bacteria have led to the notion that about one-quarter of its cell wall has a very similar composition to the cell wall of gram-positive bacteria; the other three-fourths is made up of different lipids and more normal types of protein. This is illustrated diagrammatically in Fig. 3-11. In gram-positive bacteria the muramic acid complex constitutes such an important part of the cell wall that when it is removed the cell can no longer resist the osmotic push of the cell membrane against it;

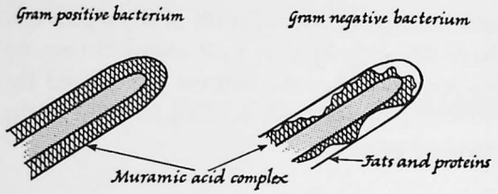


FIG. 3-11. *Diagram illustrating the composition of the cell wall in gram-positive and gram-negative bacteria.*

the protoplast, unless protected osmotically, bursts. In the gram-negative bacteria the muramic acid complex is much less a part of the wall. It is responsible for the rigidity and when muramic acid is removed, the cells are no longer cylindrical but all become round. However, there is sometimes still enough strength left in the remaining materials to prevent bursting; such cells—with muramic acid removed but with other materials still present—are called **spheroplasts.** They are not as osmotically sensitive as protoplasts.

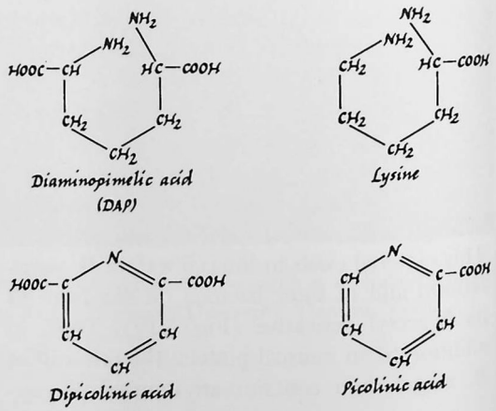
There are two experimental phenomena which ought to be related here. First, the antibiotic, penicillin, acts by inhibiting the incorporation of muramic acid into the cell wall. As such, it acts only on growing cells, i.e., those making new cell walls. It also acts largely on gram-positive organisms—those in which muramic acid is the critical part of the structure. Penicillin causes such cells to burst, but if they are protected osmotically the antibiotic will cause the formation of protoplasts. On gram-negative cells, where penicillin is less lethal, it causes spheroplasts to be formed.

Second, most cell walls of bacteria have diaminopimelic acid. This compound is closely related to lysine (Fig. 3-12) and some bacteria have lysine instead of diaminopimelic acid in the cell wall. And there are further some bacteria—*Streptococcus faecalis* is one—which have lysine in the cell wall but are unable to synthesize it themselves, i.e., it must be supplied in the medium or they will not grow. When such a bacterium is given only about half the amount of lysine it needs, it starts to grow normally but suddenly finds itself without lysine to make more cell wall. The rest of its metabolic machinery keeps on going and when it starts to divide, it has only protoplast, not cell wall. If it is not protected osmotically, the cell dissolves and, of course, is killed in the process.

The Cell Membrane of Bacteria

Protoplasts, cells free from cell walls, can be prepared from *B. megaterium* and other microorganisms. The protoplasts show the same permeability properties as the whole cell, i.e., allow certain substances to penetrate while excluding others, and, of course, burst if the external osmotic pressure is lowered. It is clear that the permeability properties, the "living membrane," is still functioning in the protoplast and is not dependent upon the integrity of the cell wall. For many years there was some uncertainty as to whether the "membrane" was a membrane, i.e., two-sided like a sausage skin, or whether it was one-sided, i.e., there was no membrane but the protoplasm formed its con-

FIG. 3-12. *Relations between the chemical structures of materials found in cell walls and spores.*



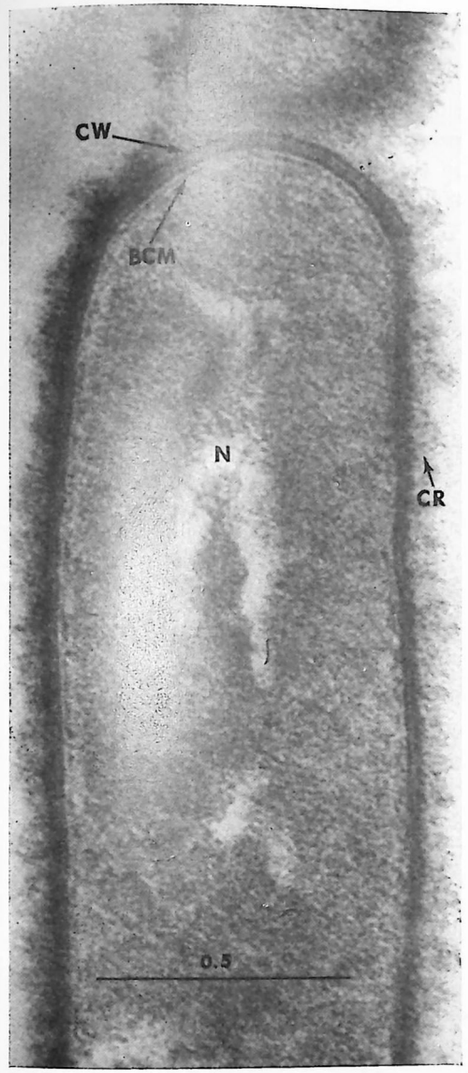


FIG. 3-13. *An electron micrograph of an ultrathin section of* Bacillus anthracis. *The line represents 0.5 μ. CR is the capsule residue after dehydration. CW is the cell wall; note how thick it is compared to the bacterial cell membrane* (BCM). *N represents a concentration of nucleic acids, i.e., the nuclear apparatus. This is a twofold enlargement of the same cell shown in Fig. 2-7.*

tact with the world at this surface. There is a membrane. This can be demonstrated and indeed the membrane isolated. If protoplasts are diluted, they burst, and the cell membranes can be isolated by centrifuging. When such membranes, or rather their fragments, are prepared from *B. megaterium*, the preparations show enzymatic activity; especially they show the presence of respiratory enzymes. The membrane is thus more than just an inert osmotic barrier. The membrane itself seems to be from 11–15 mμ thick, and in some cases (in *Spirillum* especially) seems to be composed of a hexagonically packed array of spheres rather than a rectangular packing. The hexagonal packing leaves less "open space" or "pore size." In a gram-positive organism, related to *B. megaterium*, the particles were 11 mμ; this would leave a pore size of 1.5 mμ for hexagonal packing versus 5 mμ for rectangular packing. From permeability measurements the pore size has been estimated to be about 1 mμ. In electron micrographs of *B. anthracis*, an organism causing anthrax and related closely to *B. megaterium*, shown in Fig. 3-13, the cell membrane shows clearly. (BCM = bacterial cell membrane.) In *B. megaterium* and almost all other organisms, the cell may be seen to contract away from the cell wall when intact cells are transferred to 2 *M* NaCl broth, which has an osmotic pressure high enough to pull water out of the cell. This may be observed even in the light microscope. Thus the membrane has an osmotic function and an enzymatic function. When isolated and analyzed, the cell membrane seems to be rather similar in both gram-negative and gram-positive bacteria, although it must differ in some critical way. Gram-positive bacteria are generally much more sensitive

Cell walls and cell membranes are also clearly visible in Figs. 3-6 and 4-4. [*From Roth, Lewis, and Williams.* J. Bacteriol., **80,** 772 (1960). *Courtesy of Dr. R. P. Williams.*]

to low surface tension than gram-negative bacteria, and the low surface tension seems to act on the membrane rather than on the cell wall. However, so far as can now be determined, the composition of the membrane is closely similar in all bacteria: there is 40–50% protein, which contains all the usual amino acids; 20–30% phospholipid (choline, a characteristic component of most phospholipids, seems to be absent in bacterial cell membranes); and 15–20% carbohydrate (generally glucose is the basic unit of the polysaccharide, but mannose has been found in some). While the intact membrane must be a sheet surrounding the cell, when disintegrated it readily breaks up into small particles. This "small particle fraction" comprises roughly 10% of the weight of the cell, and thus the membrane itself comprises roughly 10%.

The membrane possesses very little mechanical strength but is forced up against the cell wall with a pressure of 15–20 atmospheres. The cell wall, therefore, must be so designed as to exert a fairly even thrust over the entire surface of the plasma membrane, and the pores in the cell wall must be quite small. Yet there is evidence that the cell membrane sticks out through the cell wall in some cases. At least it reaches some kind of physical contact with the external world. There is an enzyme (see p. 149 for definition), a phosphatase, which can be recognized by its action on organic phosphates. These organic phosphates do not penetrate into the cell, yet the cell attacks them. The only way we can see that this can be done is that the cell surface contains the phosphatase. When *Staphylococcus aureus*, which possesses such a surface phosphatase is separated into cell wall and cell membrane, all of the phosphatase (90%) is in the small particle fraction (= cell membrane) and none (less than 3%) in the cell wall fraction. Either the membrane sticks out a bit (much like a balloon forced against a screen) or the organic phosphates can penetrate the cell wall, even if they do not penetrate further into the cell.

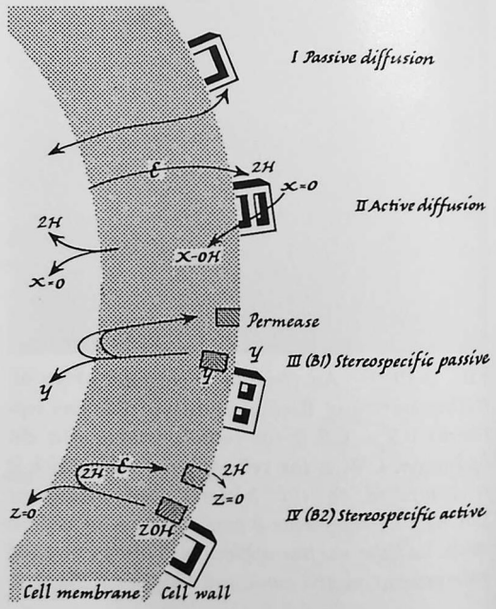
The cell membrane of bacteria is fragile, thin, has strong affinity for dyes, has semipermeability, and osmotic properties. It seems to be similar in gram-negative and in gram-positive bacteria.

Digression on Permeability

Today, there are four kinds of permeability known, in bacteria as well as in higher cells. Basically there are two characteristic types of permeability and each has an active and a passive form. These are termed active and passive diffusion, and active and passive stereospecific penetration.

One may visualize these processes, as has been done in Fig. 3-14, with the cell membrane represented on a very large scale. Some materials are soluble in the cell membrane and simply diffuse in and out of the cell because of

FIG. 3-14. *Types of permeability.*



this solubility. This is the **passive diffusion** type of permeability and requires no energy or any activity on the part of the cell. If the cell is not using up material on the inside, then at equilibrium the concentration of material on the inside and on the outside of the cell will be the same.

However, there may be other materials which require energy before they will penetrate. These may be conceived of as substances which by themselves are not soluble, but which can be, on the outside of the cell, converted into substances which are. In the case illustrated in Fig. 3-14, we have assumed that a substance X=O with a ketonic group might be reduced on the outside of the cell to form an X with a hydroxyl (X—OH) group; we have further assumed that the X (X—OH) with the hydroxyl group is now soluble in the cell membrane and can penetrate it. At this point it is capable of giving up its hydrogen again, becoming X=O, the hydrogen returning to reduce more X=O on the outside.

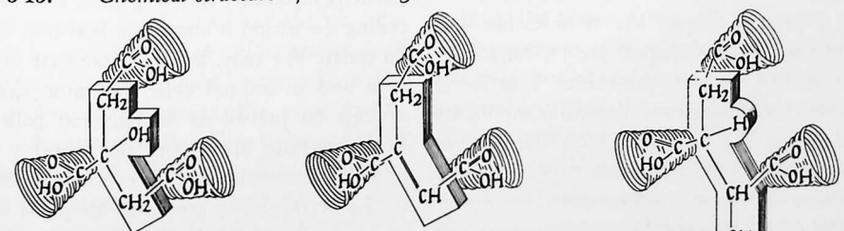
Under these circumstances, energy—that is, the addition and removal of hydrogen—is necessary before the material can penetrate. This is an example of the **active diffusion** process, and it is possible under these circumstances that the concentration inside the cell may eventually be higher than that on the outside of the cell. All the materials which penetrate by diffusion have the characteristic property that related substances act similarly.

The third kind of permeability might be represented by a substance Y, which is not soluble in the cell membrane nor can it be rendered so. But it is possible that Y can combine with a protein which is imbedded in the cell membrane. This protein we have indicated by a small cross-hatched block; on Fig. 3-14 it has been labeled "permease." (This name permease will have a meaning to you later as we explain what the situation is.) Substance Y can combine with this protein and the combination of protein plus Y is now soluble. It diffuses through the cell membrane and on the inside, gives up Y to the inside of the cell; the protein is free to return to the outside and pick up more Y.

In this fashion Y is able to penetrate the cell membrane. That is, there is imbedded in the cell membrane a specific mechanism for its penetration. This would be called **stereospecific passive permeability.** (We will define and talk about the meaning of "stereospecific" on p. 74.)

The final type of permeability is somewhat similar to active diffusion in that we have, for example, a Z=O, a material which will not penetrate the cell nor combine with a specific protein carrier Z. Hence something else must be done to it. In this case, we have illustrated that it can be converted to Z—O—H; Z—O—H would combine with such a carrier protein; this would diffuse through the cell wall, liberating Z=O on the inside, and the

FIG. 3-15. *Chemical structure of related organic acids.*



hydrogen going back to reduce more Z=O. This process requires energy. This would be an example of **stereospecific active transport.**

All substances exist in space and occupy particular forms within space. In Fig. 3-15 we have drawn a series of related organic acids showing how they exist as spatial molecules, i.e., how they exist in three dimensions. If such substances combine with protein, or combine with anything else, that does not alter their fundamental nature, they must do so in a spatial relationship; there must be a fit. For example, a molecule shaped like A in Fig. 3-16 will not

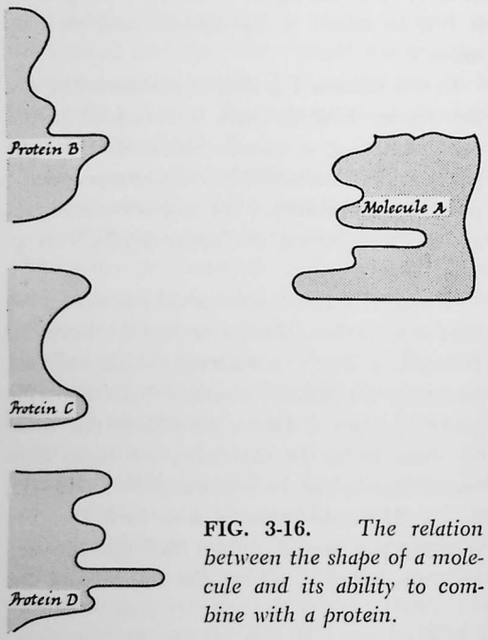


FIG. 3-16. *The relation between the shape of a molecule and its ability to combine with a protein.*

fit on a protein shaped like B or C but will only fit on a protein shaped like D. Therefore, if in Fig. 3-14 we wish substances Y or Z=O to combine with particular protein components imbedded in the cell wall, both the protein components and the substance to be combined with them must have a proper spatial configuration. Otherwise they will be unable to react and the material will be unable to penetrate. Although we do not always know what the configuration of the protein is or just how the substance combines with it, we can distinguish **stereospecific transfer** by the fact that related substances do not necessarily act alike. A series of substances may have discontinuous permeability properties. For example, citric acid, isocitric acid, and cis-aconitic acid may not all penetrate a given cell even though their chemical structure and their configuration in space is quite similar; see Fig. 3-15. In fact, it is frequently found that citric does not penetrate, that isocitric does, and that cis-aconitic does not. These things are explained by assuming that the carrier protein which is in the membrane has a configuration which will fit only the isocitric and not the citric or the cis-aconitic acid. At any rate, we do know that in many stereospecific transformations the related members of a series show completely different permeability properties. The best way to explain this phenomenon is to assume that they are adsorbed on or combined with particular configurations in particular carrier proteins. Inasmuch as the action of enzymes, which we will describe later, also shows this stereospecificity, it has been assumed that in the cell wall there are enymes whose function it is to combine with materials on the outside and transport them to the inside of the cell. Many enzymes are named by adding "ase" to the name of their substrate, and since these enzymes are concerned with permeability, a popular name for them is **permease.** There is much to be learned about permeability in living cells, and the preceding discussion is about the best that we can do today. We may, however, say that in plant cells and in animal cells the same situation appears to prevail as in bacterial cells. We know nothing about the permeability properties of bacterial cells that is not also reflected in the permeability properties of plant or animal cells. We know further that the cell membrane is not an inert material but contains

enzymes of one sort or another, many of which are concerned with the respiratory activity of the cell.

Protoplasm

In bacteria the **protoplasm** itself lies beneath the rigid case of the cell wall and beneath the membrane which possesses the permeability properties attributed to the cell. The word "protoplasm" started out to mean *the life substance.* Today its meaning is considerably more vague; a present definition is "the permanent and transitory elements of metabolism encased within a membrane." We are faced with this problem in bacteria: Is there any internal organization? Chemically, there must be. This is not an indiscriminate soup in which all things mingle without order but it is the living substance itself. In higher cells, in both animals and plants, there is a definite organization of the protoplasm. Photographs (Fig. 3-17) illustrate the internal structure of animal cell (from the spleen) as revealed by thin sections and photographed in the electron microscope. Present are mitochondria, microsome, nucleus, and other material. This same photograph shows bacteria within or adjacent to the animal cells. The bacteria show considerably less internal organization—certainly not an organization similar to that of the animal, but still not a completely uniform mixture.

There are at the outset a few basic problems. One is that all animal and plant cells possess a nucleus, which carries the hereditary factors, the **genes;** these decide the kind of a cell it is going to be and the kind of cell its progeny will be. Bacteria certainly have within them the same genetic determinants, the same factors which influence what the cell is to be and what its progeny shall be. But it has been most difficult to see an organized nucleus within a cell. After all, in many ways this is not too surprising, since the bacteria are about the size of the mitochondria of higher cells; indeed, the bacteria are smaller than the nuclei of higher cells (compare Fig. 3-17) and hence it may well be that the bacterial nucleus is just too small to be seen by today's methods, even in the electron microscope. Therefore any present evidence for a nucleus in bacteria must almost of necessity be somewhat indirect.

There is some information that leads us to believe that bacteria possess a nucleus. First, bacteria possess **DNA** (deoxyribose nucleic acid or deoxyribonucleic acid), a material characteristic of the nuclei of higher organisms and the bearer of the hereditary carrier. This is present in every bacteria in which it has been looked for and under some circumstances it can be seen to be in particular parts of the cell, as though it were organized into a nucleus although more diffuse than in the animal (Figs. 3-13, 3-17). Second, bacteria do possess hereditary character; the kind of cell which is derived from *Bacillus megaterium,* for example, is always *B. megaterium* and not some other bacteria. The forces within the cell when it divides produce another cell of the same nature as before. Hence, even though we may be unable to see a nucleus under any but the most special circumstances, there is no doubt but that a nucleus does exist.

In higher cells there is a division, a relatively sharp division, indeed, between nucleus and cytoplasm. The nucleus comprises those materials enclosed within a nuclear body, and cytoplasm those materials in the rest of the cell and enclosed within the cell membrane. It is, as the above paragraph illustrates, somewhat uncertain as to whether the bacteria possess an organized nucleus or whether the nuclear material is within the cytoplasm but not separated from it by a membrane. However, in the cytoplasm of higher cells the cytoplasm is not just a continuous soup, but contains particles of varying sizes and function. These particles are called mitochondria and microsomes. In

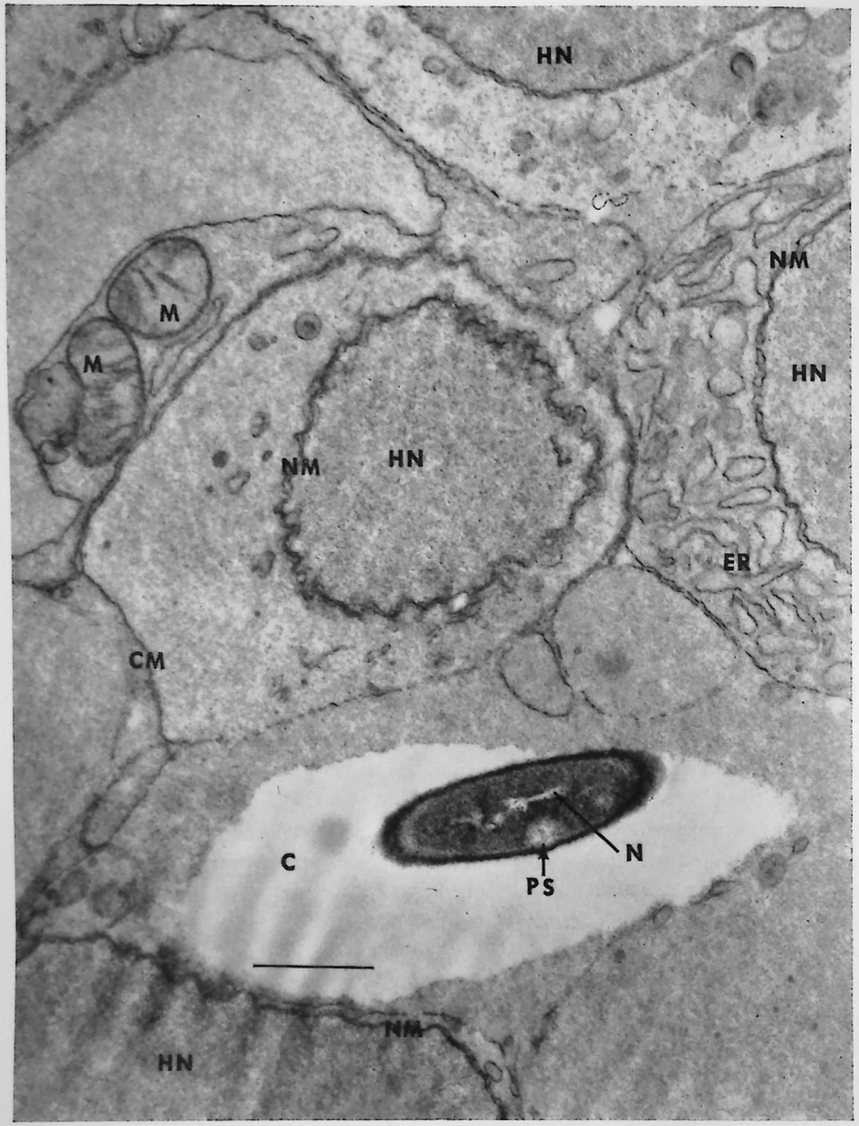


FIG. 3-17. *An electron micrograph of an ultrathin section of mouse spleen containing a bacterium. The line represents 1 μ. The darker area is a bacterial cell* (Bacillus anthracis) *showing nuclear material (N) and a pre-spore (PS). It is surrounded by an area of tissue damage (C). Immediately above is a normal spleen cell with a nucleus (HN), a nuclear membrane (NM), a cytoplasmic membrane (CM), and mitochondria (M). Note how the mitochondria have a further internal structure. ER represents a structure characteristic of most lymphoid tissue; spleen is one type which has to do with blood formation and does not concern us here. Note that the bacterial cell is hardly larger than the mitochondria and is considerably smaller than the nucleus of the animal cell.* [*From Roth, Lewis, and Williams,* J. Bacteriol., **80,** 772 (1960). *Courtesy of Dr. R. P. Williams.*]

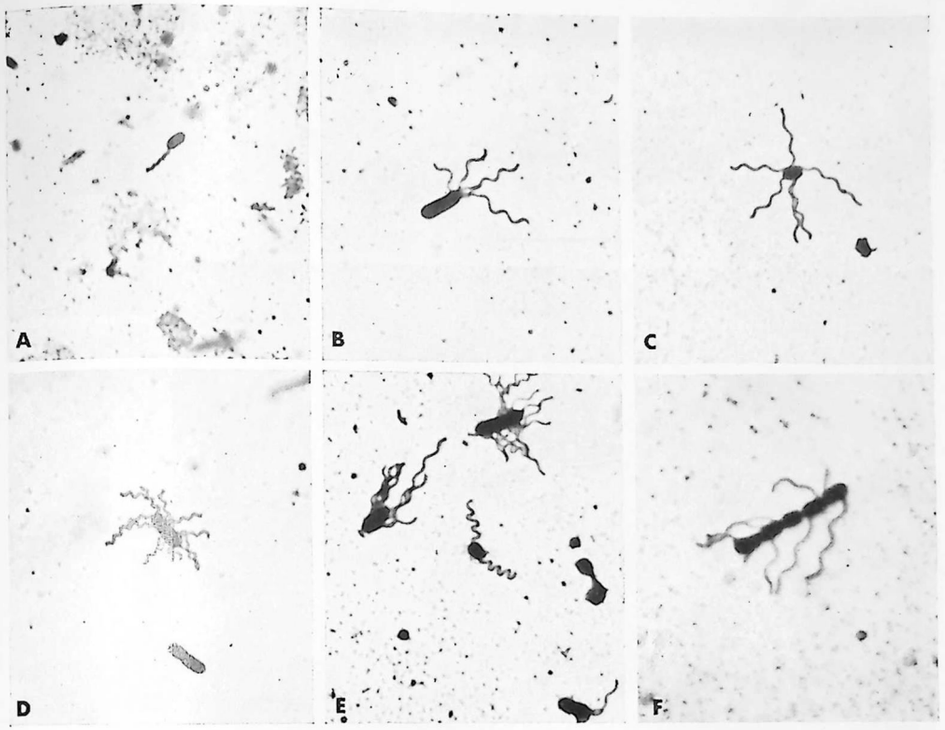


FIG. 3-18. *Types of flagella. A is a* Pseudomonas *sp. (monotrichous flagella). B is a* Pseudomonas *sp. with polar multitrichous flagella. C, D, E are* Proteus *sp. with various types of peritrichous flagella. F is a rare form of* Streptococcus *which is motile; most cocci do not have flagella.* [*From Leifson* Atlas of Bacterial Flagellation, *Academic Press, 1960. Courtesy of Dr. E. Leifson.*]

general, the **mitochondria** (Fig. 3-17; note the laminated structure) possess the respiratory enzymes, which are concerned with the oxidation of foodstuffs of the cell to provide energy. The question which we have in respect to bacteria is whether they possess mitochondria. It is true that it is possible to isolate from crushed cells of bacteria fractions containing very small particles which possess the respiratory enzymes of the cell. The difficulty is that we are not certain that these came from the cytoplasm, since we know that under some circumstances such particles can arise from the cell membrane. The problem is an exceedingly difficult one, based primarily on the fact that the particles with which we are dealing are so very small.

Other Structures Found in Bacteria

While all bacteria have capsules (or at least traces of an outer layer), cell walls, cell membranes, and cytoplasm, some bacteria have additional structures. The two principal ones are flagella, which are responsible for motion, and spores.

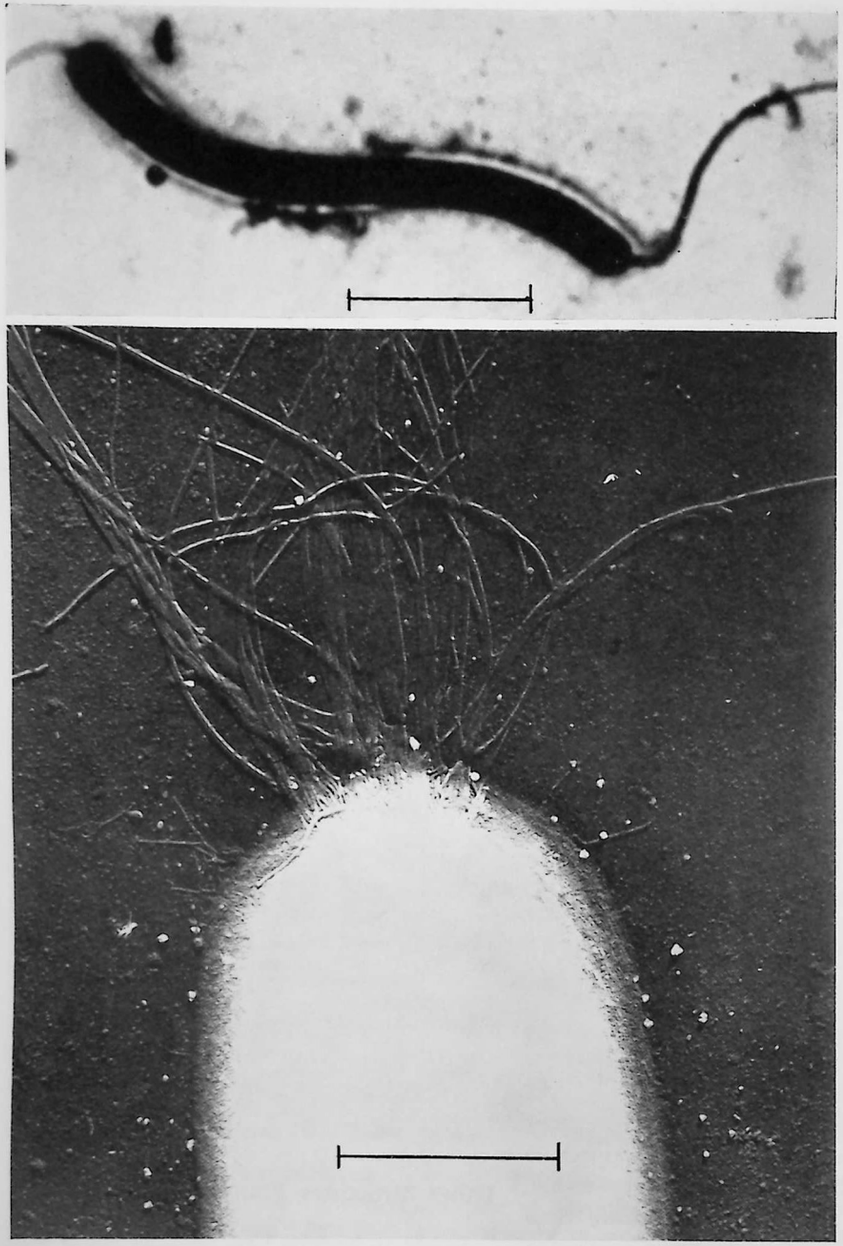


FIG. 3-19. Spirillum volutans, *showing flagella. The upper photo is a flagella stain, showing a single stranded flagellum. The line represents 10 μ. Below, is an electron micrograph of the same organism, showing that the flagellum is composed of several strands. Note that the flagellum strands originate below the cell wall and pass through it. The line represents 1 μ. Note also how much thicker the stained flagellum is than it ought to be.* [*From Williams and Chapman,* J. Bacteriol.; **81,** *195 (1961). Courtesy of Dr. Chapman.*]

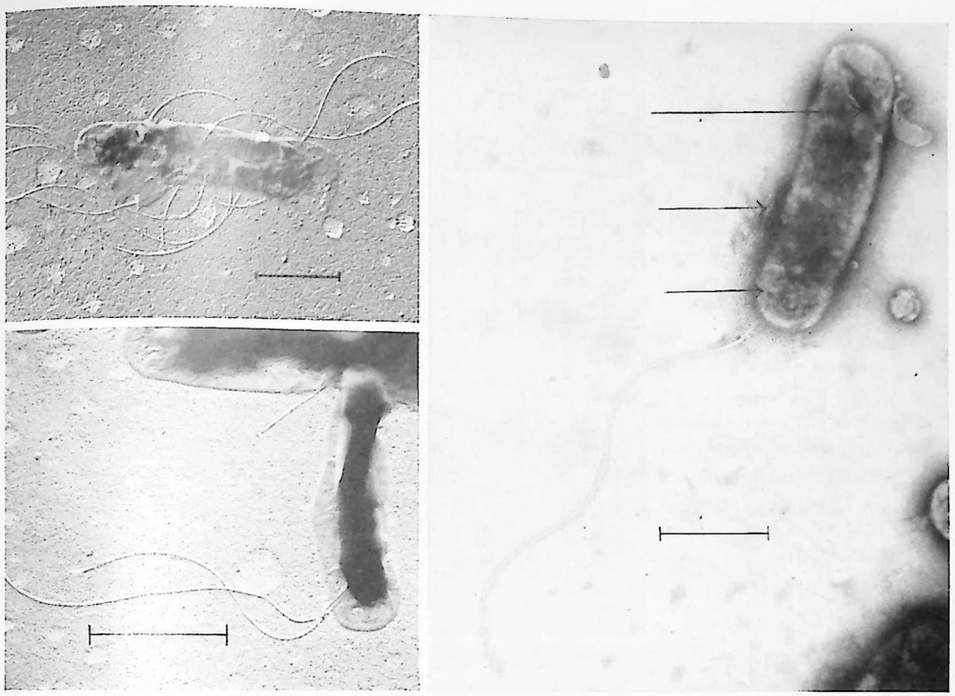


FIG. 3-20. *Origin of flagella. The scale marked on the figures is 1 μ. All are prepared from* Xanthomonas uredovorus. *Upper left, a partially autolysed cell with flagella originating beneath the cell wall. Right, a flagellum from its origin within the cell to its final emergence. Lower left, the emergence of flagella through the cell wall.*

Flagella

About one-fifth to one-tenth of the known bacteria can move in a movement different from Brownian movement. Bacteria are so small in size that they are subject to movement by the bombardment of particles of molecules in their environment (Brownian movement), but definite motion for any distance is achieved by the **flagella** (singular, flagellum). The flagella are long, whiplike appendages, illustrated in Fig. 3-18. In the electron microscope (Figs. 3-19 and 3-20) they sometimes look like braids of rope. It is not exactly certain just how they propel the cell; some people believe that they push it, others that they pull it along. Cocci generally do not possess flagella. Among the rods or cylindrical forms, roughly about one-half of them are motile and possess flagella. Among the spirals most of the organisms are mobile. Flagella are variously termed: a single one is monotrichous, more than one, multitrichous and peritrichous (Fig. 3-21). Mere mechanical agitation breaks them off. If, for example, a suspension of a motile strain is put into a mixer and stirred for a few moments, the flagella are broken off; the cells may then be centrifuged down and pure flagella isolated from the supernatant fluid. They are too small to be seen on normal staining procedures although they are

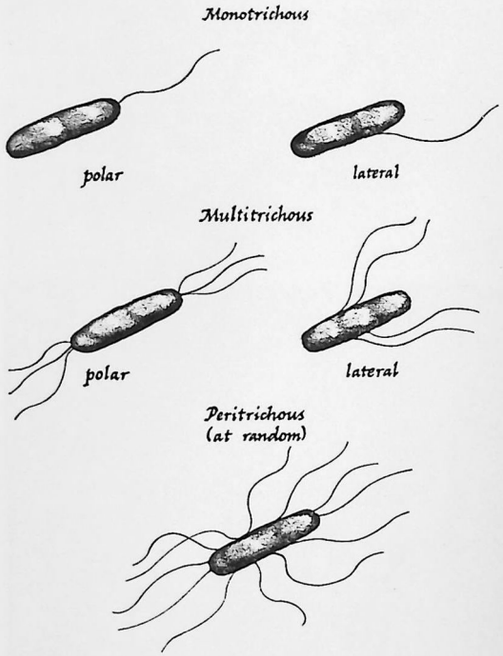


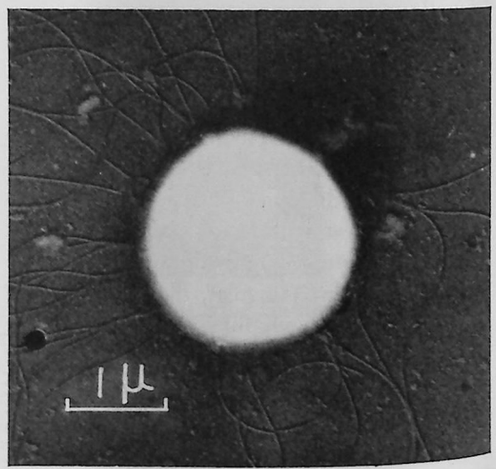
FIG. 3-21. *Names applied to the number and location of bacterial flagella.*

readily visible in the electron microscope. Before electron microscopes were available, flagella could be stained by precipitating materials on them (generally tannin mixed with alum) and staining this. A typical flagella stain is as follows.

Leifson flagella stain: Mix, just before use, 2 ml of a saturated water solution of ammonium alum, 1 ml of a 20% tannic acid solution, 1 ml water, 1.5 ml 95% alcohol, and 0.3 ml of a saturated alcoholic solution of basic fuchsin (as the dye), in the order given. Flood an *unfixed* smear with this solution and let stand 10 minutes, or until a "metallic scum" forms over the surface, due to the precipitation of the aluminum tannate. Wash in water; counterstain if desired with methylene blue. Such an unstable solution will precipitate aluminum tannate on any scratch, projection, or even piece of protein on the slide; hence, the secret of flagella staining is to have all equipment scrupulously clean. When you see "stains of flagella," it is necessary to remember that you are not looking at the flagella but at an aluminum tannate precipitate caused by the presence of flagella.

Flagella are protein and are denatured by heat. They are not essential to the life of the cell: in the first place, there are nonmotile strains; second, strains possessing flagella may be grown under conditions where no flagella develop. There is further a "phase variation," in which flagella are present during one phase and absent during another. All these things are alterable without influencing the life of a cell. Similarly, flagella once formed may be broken off, but the cells will remain viable. Flagella seem to be attached not to the cell wall but to the cell membrane or below it; protoplasts or spheroplasts, freed of the cell wall, still retain their flagella (Fig. 3-22).

FIG. 3-22. *Spheroplast of* Bacillus megaterium. *The normally cylindrical cells become spherical. Cells fixed with formaldehyde and shadowed with palladium.* [*From Kawata, Asaki, and Takagi,* J. Bacteriol., **81**, *160 (1961). Courtesy of Dr. T. Kawata.*]



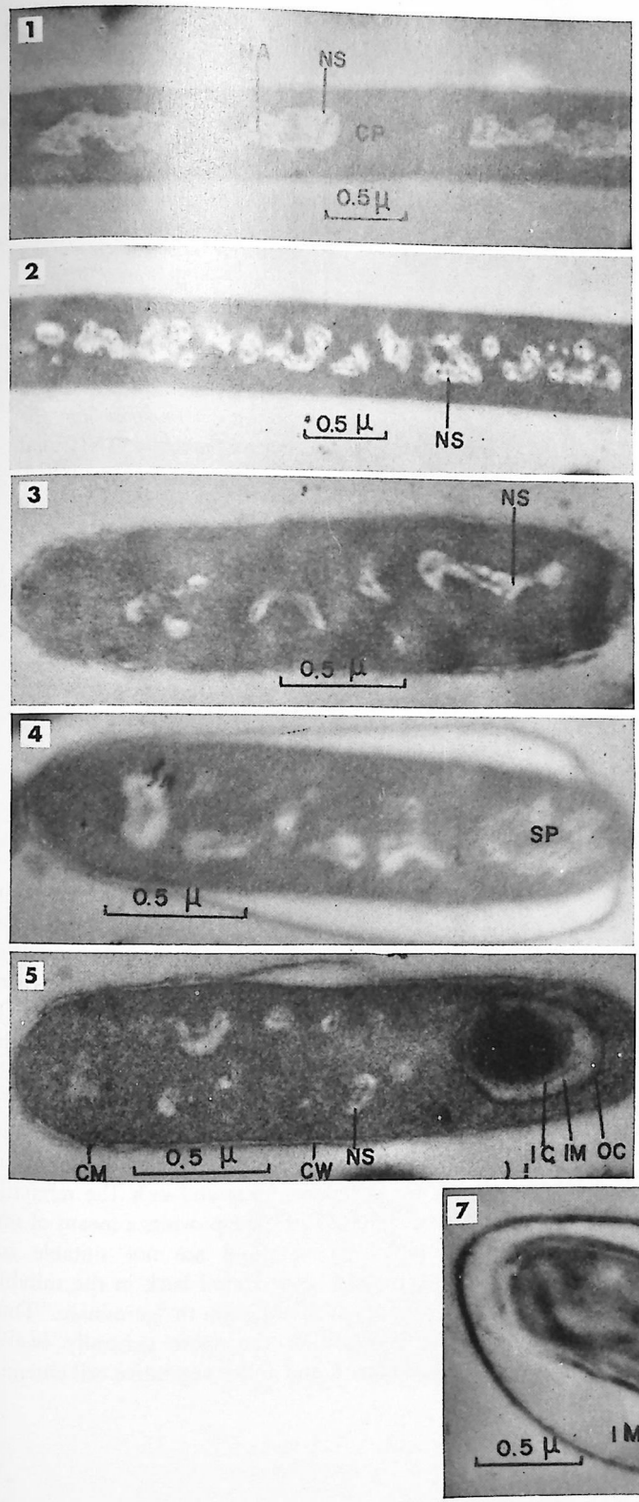


FIG. 3-23. *Electron microscope photographs of the process of spore formation in* Clostridium butyricum. *The original vegetative cell (1) in a 7-hour culture with nuclear material and cytoplasm (cp). At 8.5 hours (2) there is a serpentinelike arrangement of the nuclear material. At the beginning of sporulation at 17 hours (3) there appears to be an enlargement of the nuclear material at one end of the cell and around this nuclear site (4) a spore begins (sp). Soon (5) an outer coat develops about the area which thickens (6) and the inner contents shrink. Finally, at 72 hours, a liberated mature spore (7) may be seen.* [*From Takagi, Kawata, and Yamamoto,* J. Bacteriol., **80**, *37 (1961). Courtesy of Dr. T. Kawata.*]

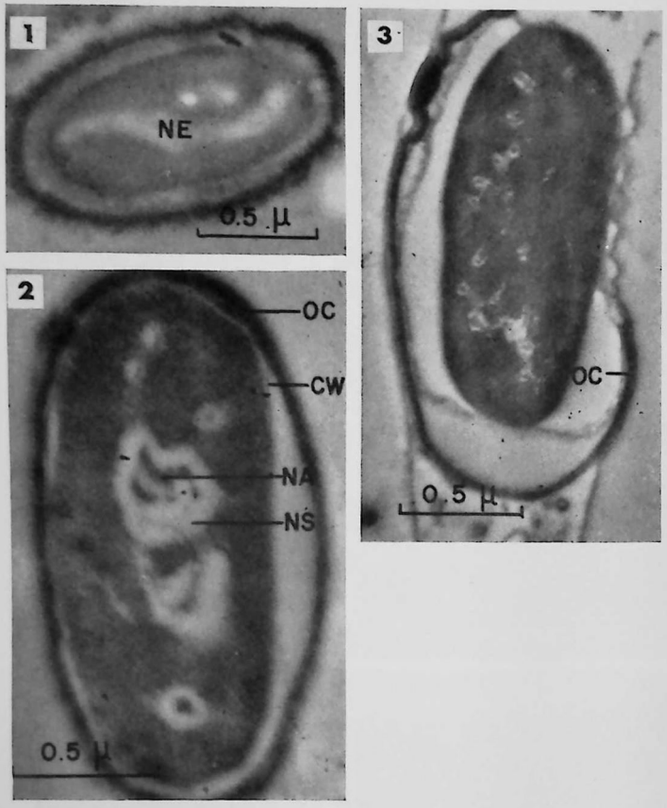


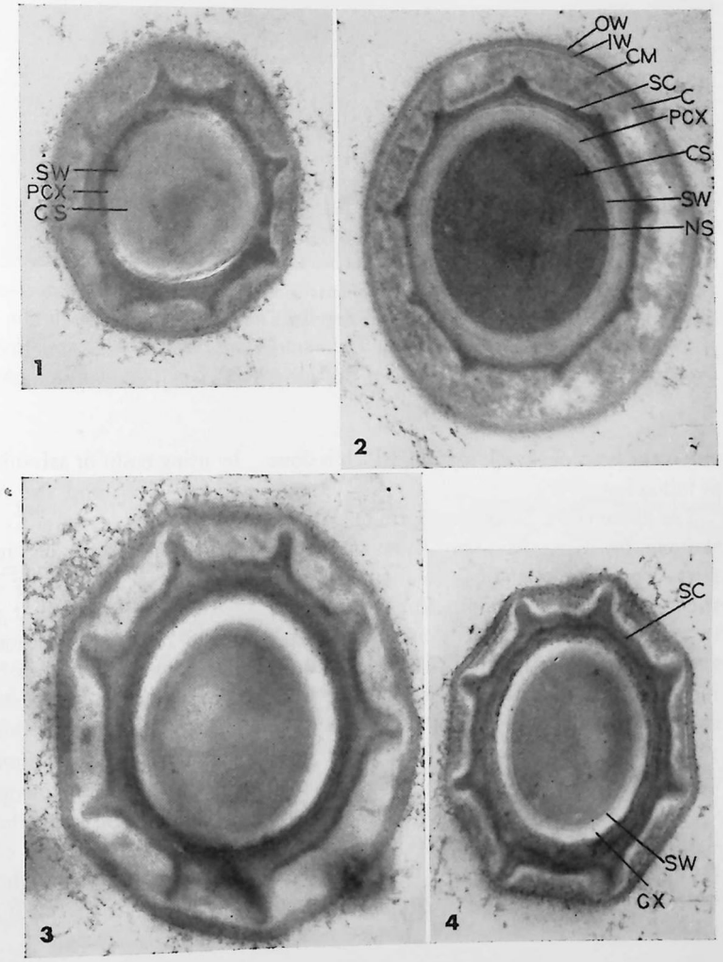
FIG. 3-24. *Electron micrographs of ultrathin sections of germinating spores of* Clostridium butyricum. *No. 1 is after 5 minutes' incubation. The spore has decreased in density. NE = nuclear element. No. 2 is after 30 minutes; the spore has swollen, showing the 3-layer outer coat (OC). The cell wall (CW) of the vegetative cell is visible inside the spore and the nuclear material (NH and NS) has been concentrated. No. 3 shows the vegetative cell escaping from the outer coat (OC) which has ruptured. [From Takagi, Kawata, and Yamamoto, J. Bacteriol., 80, 37 (1961). Courtesy of Dr. T. Kawata.]*

Spores

Several kinds of bacteria have the ability to form **spores.** The spore is usually refractile and is quite noticeable in the light microscope. There is one spore formed per cell; hence it is called an **endospore** (within the cell). The spores are capable of remaining alive under conditions where the vegetative cell—i.e., the cell that has not formed a spore—would be killed. Most spores can be dried; many are not killed even by boiling for several minutes. The organisms capable of producing spores are almost all cylindrical organisms and are placed in two genera: those that are aerobic, that require air, are placed in the genus *Bacillus.* The second group of rod-shaped organisms which possess spores are anaerobic, or grow without air, and they are all placed in the genus *Clostridium.* The common mode of spore formation is the following. In the vegetative cell there appears a tiny **refractile** granule (Fig. 3-23). ("Refractile" means that it will bend the light rays in the microscope.) This refractile granule gradually enlarges, or the spore "matures." Finally, a structure results which is refractile and resistant to dyes. Frequently the vegetative part of the cell drops away and only the refractile spore is left. This spore provides a means of survival when conditions are not suitable for growth, and when placed back in the suitable environment it will begin to "germinate." During germination the spore generally swells, breaks apart, and a new vegetative cell emerges

(Fig. 3-24). The manner in which the cell comes out of the spore differs from one species to another and can be used to characterize such species. In most cases the spore can be considered comparable to the seed of a plant except that there is only one spore per bacterial cell. Spores are not always formed. Spore formation depends on the conditions of the growth and is not yet well understood. Further, the cause of the resistance to adverse conditions is not known. The spore itself has a heavy coat (Fig. 3-25) which resists staining by dyes. This prop-

FIG. 3-25. *Electron micrograph of a thin section of a spore. The organism is* Bacillus polymyxa *and the photographs are arranged in an order thought to represent the sequence of stages in spore maturity. SW is the spore core wall, CS is spore cytoplasm, OW and IW are the outer and inner cell walls, SC is the spore coat; the remainder of the designations do not concern us. [From Holbert, J. Biophys. Biochem. Cytol., 7, 373 (1960). Courtesy of Miss Pauline E. Holbert.]*



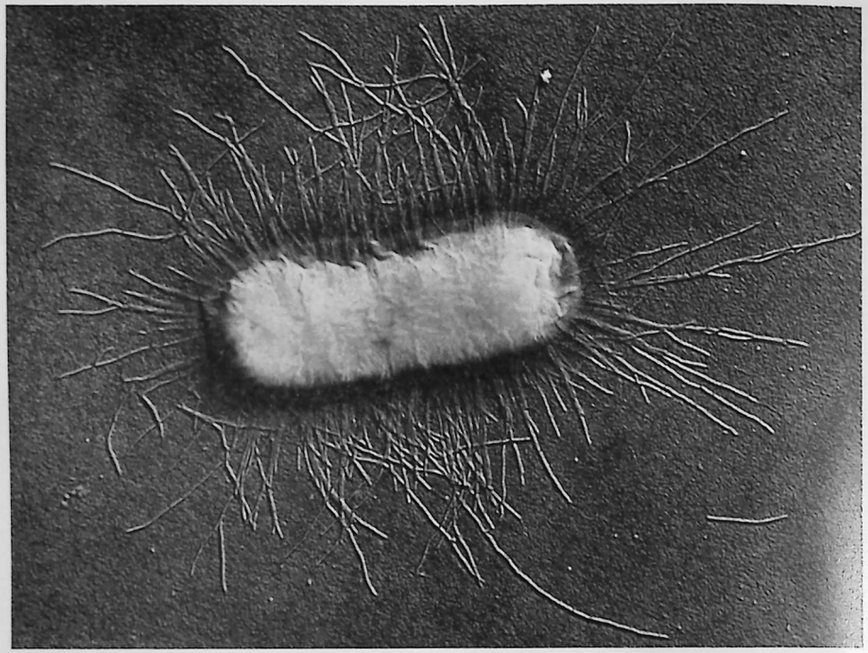


FIG. 3-26. *Fimbriae in bacteria. This is a cell of* Klebsiella *sp., shadow cast with gold-palladium. The strain is* nonmotile, *possessing no flagella. The capsule has been shrunk by drying and partially obscures the margins of the cell wall. The fimbriae are the threadlike projections from the cell.* [*From Duguid,* J. Gen. Microbiol., **21,** *271 (1959) ×40,000. Courtesy of Dr. Duguid.*]

erty is the basis of the spore stain, which is done as follows.

The spore stain: Since spores are resistant to staining by dyes, they may show up as unstained portions of the cell in simple stains—the gram stain, for example. But once the dye is applied—by somewhat drastic methods—the spores resist its removal. The cells containing the spores are heated with a very water-soluble dye such as Malachite green. The preparation is then washed with water until all the water-soluble dye has been extracted from the cells—but not from the spores. Since it takes heat to get the dye to combine with the spores, it takes heat to get it out. The cells can be stained with a contrasting color, red, by using eosin or safranin; the spores will then appear green and the remainder of the cells red.

The spore coat has in it the same type of muramic acid structure as the cell wall, but it has also a high content of dipicolinic acid; see Fig. 3-12 for the relation of this substance to diaminopimelic acid or lysine, which occurs in the cell walls of vegetative cells. This acid absorbs strongly in the ultraviolet and one method of studying spore formation is to use the ultraviolet microscope since spores, due to the dipicolinic acid they contain, are easily visible. This acid also combines with calcium and spores are very high in calcium. They only have about half as much DNA as the vegetative cell and, when the spore is cut open, there is no structure that one can discern inside; the material appears to be uniform.

Other Structures

There are a few other organisms that have resistant cells which are not exactly spores. *Azotobacter,* for example, forms a thickly coated cell, called a **cyst,** which is resistant to drying and to heat, but in general most other bacterial cells do not possess this property.

There are some further structures being revealed by the electron microscope which would otherwise be probably unsuspected. One of the most interesting of these are filamentous appendages, smaller and more numerous than flagella. They are called **fimbriae,** illustrated in Fig. 3-26. It was first thought that they might be dried strands of capsular substances, but they have now been demonstrated on noncapsulated mutants and they appear to be distinct organs. Figure 3-27 shows a noncapsulated mutant of the same organism as shown in Fig. 3-26. Fimbriae are widely distributed among the gram-negative organisms.

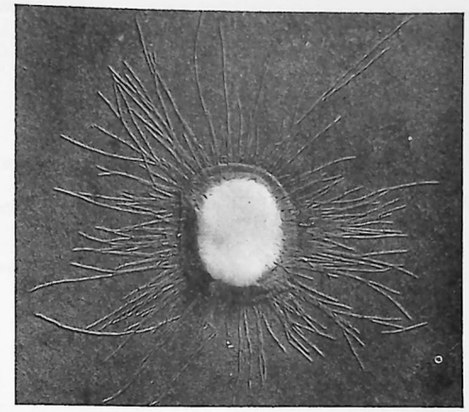


FIG. 3-27. *Fimbriae in a noncapsulated mutant. This noncapsulated mutant is derived from the same strain shown in Fig. 3-26. Note how the lack of a capsule permits the clear appearance of the cell wall.* [*From Duguid,* J. Gen. Microbiol., **21,** *271 (1959). ×40,000. Courtesy of Dr. Duguid.*]

The presence of fimbriae causes the cells to adhere to other cells, so fimbriae are thought to be organs of attachment.

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For background information, see page 491.

QUESTIONS

1. Define or identify:

cells
coccus, cocci
bacillus, bacilli
spirillum, spirilla
micron
flagellum, flagella
spore
capsule
cell wall
cell membrane
protoplasm
cellulose
supersonic vibration
microdissection
lysozyme
0.6 *M* solution
osmotic pressure
arginine
proline
tyrosine
tryptophan
phenylalanine
cystine
cysteine
methionine
diaminopimelic acid
lysine
complex polysaccharide
hexosamine
muramic acid
N-acetylmuramic acid
aromatic amino acid
small particle fraction
semipermeable
active permeability
passive permeability
stereospecific permeability
diffusion permeability
ketonic group
hydroxyl group
permease
citric acid
isocitric acid
cis-aconitic acid
mitochondria
microsome
nucleus
genes
genetic determinant
DNA (deoxyribonucleic acid)
cytoplasm
slime layer
glutamic acid peptide
fructose
Brownian movement
sphaeroplast
cyst
monotrichate
amphitrichate
lophotrichate
peritrichate
phase variation
endospores
genus *Bacillus*
reducing sugar
lipid
respiratory enzymes
hexagonal packing
pore size
phospholipid
choline
protoplast
mannose
phosphatase
genus *Clostridium*
refractile granule
vegetative cell
germinate
negative stain
nigrosine
malachite green
dipicolinic acid
penicillin
fimbriae

2. The study of cell structure has been called "bacterial anatomy." Why is this study of importance in microbiology?
3. What are the approximate dimensions of a coccus? a bacillus? How can their dimensions be accurately determined?
4. Give two methods of preparing specimens for microscopic examination. For what kind of specimen could you use each?
5. What are the functions of a capsule or slime layer?
6. What is the importance of a spore to a bacteria? to man?
7. List three structures which are always present in any bacterial cell. Which are removable if the cell is killed?
8. List three structures which may or may not be present on a bacterial cell. What is their function?
9. How does the cell wall of gram-negative and gram-positive bacteria differ?
10. What specific methods would you employ to stain:
(a) spores, (b) capsules, (c) flagella.

Chapter 4

GROWTH

"No single thing abides, but all things flow. Fragment to fragment clings; the things thus grow until we know and name them. By degrees they melt, and are no more the things we know."

LUCRETIUS FROM MALLOCK, *Lucretius on Life and Death*

BACTERIA REPRODUCE by binary fission; one cell becomes two. Between divisions the individual cell cannot really "grow" very much, i.e., it can only grow to about twice its size, then the cell divides (Fig. 4-1). When we speak of bacterial "growth" we mean an increase in the numbers of cells. When bacteria have grown, it is not because each bacterium is larger, but because there are more bacteria.

In one sense, growth means the same thing in higher organisms. A small rabbit grows into a large rabbit not because each of its cells is larger, but because the larger rabbit has more cells. While the growth in size of a rabbit is not exactly comparable to the growth of a bacterial colony, one should realize that under most circumstances the word "growth" means the development of more cells.

If growth means increased numbers of cells, then growth and reproduction cannot be separated. All true bacteria reproduce by binary fission, a process not involving sex. Some true

FIG. 4-1. *"Growth" and division in binary fission.*

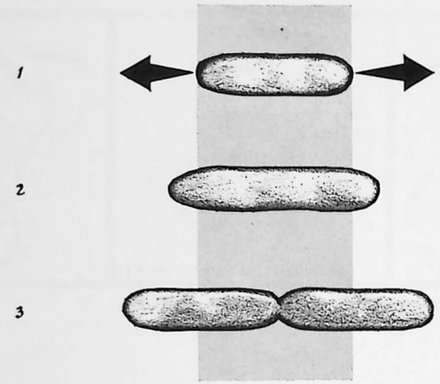


FIG. 4-2. *Diagram illustrating cell elongation in two directions.*

bacteria may have, in addition to binary fission, a "sexual" process (to be discussed in Chapter 11) but in these cases most of the cells reproduce by binary fission and the sexual reproduction is a rare event. There are a few microorganisms that employ other kinds of cell division; these we classify as "higher bacteria."

Nature of Binary Fission

There are two broad theories as to the nature of binary fission. In the first theory, the cells are assumed to have grown in two directions, and after such growth there is the formation of a membrane and a cell wall down the center of the elongated cell. It is supposed that division takes place as illustrated in Fig. 4-2. If one watches many bacteria under the microscope, or better, takes time-lapse movies of the growth of individual cells (as shown in Fig. 4-3) one obtains the impression of elongation in two directions. Certain electron micrographs (as shown in Fig. 4-4) also appear to show the development of the cell wall, starting at the edge of the cell section and progressing inward.

Such evidence is also compatible with the second theory of binary fission—that the cell grows longitudinally in one direction only, as diagramed in Fig. 4-5, a process which resembles "budding" in yeasts. The evidence for such a concept is sketched in Fig. 4-5—that many times flagella may be found attached only to *one* cell of a recently divided pair (Fig. 4-5). Theoretically, the mother cells retain the major part of the original cell walls and flagella, and, since the development of flagella takes time, the daughter cells do not possess them. Time-lapse photographs of cell division in *Spirillum* (Fig. 4-6; compare 2, 4, and 5), do not seem to support the idea of budding in this group. Perhaps a similar argument could be used with fimbriae (Fig. 4-7), but one is not sure which is the "mother" and which the "bud" in such photographs.

We might further ask the question as to whether there is a direction to the growth and division of individual cells. For rod-shaped organisms, illustrated in Figs. 4-3, 4-6 and 4-7, there is obviously such a direction. The cell does not divide or "bud" at its side. There are

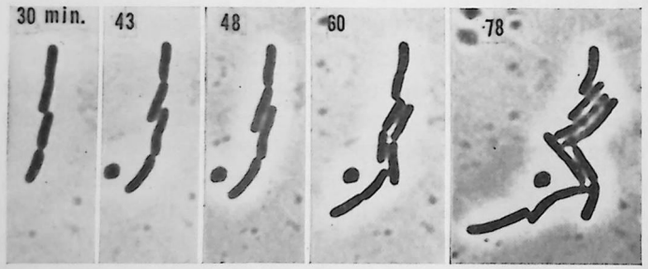


FIG. 4-3. *Time-lapse photographs of the growth of* Escherichia coli. *The numbers at the top represent minutes. This is the same group of cells in the phase-contrast microscope. Note especially the four cells at 43 minutes and 5 minutes later, which gives the impression of elongation in two directions. The organisms are growing on a solid surface which restricts movement. [From Hoffman and Frank,* J. Gen. Microbiol., **25,** 353 *(1961). Courtesy of Dr. Hoffman.]*

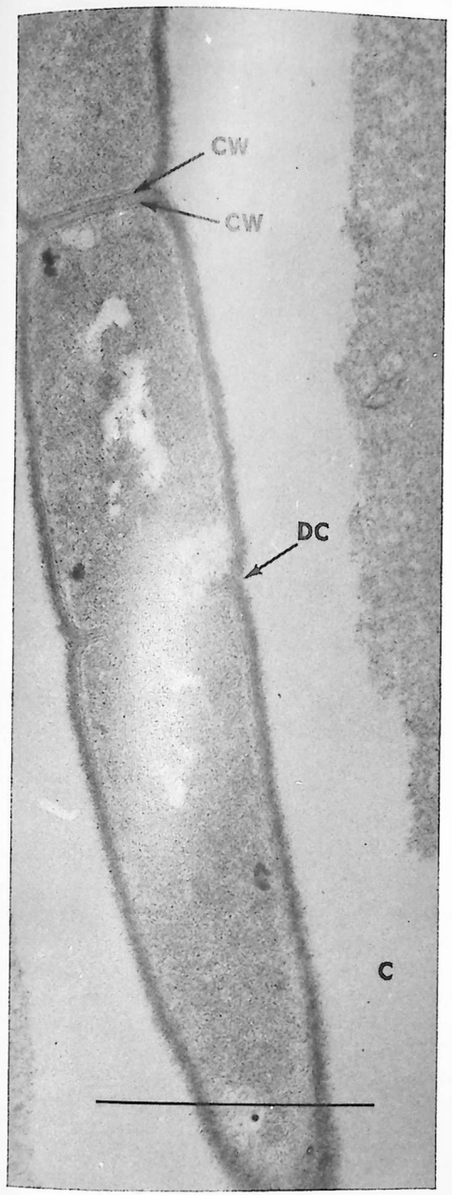


FIG. 4-4. *An electron micrograph of* Bacillus anthracis *in the process of cell division. The cell walls* (CW) *between the two cells are clearly evident. The invagination of the dividing cell* (DC) *makes it seem likely that cell wall formation starts from the edge of the cell after longitudinal growth. This is taken from a section of mouse spleen. C represents an area of the mouse tissue which shows damage due to the bacterial cell. [From Roth, Lewis, and Williams,* J. Bacteriol., **80,** 772 *(1960). Courtesy of Dr. R. P. Williams.]*

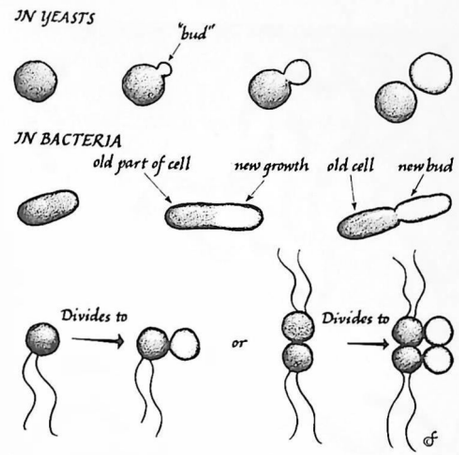


FIG. 4-5. *Budding as a form of binary fission. In yeasts (top line), the process of cell division is budding, as shown. The bacteria, smaller than the yeasts but shown here the same size for comparison, could grow laterally in one direction only, then split off the bud. Evidence favoring this idea is shown in the third line. In some dividing bacteria, only the mother cell retains the flagella; the daughter cell (or bud) must grow its own later. If the process were simple division, one might expect each daughter cell to have flagella.*

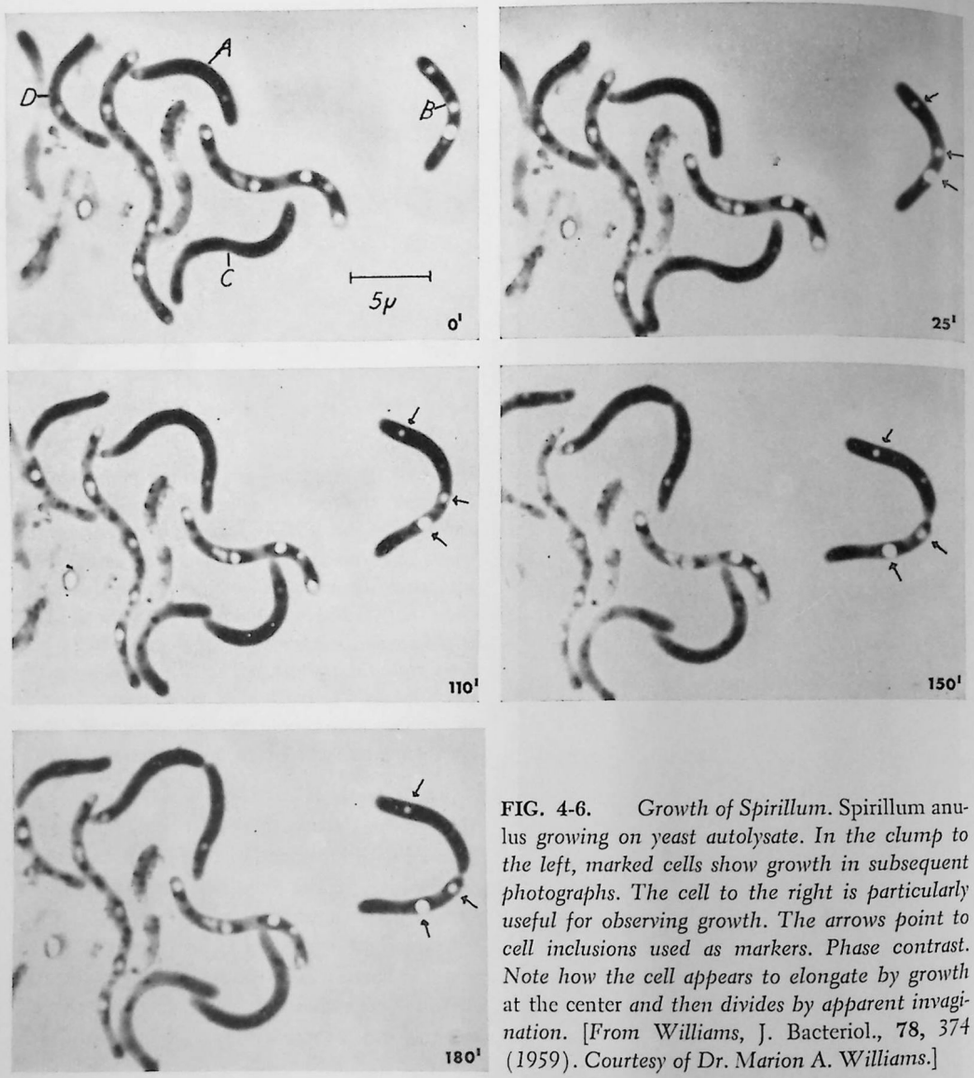


FIG. 4-6. *Growth of Spirillum.* Spirillum anulus *growing on yeast autolysate. In the clump to the left, marked cells show growth in subsequent photographs. The cell to the right is particularly useful for observing growth. The arrows point to cell inclusions used as markers. Phase contrast. Note how the cell appears to elongate by growth* at the center *and then divides by apparent invagination.* [*From* Williams, J. Bacteriol., **78,** *374 (1959). Courtesy of Dr. Marion A. Williams.*]

organisms that do this, but we do not think they are true bacteria. At least most bacteria do not act in this irresponsible fashion.

But what about spherical organisms? Do they know which way is up? Is there a directional force in division, or, if all sides of the sphere are equal, does it matter which side they divide from? The answer, as shown in Fig. 4-8, is both yes and no. Some spheres have a definite direction and pattern of division; others do not.

The consequences of binary fission, this "one dividing into two," are fantastic; Fig. 4-9 shows what happens when one cell divides, and each cell so formed divides again, and each again,

and each again, and so forth, and so forth. One cannot plot this deluge reasonably in an ordinary way; the numbers become astronomical.

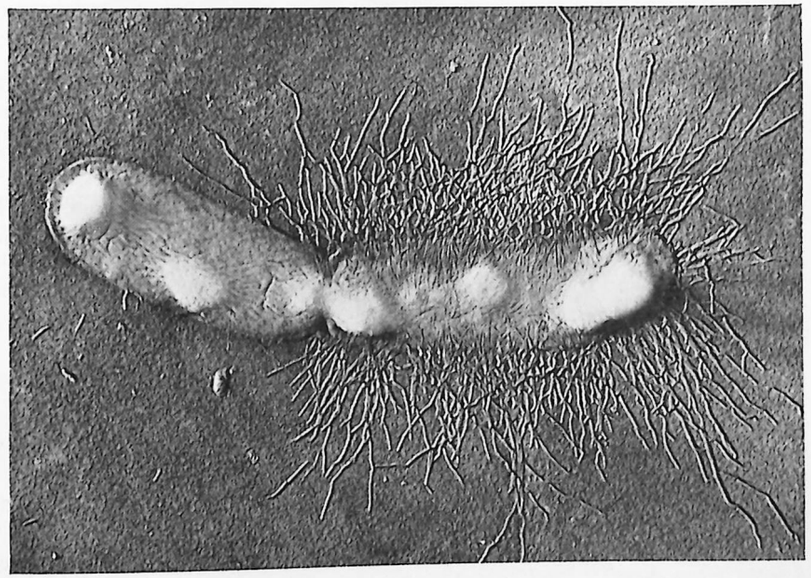
The Growth Curve

If we plot the numbers of organisms in a bacterial culture at various times, or after several generations, as has been done in Fig. 4-9, we soon run out of space because of the large numbers of bacteria. But we may observe that if we start with one cell (the zero generation, i.e., $2^0 = 1$) at the end of the first generation there will be 2 cells ($2^1 = 2$), the second generation 4 (2^2), the third generation 8 (2^3), etc.; the nth generation will have 2^n cells. This is a simple exponential increase, which can be handled by plotting the logarithm of the number of cells against time. In case you have forgotten how to handle logarithms, we have provided an elementary digression on logarithms, their care and use, at the end of this chapter (p. 108). If you are not familiar with logarithms, it would be best to study that section now; if you are familiar with them, skip that section when you come to it.

When we plot the logarithm of the number of cells against time, we obtain a curve like that of Fig. 4-10. This curve is called a **growth curve.** Frequently it is divided into eight sections, called **growth phases;** they have been named as follows (the numbers correspond to those of Fig. 4-10).

1. Lag phase
2. Accelerated growth phase
3. Log phase
4. Decelerated growth phase
5. Stationary phase
6. Accelerated death phase
7. Death phase
8. Survival phase

FIG. 4-7. *Fimbriae on recently divided cells.* Klebsiella *sp., shadow cast with gold-palladium. One daughter cell is fimbriate, the other nonfimbriate.* [*From Duguid,* J. Gen. Microbiol., **21,** *271 (1959). ×25,000. Courtesy of Dr. Duguid.*]



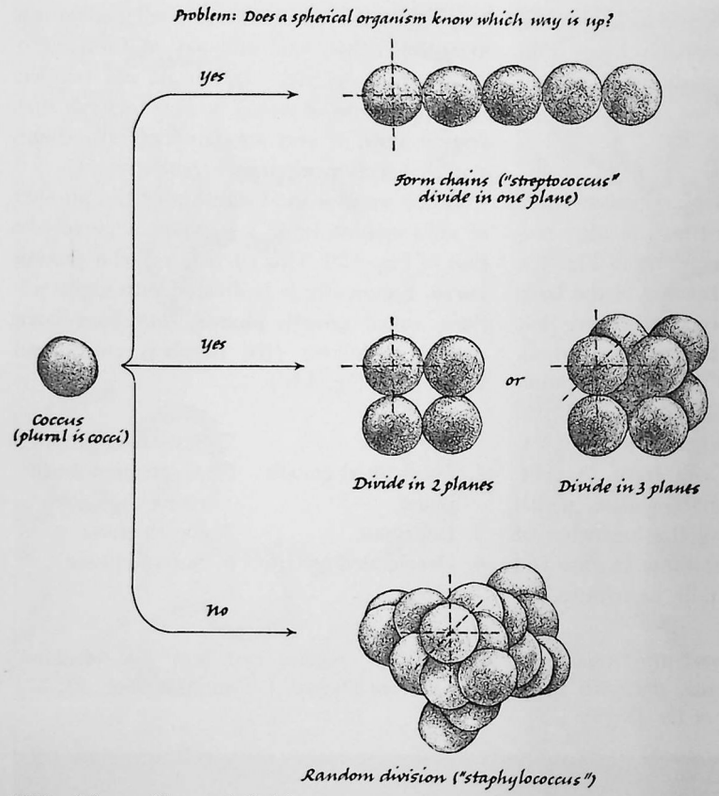


FIG. 4-8. *Types of division in cocci.*

We have the distinct feeling, however, that the division into eight phases is a little too many, and that phases 2, 4, and 6 are merely intermediates between two distinct phases, or are caused by mixing two phases. We shall therefore divide the growth curve into five phases, as shown in Fig. 4-11. We shall discuss each major phase separately.

The **lag phase:** During this period, after transfer to a new medium, there is a period of no apparent growth. The lag phase occurs when:

1. The inoculum used for the new culture was obtained from a previous culture in the stationary phase or beyond, and the inoculation is made into a suitable medium. It does not generally occur when the inoculum comes from the log phase.

2. When the inoculum, which may come from any phase, is placed in an unsuitable medium.

The length of the lag phase is dependent upon:

1. The kind of bacteria.
2. The medium the inoculum was taken from.
3. The medium into which it was inoculated.

(In both 2 and 3, the better the medium, the shorter the lag.)

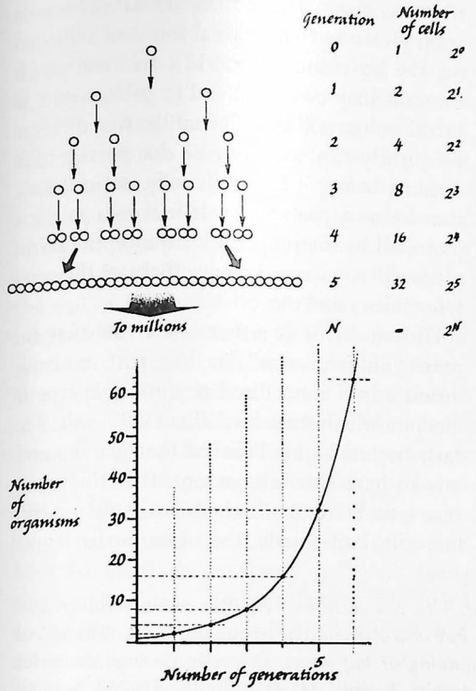


FIG. 4-9. *The numerical consequences of binary fission.*

4. The phase of the culture from which the inoculum came (the closer to the log phase, the shorter the lag).

5. The size of the inoculum. The larger the inoculum, the shorter is the lag phase.

In an effort to explain the existence of a lag phase, and to account for the circumstances that influenced it, certain theories have been proposed:

1. The organism must modify itself.

(a) To recover from injury which occurred in the stationary phase or during the transfer from the old medium.

(b) To adapt itself to the new medium.

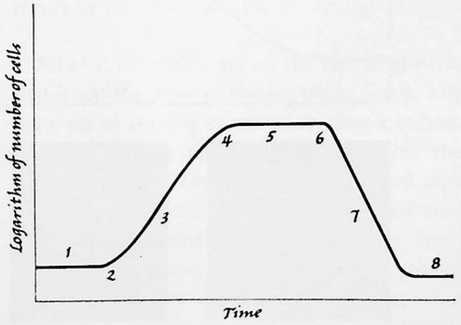
2. The organism must change the medium.

(c) It has been supposed that the organism had to secrete a substance into the medium necessary for growth.

For all of these theories there is some support, but most of the evidence was obtained before knowledge of the nature of the bacterial cell wall and the process of division was very clear. Hence it is best to describe the actual situation in beginning growth. From this information it is relatively simple to see how these theories arose, and to understand the nature of the lag phase.

Let us visualize a situation which has few variables: a bacterial cell inoculated into a good medium but inoculated from a culture (in the same medium) from the stationary phase or beyond. The organism from the stationary phase has stopped growing for any of a variety of reasons (discussed in the section on the stationary phase). When it is placed in the new medium, the food materials of the new medium penetrate it, and the cell's machinery starts to function. The metabolism per cell increases—note that this increase is per cell, not per unit nitrogen or per unit mass—and the cell becomes larger. At a certain point another stimulus causes the cell to divide; we are not sure just what the stimulus is. At the start there may be a delay in this because, before a cell can divide, it must not only have the proto-

FIG. 4-10. *The logarithmic growth curve.*



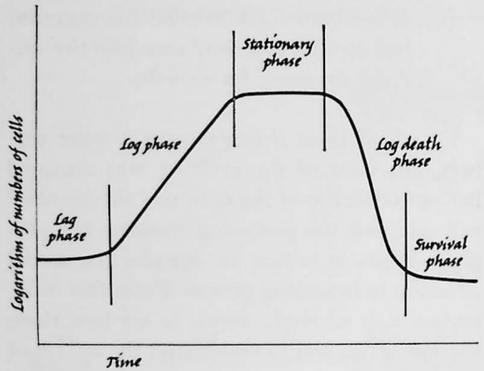
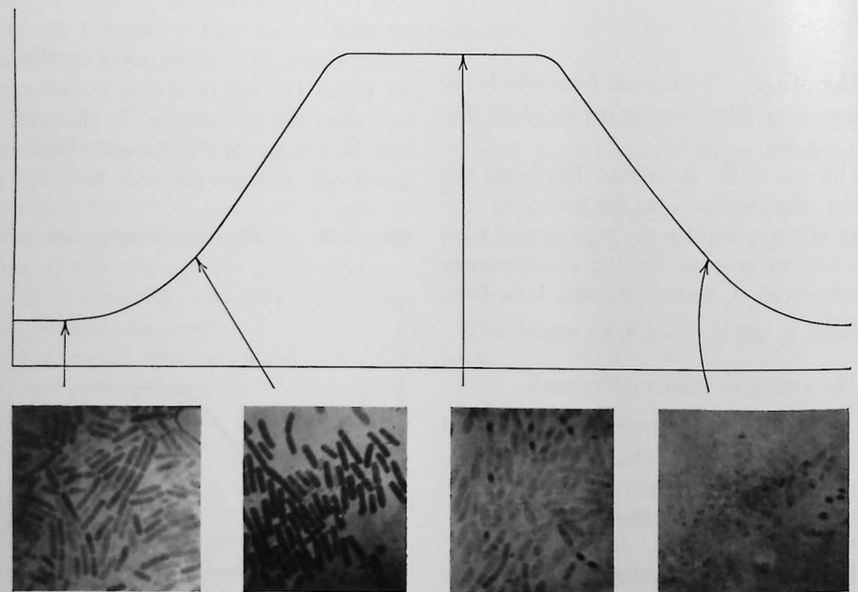


FIG. 4-11. *The five phases of the log growth curve.*

plasm for a new cell but a new cell wall too. Sometimes these processes are not synchronized: the cell may divide before it has enough cell wall for two cells, or it may not divide until it has grown larger than normal. This situation gives rise to abnormal forms of cells during the lag phase (Fig. 4-12). In some media the cells may even die, and so a large part of the inoculum will be killed on the first division. Frequently this seems to be due to the cell's dividing before it has built sufficient cell wall, thus forming protoplasts. If it is in a medium protected by the proper salt balance, the protoplasts will survive and grow; otherwise they may lyse (burst) and die.

Historically, it is rather interesting that the term "nutrient agar" in the past generally meant a beef extract and peptone agar type of medium which contained about 0.3% salt. The early bacteriologists believed that it was necessary to have this salt present, that the organisms grew better; but today's media do not contain salt. Particularly, the older media tended

FIG. 4-12. *Size of organisms in the log phase. This is a culture of* Bacillus cereus *photographed in ultraviolet light, to show not only the cell size but also the content of nucleic acids. The size of the cells becomes larger in the log phase; at the beginning of log phase, the cells are high in nucleic acids.* [*From Malgren and Heden,* Acta Path. Microbiol. Scand., **24,** *417 (1947). LS-232.* ×*1500.*]



to develop many bizarre forms during the late lag phase and so was regarded by many as toxic. Today we have a better understanding of what was happening. The older bacteriologists were right in saying that an organism grew better, that the salt was helpful and not toxic. What it did was to provide osmotic protection; protoplasts, then had a chance to develop, whereas in beef extract peptone without salt they were generally lysed. These strange aberrant forms at the end of the lag phase can be considered as adaptations, or as caused by injury or lack of synchronization of cell and cell wall growth. In a good medium the cell would have a chance to obtain all the things it needed and so could become synchronized earlier. In a poor medium it would take longer to achieve this synchronization. In a medium without the amino acids necessary to make the cell wall, the cell would have to build the enzymes for synthesizing these amino acids. Another finding which led to the idea that the cells in the lag phase had to secrete something came from the following kind of experiment. If very few cells were put in a poor medium, there was generally no growth. If exactly this same medium were used with the same number of cells, but with sterile sand or powdered agar added after the cells were inoculated, growth frequently occurred. It would not work if the sand or agar were added beforehand. It was thought that the added sand or powdered agar had swept down the organisms with it as it settled to the bottom. Thus, each organism was growing in a smaller amount of medium than when it was dispersed in the entire tube. If it were secreting something, it would have to secrete much less and could grow much faster. Since it did grow much faster, it was concluded that it must secrete something. The explanation, of course, could also be that the cell had to adjust the *p*H or the oxidation reduction potential or any of a variety of other things.

The **log phase:** Let G represent the time from one division to another and call G the generation time: the time required for one generation is obviously the total time required for n generations, divided by n; or $G = t/n$.

The generation time (G) may be calculated, but only in the log phase. (Why?)

$$G = \frac{t}{n} = \frac{\text{total time elapsed}}{\text{number of generations in that time}}.$$

If the number of bacteria at n generations is B_n and the number of bacteria at the start is B_0

$$B_n = B_0(2^n),$$

$$\log B_n = \log B_0(2^n) = \log B_0 + n \log 2,$$

$$n = \frac{\log B_n - \log B_0}{\log 2}.$$

Hence

$$G \text{ (generation time)} = \frac{t \log 2}{\log B_n - \log B_0}.$$

The factors which control the generation time are

1. The nature of the organism. Nobody, for example, can make the organism causing tuberculosis (*Mycobacterium tuberculosis*) grow as fast as the organism causing diphtheria (*Corynebacterium diphtheria*)—in the laboratory, at least.

2. The temperature. Within the possible limits, for each 10°C rise in temperature one can expect to double the rate of growth.

3. The rate of penetration of the medium. This is influenced by the concentration of materials in the medium and by stirring.

All of these effects are on the rate of growth, that is, on the *slope* of the log phase curve. The duration of the log curve—how long a culture stays in the log phase—has to do with the question of why the log phase ends and why the stationary phase begins. These we shall consider under stationary phase. Eventually, however, growth slows down and a log growth ceases. Growth as such may continue for some

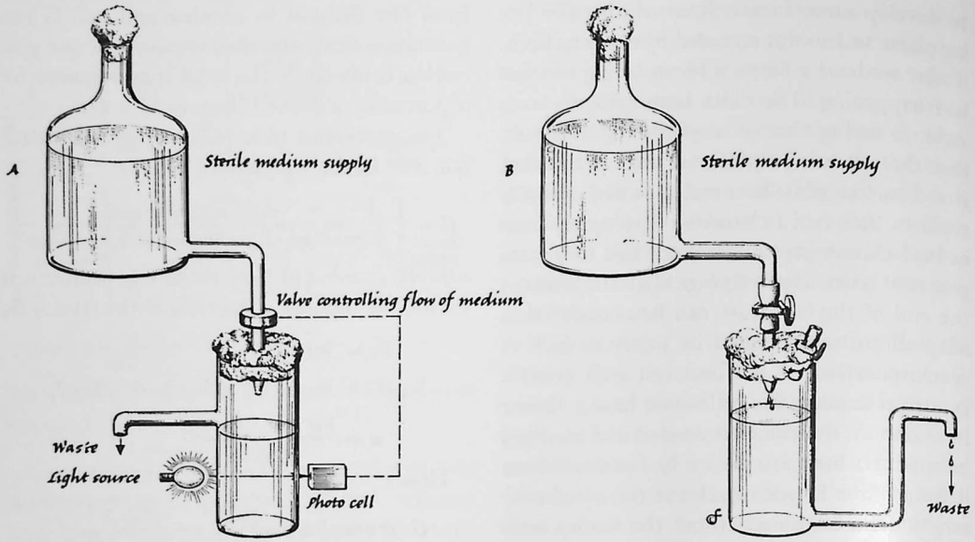


FIG. 4-13. *Examples of chemostats. At the left the flow of medium into the culture is controlled by the turbidity. On the right the medium flows in continuously but at such a rate that the turbidity remains constant.*

time, but not at a logarithmic rate, or it may cease abruptly.

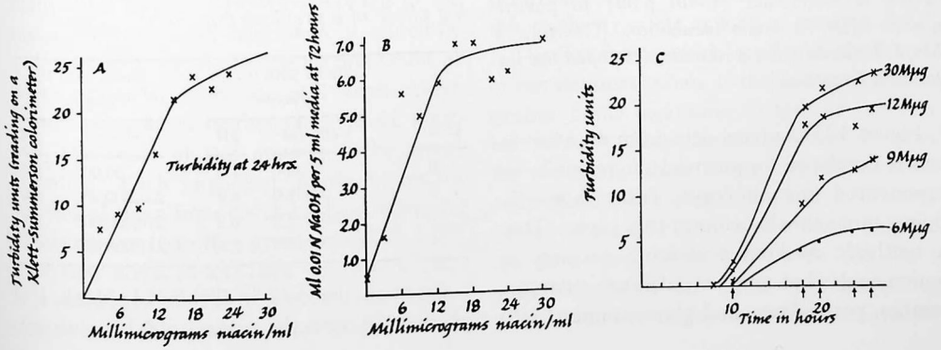
It has always been something of a theoretical problem as to whether a bacterium could stay in the log phase "forever" or whether it had to go into a stationary phase. If we could inoculate an ocean of medium, would the organism keep on growing in the log phase until the ocean was full? We can try this experiment in a slightly different fashion in an instrument called the **chemostat.** (We agree that the name does not seem appropriate.) In this instrument (Fig. 4-13) the population is kept at any desired level by the addition of fresh sterile medium. If we wish to keep a culture in the log phase, we need only set the controls to a turbidity equivalent to half the turbidity of the log phase. As this level of growth is reached, more media is supplied so that the organism is always at this turbidity. (Why does this mean that it is maintained in the log phase?) There is, of course, an outlet tube which permits the excess medium to escape, and the outflow rate is synchronized with the inflow rate. There are two kinds of instruments employed. One, as described above and illustrated in sketch A, Fig. 4-13, has the flow of media controlled by the turbidity. The other type, shown in sketch B, Fig. 4-13, has the media coming in at a preset flow rate, but some nutrient in it is limiting so that the bacteria can grow only as fast as the media enters. With such instruments it is possible to keep cultures growing logarithmically for months on end. What happens to such cultures? Some suffer some degeneration in the process, growth eventually slows, aberrant forms appear, even the products of metabolism may change. Other cultures seem to suffer no ill effects and grow just as merrily as when they started. While some cultures undergo a relatively rapid change, others are relatively stable. Since this is related to their genetic stability (see Chapter 11), the

chemostat is useful for studies of bacterial genetics. The chemostat, or various simplified versions of it, is finding wide use in studies of continual fermentation and in bacterial physiology, for the instrument is capable of providing cells in quantity from essentially any stage of the growth curve.

The **stationary phase:** Under ordinary conditions, when the medium is not continually replaced, log phase growth eventually comes to an end and the stationary phase appears. Why is there a stationary phase? If the medium is continually renewed, as in the chemostat, there need not be a stationary phase. Evidently it is the medium which controls and conditions this phase. Several factors are involved, any one of which is capable of causing the onset of the stationary phase. These are a limited food supply, the accumulation of toxic waste products (such as acids and alcohols), and the effects of crowding. They have the following importance.

1. *Limited food supply.* When the food is exhausted, the organisms of course can no longer grow. We may demonstrate that a food material is a limiting factor for growth by supplying more food, and so obtaining further growth. The "food" may be anything the organism requires—sugar, an amino acid, a vitamin, or even a mineral. So long as *one* of the food materials required for growth is limited, even though all the others are in excess, growth will stop. Within limits, the *amount of growth* will be dependent upon the *amount* of material. These facts make it possible to use bacterial growth to assay for (i.e., to estimate) the amount of amino acids, vitamins, proteins, or any of a variety of materials in complex materials. The basis of a microbiological assay is shown in Fig. 4-14. These are data obtained by a student, after one course in microbiology, who was attempting to devise a simple method of determining niacin (nicotinic acid). He had found an organism of the *Escherichia* genus which required niacin, and he could grow the organism on a very simple medium—merely glucose and mineral salts—providing niacin was added. The amount of niacin required was very small, and near-maximum growth was achieved at about 20 mμg (0.02 μg) niacin per milliliter when the response was measured by turbidity at 24 hours (part A, Fig. 4-14). The organism also produced acid which could be measured (at 72 hours) by titrating with NaOH (part B, Fig. 4-14). With this measurement and with a longer incubation time, near-maximum growth was obtained at 12 mμg/ml. The growth curves at several selected levels of niacin are given in part C

FIG. 4-14. *Simplified assay for niacin. Student results on a mutant of* Escherichia coli. *Turbidity at 24 hours in part A, acid produced in 72 hours in part B; rate of growth shown in part C.*



of Fig. 4-14. They show that not only is the final growth less with lower levels of niacin but the rate of growth is slower and the lag phase is longer. The student found that 0.01 ml of milk gave, after 24 hours incubation, a turbidity of 9 units (when corrected for the turbidity of the milk itself). Thus, 0.01 ml milk = 6 mμg niacin, and 1 ml milk = 600 mμg = 0.6 μg/ml.

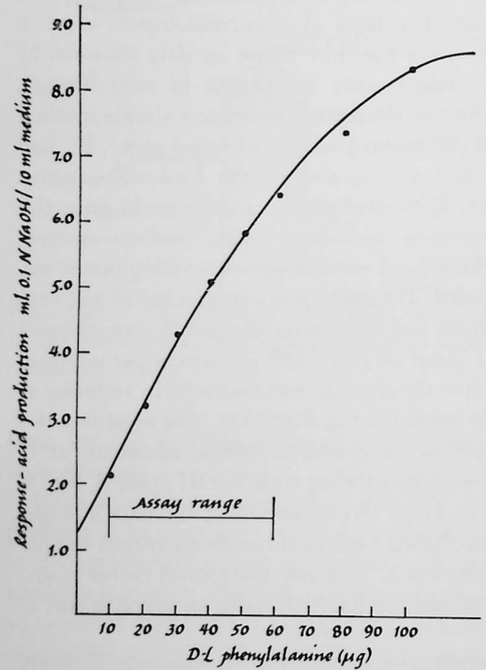


FIG. 4-15. *The assay response curve of* Leuconostoc mesenteroides *(strain p-60) to phenylalanine after 72 hours incubation. [Courtesy of Mr. J. J. Gavin from a recently developed but unpublished method.]*

Figure 4-15 contains data from an assay recently developed for phenylalanine done by an experienced microbiologist (note how the points more closely conform to a curve). Here a synthetic medium containing an array of amino acids (but no phenylalanine), vitamins, purines, pyrimidines, and glucose-mineral salts is the base medium to which increasing quantities of phenylalanine are added. If we have an unknown material, we can use it as a source of phenylalanine (or of any other required nutrient for which we have devised an assay). The amount of growth or the acid produced over the range where phenylalanine is the growth-limiting factor, called the assay range in Fig. 4-15, will give us an estimate of the amount of phenylalanine in the unknown material.

A wide variety of assays of this sort have been devised. The relationship between the concentration of the limiting nutrient and the amount of growth may be linear (a straight line) as in the case of niacin or phenylalanine, or it may be logarithmic, but whenever there is a response of either type it can be used for assay purposes.

There are, then, countless examples where the amount of food available in a medium limits its growth. One cause of the end of the log phase and beginning of the stationary phase is exhaustion of the medium in at least one necessary constituent.

2. *Accumulation of toxic waste products.* Exhaustion of the medium is not the only cause of the stationary phase, because if it were we would expect that a doubling of the concentration of the medium would double the growth.

TABLE 4-1. *Growth of* Streptococcus faecalis *in 1% yeast extract, 1% peptone, 0.5% K_2HPO_4 broth, inoculated 50,000/ml from the log phase of a previous culture*

	Media with 0.1% glucose		Media with 1% glucose	
Hours	Cells/ml	*p*H	Cells/ml	*p*H
0	50,000	7.4	50,000	7.4
6	175,000,000	6.9	268,000,000	5.6
12	320,000,000	6.8	510,000,000	5.1
24	609,000,000	7.6	221,000,000	4.8

Examine the data in Table 4-1. With 1% glucose (10 times the amount of 0.1% glucose)

the culture grows a little faster but never reaches the same high population, largely because it kills itself off by acid production. Obviously in this case the accumulation of toxic products stopped growth. Figure 4-16 provides

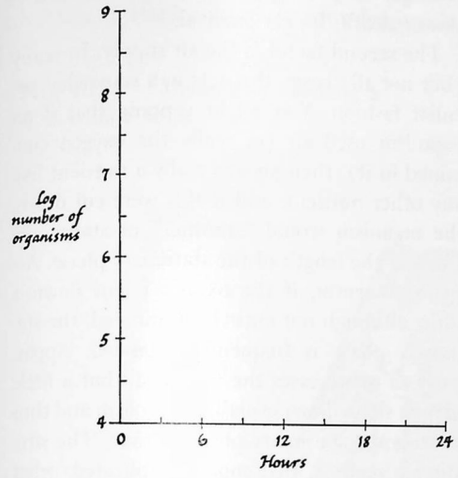


FIG. 4-16. *Graph for plotting the data of Table 4-1 (see facing page).*

an empty graph space in which you should plot the logarithm of the number of cells against time, using the data of Table 4-1 for both concentrations of glucose, to illustrate the kind of a growth curve you obtain. When is the log phase over?

3. *The effects of crowding.* While there are many cases in which growth is limited or the transition to the stationary phase is caused by a limited food supply or the accumulation of waste products, there are still cases where growth stops when neither of these are limiting. The cause of this appears to be actual crowding. Each organism needs a certain amount of space from which to draw nutrients; if there is less than this, growth will stop or at least slow down to less than logarithmic rate. It is sometimes possible to increase the population or raise the level at which the stationary phase occurs by stirring the medium and keeping it well mixed.

How long the stationary phase lasts—i.e., its duration rather than its level—depends on the organism and the specific conditions found in any particular medium after growth to the stationary level. The conditions result from the composition of the medium, the physical conditions in the medium, and the growth products. In a very real sense the population in the stationary phase is not inert. Some cells are growing, some are dying, but they balance each other so that the overall population remains relatively constant. So far as can be judged, however, only a relatively few are growing and only a few dying. Half do not die and half divide each generation time, but only about one in ten; even this rate of turnover can be slowed down.

Preservation of Cultures

To extend the stationary phase at population levels is a matter to which much study has been given. It has two practical elements of importance. First, extension is needed for the preservation of bacterial or other cultures so that we do not need to transfer them to fresh media as often. Second, if the cells are producing something valuable as they metabolize—such as yeasts producing alcohol, bacteria making butylene glycol, molds producing citric acid or penicillin—we should want to keep them in this productive state as long as possible.

The first factor which conditions the length of the stationary phase is the nature of the organism. Some organisms, in spite of our very best efforts to keep them, die off rapidly after the stationary phase is reached. *Diplococcus pneumoniae*, for example, has a very short stationary phase and there is not much that we can do to prolong it. *Escherichia coli* has a much longer stationary phase—perhaps as long as a day or two under favorable conditions—

and while we can shorten the phase markedly by adverse conditions, we cannot lengthen it beyond a given value. This "maximum length" of stationary phase varies from organism to organism. The actual length depends upon the actual conditions. For example, compare curves A and B of Fig. 4-17. The stationary phase with

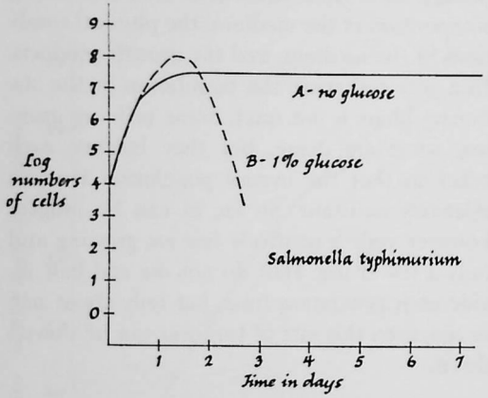


FIG. 4-17. *The influence of sugar, and the acid derived from it, on the length of the stationary phase.*

1% glucose is considerably shorter, for the organisms are killing themselves off with acid. The organism as such is capable of a longer stationary phase, and if we neutralize the acid it will last much longer, but the actual length of this phase is determined by the toxic factor, the acid present. The factors which influence the duration, besides the inherent longevity of the organism itself, are the acidity, the temperature, the air supply, the nutrient supply, and, in addition and perhaps surprisingly, the colloidal condition of the environment.

The effect of acidity is fairly obvious, but is further discussed in Chapter 6, under *p*H. Temperature is also discussed in the same chapter but its action is general enough that we can say here that the lower the temperature, the slower the growth and the slower the deterioration, and thus the longer the stationary phase. In fact, over the biological temperature range, the time over which the pattern of a growth curve occurs is dependent on temperature, although the growth curve looks much the same. Lower temperature is a way of extending time —longer lag, longer log, longer stationary, slower death, longer survival.

The second factor is the air supply. In many (but not all) cases, this acts in a somewhat peculiar fashion. You might suppose that if an organism used air (or really the oxygen contained in it), then air was really a nutrient like any other nutrient; and if this were cut down, the organism would "smother" or starve, decreasing the length of the stationary phase. Actually, however, if the oxygen is cut down a little, although not entirely eliminated, the stationary phase is frequently extended. Apparently in many cases the lack of all but a little oxygen slows down overall metabolism and thus extends the stationary phase in time. The situation is really a little more complicated: what lack of oxygen appears to do is to slow down the death phase and thus permit extension of the time a culture will stay alive, even if not preserving it at its maximum population.

Maintenance of Cultures

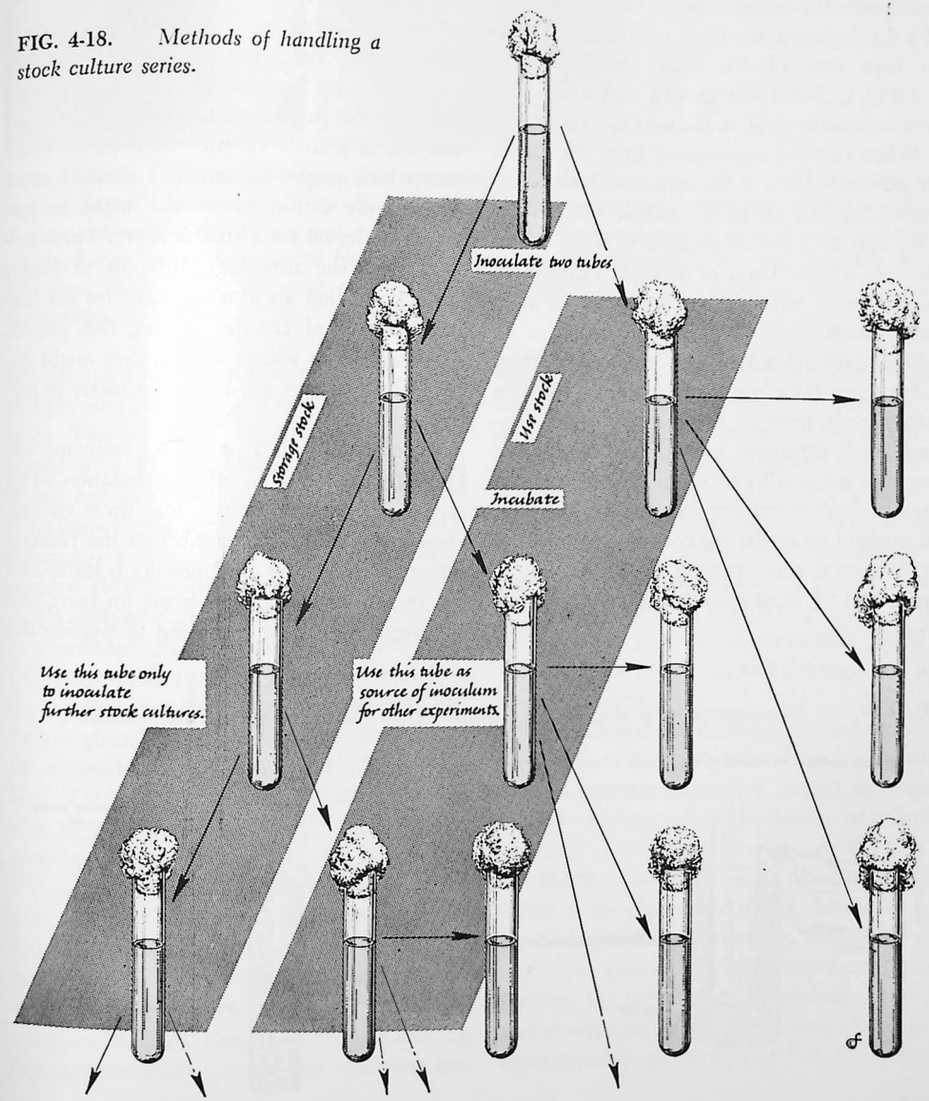
In **slant cultures** we simply make a streak on a slant of suitable medium, allow the organism to grow, and after a suitable interval transfer it to another slant. Of course, some organisms (anaerobes, many streptococci, etc.) do not grow well on slants, and for these organisms deep stabs are used. In general, we choose a medium which is good for the organism involved, prepare a slant, refrigerate the slant after initial growth, and retransfer it after an interval chosen by experience. Some organisms do not survive long, and transfers must be made every 2–3 days; for others, transfers once a month are adequate.

Stock cultures are cultures that are held as a source of organisms, i.e., used to inoculate other

media. In making them, we generally inoculate two tubes. After growth one of them is used as a source of inoculum for any "use" we may wish. The other is never opened until the next stock culture is made; it is used solely for the stock culture line (Fig. 4-18). Slants have some advantage in stock cultures because frequently contamination is readily seen. A streak with a needle is generally better than a broad streak with a loop because it provides more media per organism.

The medium employed is not necessarily that which provides the "best" growth, since "best" may be defined as fastest or as most growth. In

FIG. 4-18. *Methods of handling a stock culture series.*



the case of stock cultures, we wish to have a medium on which the organism survives longest. For example, *Escherichia coli* will grow well on **nutrient agar**, a beef extract with peptone base. If we add 1% glucose to this medium, the *E. coli* will grow faster and produce much more growth, but the medium becomes acid and this acid causes the organism to die off more rapidly. If a 0.1% glucose is added, with enough buffer to take care of the acid (roughly 0.4% K_2HPO_4), then more growth and longer survival is possible than on the nutrient agar alone.

When cultures are removed from stock, they are generally given 2–3 transfers without refrigeration to pep them up before use. While there may be a certain element of habit in this process—and we know of no ready explanation for the result obtained—it does seem to be a useful practice.

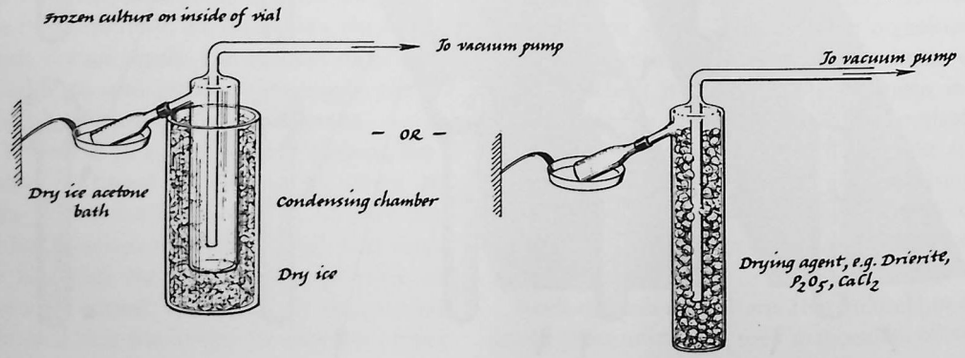
Stock cultures take much space in a refrigerator when there are many of them. Further, cotton plugs tend to become damp and contamination, especially molds, may then enter. One may use smaller tubes and plastic plugs or screw-cap tubes; but the size of tube and thus the amount of media cannot be cut down too far. We want the organism to survive, not to starve, and we must provide adequate medium per cell.

Deep stabs are not only suitable for organisms to which air is somewhat harmful, but also are useful to organisms which thrive on air—partly because of the greater amount of media per organism. Such cultures, having less surface exposed to air, do not dry out as readily as slants.

Mineral oil. It has been found that if a layer of sterile mineral oil is put over a slant or a stab culture, the organisms beneath will survive (at room temperature) for a very long time. By this process we can increase the effective life of a stock culture enormously. A culture which might die out in a slant at room temperature within two weeks might be preserved under oil for a year or more. These cultures have the advantage not only of longer survival but they are also accessible for any limited number of transfers during this period. They are not as widely used as they might be, partly because they are a little bit messy to prepare and use.

Lyophilization is a process of removing water from a bacterial or other preparation when it is in the frozen state. Under these circumstances a considerable number of the bacteria present survive. The dried powder is stable and can be kept at room temperature for years, and the organism can be recovered by placing the

FIG. 4-19. *Two systems for drying bacterial cultures from the frozen state (lyophilization).*



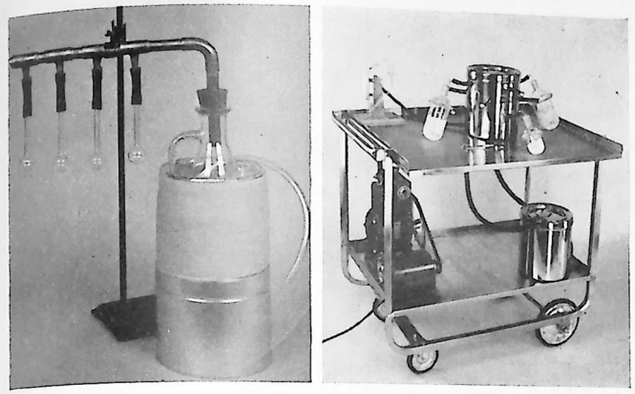


FIG. 4-20. *Laboratory apparatus for lyophilization. On the left is a simple system for lyophilizing vials of bacterial cultures for preservation. The flank is connected to a vacuum pump. On the right is a more elaborate setup: dry ice and acetone are put into the metal cans as cooling and condensing agents. Larger quantities may be lyophilized under these circumstances.* [*From Heckley*, Adv. Appl. Microbiol., **3**, *1 (1961). Courtesy of Dr. Heckley. The photo on the right originally from the VirTis Corporation.*]

powder in a medium suitable for growth. Hence this provides an excellent way of preserving cultures. The culture of bacteria is frozen and placed in a high vacuum, and the water is drawn out of it, generally by being adsorbed onto some drying agent as shown in Fig. 4-19. Apparatus is shown in Fig. 4-20. Such dried powders then are made in quantity and whenever a culture is desired, the powder is placed in a medium suitable for growth and the organism is recovered. It is true that under such drying conditions, a great many of the cells die; for any given culture, perhaps less than 1% of the organism survive. But this 1% is in some kind of state of "suspended animation" and is able to survive for exceedingly long periods, years in fact.

Preservation on **colloid dispersions.** There is evidence to show that when bacteria are adsorbed onto finely divided particles or colloidal dispersions, their metabolic activity slows down and they survive much longer. After being so adsorbed, they are also resistant, especially to drying; presumably a water film of almost molecular dimensions is always present on the colloid. It therefore is possible to preserve organisms grown elsewhere by adding them to sterile colloid dispersions (Fullers earth, sterile soil, or colloidal clays) and allowing them to dry slowly at room temperatures. After drying, the tubes are stored at room temperature. Frequently a loopful of the colloid powder added to fresh medium will permit growth; if this does not suffice, the fresh medium is added to the stock tube of colloid, and the organism usually is recoverable.

The colloidal condition as an influence on the length of the stationary phase is somewhat difficult to understand; it is indeed not experimentally understood. The addition of colloidal materials during lyophilization or the use of soil and its colloids as agents of culture preservation is an experimental fact. Similarly a favorable colloidal environment, not always easy to specify, promotes a longer stationary phase. Possibly the colloid acts by adsorbing the cell and slowing down its metabolism. This is an experimentally demonstrable fact, but why the

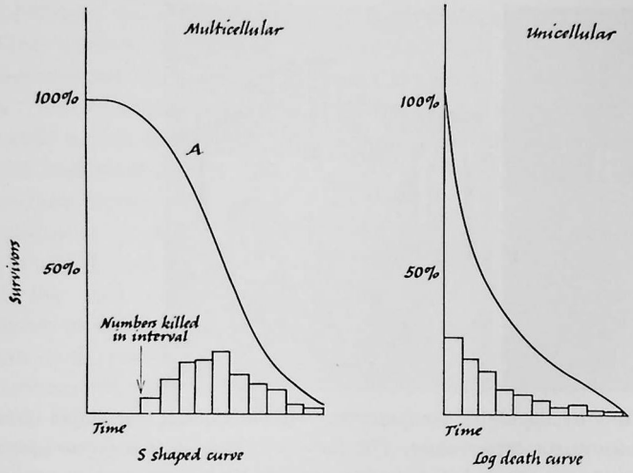


FIG. 4-21. *Death curves of multicellular organisms compared to unicellular organisms.*

adsorption to a colloid *should* slow down metabolism is not clear. Possibly the colloid also acts to adsorb some toxicity, but as of the moment the exact effect of the colloid is not clear.

The **death phase:** Eventually, even under the best of circumstances, the cells of a bacterial population begin to die. If the logarithms of the numbers are plotted, a straight line results. There is a log death phase. Now it is easy to understand a log growth phase: 2 organisms to 4, to 8, to 16, to 32, to 64, etc. But why a log death phase? Of 64 organisms, 32 die the first interval, 16 the next, 8 the next, 4 the next, and 2 the next. Really, this is most peculiar. Many multicellular organisms—seeds of plants, for example—or even many single-celled populations composed of different kinds of cells die off quite differently.

In Fig. 4-21 we have plotted the usual death curves for multicellular organisms compared to bacteria; the insert bars give the number of organisms killed during the respective interval. Why is there this difference?

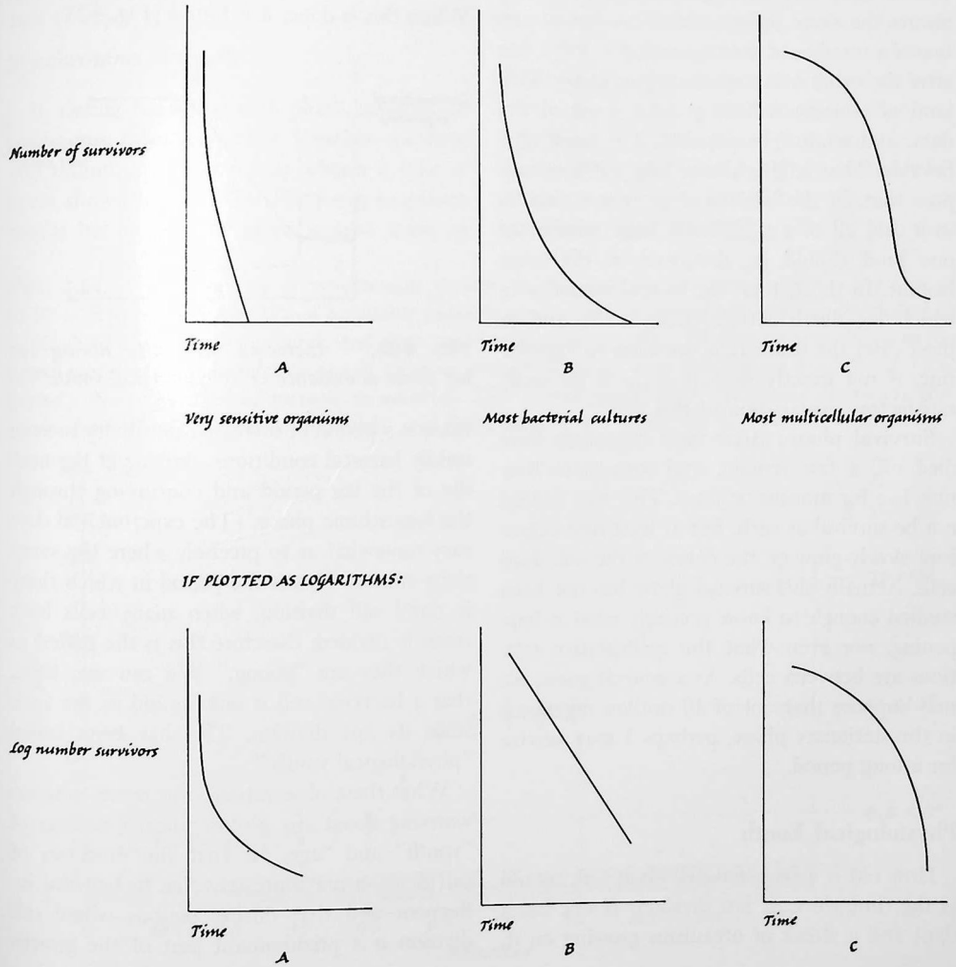
There are people who say that there really isn't any difference. They believe that there are reasons for what they regard as an error in speaking of a log death rate. One reason, so these people say, is that in order to get the bacterial type of curve, one is really only selecting out the steep part of the S curve that "all organisms die by," as if, for example, we took only the data from point A on, and plotted it. But careful analysis of the actual data shows that this cannot be correct. If we compare the death rate of a suspension of bacteria to the death rate of a suspension of mustard seeds—a very small seed indeed but multicellular—the mustard seeds die by way of an S curve, the bacteria by way of a log death curve.

Another error, so some say, is that while experimentally the log death curve is obtained all right, it is due to the "fact" that a bacterial culture is a mixed population—some cells are young, some are old, some are more sensitive than others. The death curves for each kind of cell are S-shaped with log, but when they are all averaged out they become log. There are three kinds of curves possible due to different sensitivities, shown in Fig. 4-22. Very sensitive organisms give curves similar to A, plotting number alive against time; the more common bacterial curve is B. The usual curve for multicellular

organisms is C. But it turns out experimentally that if you take two bacteria of different sensitivities and mix them together, you will then obtain curves of type A, which when plotted log number against time does not show the log type death found in bacteria. Therefore, when curves of type A are found they appear to be due to just this mixture of sensitive and resistant cells. Since, however, bacterial cultures by and large die by curve B, the log death is not due to mixtures of organisms of vastly different sensitivity. There is indeed such a thing as log death and it differs from the death of higher organisms.

There are also people who admit that log death is experimentally demonstrated and is a real thing, and some of them have made an effort to explain it. This is, of course, difficult. The "die-away" curve can be written $-dn/dt = kn$ or $n_1/n_2 = e^{-kt}$; i.e., the loss of num-

FIG. 4-22. *Death curves of various kinds of organisms.*



bers of bacteria per unit time is directly proportional to their number. This rate is very much like that of a "unimolecular reaction," i.e., a rate dependent solely on one molecule, as with C^{14} (radioactive carbon). This corresponds to a "first-order reaction rate," similar in particular to the curve of denaturation (= partial destruction) of proteins in pure solutions, and has led some to assume that the death of bacteria results because of the loss of a particular protein in the organism.

This does not necessarily mean that it is always the *same* protein, since the loss of any one of a number of proteins acting in series one after the other will stop the whole chain. This kind of concept will fit at least some of the data, and while it is possible, it is hardly believable. That is, it is almost impossible to suppose that, of the billions of protein *molecules* in a cell, all or a sufficiently large number of one kind should be destroyed in the same instant. In short, there are several instances in which log death actually does occur, and in most cases the death rates are close to logarithmic, if not exactly so, but there is no really satisfactory explanation of this fact.

Survival phase: After most organisms have died off, a few remain, and sometimes they may live for months or years. This may indeed not be survival as such, but at least new organisms slowly grow on the debris of the old, dead cells. Actually this survival phase has not been studied enough to know precisely what is happening, nor even what the quantitative relations are between cells. As a general guess, we may suppose that out of 10 million organisms in the stationary phase, perhaps 1 may survive for a long period.

Physiological Youth

How old is a bacterial cell? Is it only as old as the time since its last division? If one has a slant and a streak of organisms growing on it, are cells at the edge older, younger, or the same age as those in the center?

These questions are not decidable by mere arbitrary opinion; some other approach must be employed. It has been pointed out that the young of any creature are generally more susceptible to harmful influences and therefore the question can be changed. Instead of asking, "Are there youth and age in bacteria?" we ask, "Are bacteria more sensitive to harmful effects at some stage of the growth cycle?" The latter question can be answered experimentally. When this is done, it is found (Fig. 4-23) that

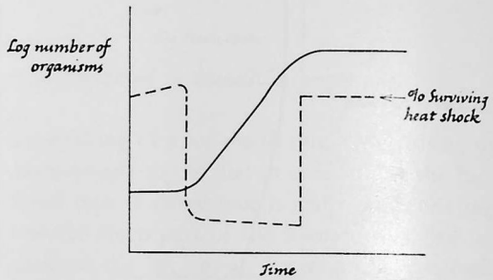


FIG. 4-23. *Increased sensitivity during the log phase as evidence of "physiological youth."*

there is a period of increased sensitivity to even mildly harmful conditions, starting at the middle of the lag period and continuing through the logarithmic phase. (The experimental data vary somewhat as to precisely where the sensitivity starts.) This is the period in which there is rapid cell division, when many cells have recently divided; therefore this is the period in which they are "young." We can say, then, that a bacterial cell is only as old as the time since its last division. This has been called "physiological youth."

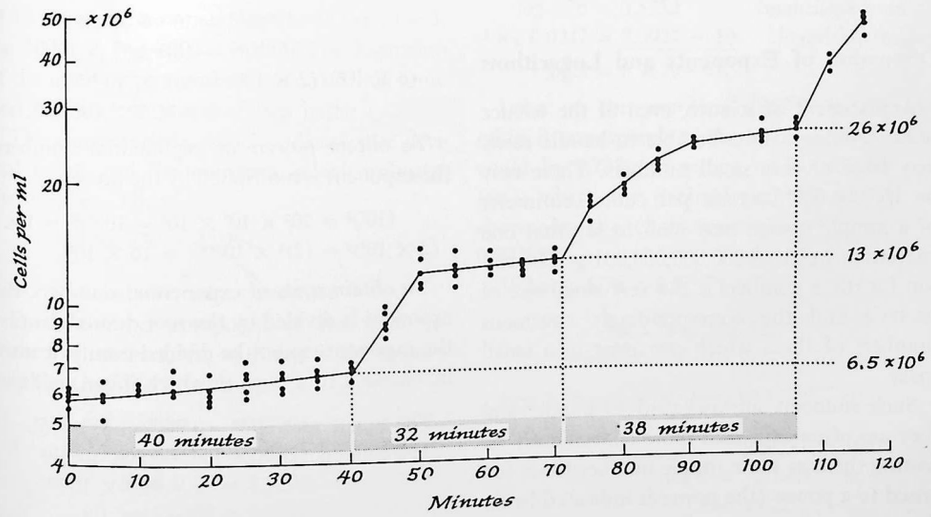
What these observations also mean, without worrying about any philosophical meanings of "youth" and "age," is that the processes of cell division are more sensitive to harmful influences and that during periods where cell division is a predominant part of the growth process, the cells are therefore more sensitive. What happens when cell division is harmed but growth and metabolism of the cell are not? One obtains cells which grow but don't divide. These bizarre and **pleomorphic** (= many forms) cells are characteristic of **sublethal injury** (= injury which harms but does not kill), whatever its cause, and may be seen in cells subjected to heat, to antiseptics, to antibiotics, to poor media, etc. When such cell forms occur, it is most frequently evidence of sublethal toxicity.

Synchronous Growth

If, during the log growth phase, samples for plating are taken very close together—perhaps one minute apart—one may obtain a type of curve shown in Fig. 4-24. The jump in growth results because at any given instant many of the cells divide; thus at the end of this instant there are almost twice as many cells as there were before. This is called **synchronous growth:** the cells in a culture all divide at the same time, grow for a generation time, and all divide again at the same time, giving the stepwise growth illustrated in Fig. 4-24. Growth and division are synchronized among a group of cells.

To achieve this synchronous growth, certain methods are available. First, in certain cases there are organisms which require thymine for their growth. This thymine is a constituent of the DNA (= deoxynucleic acid), a material characteristic of the nucleus of cells and without which the cell cannot divide. Such thymine-deprived cells, when placed in a suitable medium, can do all the other things necessary for division, except actually divide. After an interval they are all ready to divide, but they

FIG. 4-24. *An example of synchronous growth. In this case,* Bacillus megaterium *was grown at 34°C in a nutrient broth. When a suitable population was reached the culture was rapidly chilled to 12°C, held there for 30 minutes, and then rapidly rewarmed to 34°C. The time course shown in the figure represents the population found (plating every 5 minutes, after the start of the second 34°C period). Note the stepwise increase in numbers. The normal generation time is 33.6 minutes.* [*Redrawn from Hunter-Szybalska, Szybalski, and DeLamater,* J. Bacteriol., **71,** 17 (1956).]



cannot because they lack thymine. When thymine is added, the cells all begin simultaneously to incorporate it into the DNA; as soon as they have enough DNA to divide, they all divide simultaneously, thus giving synchronous growth.

A second method is to actually separate, from a mixed population, those cells which have just divided, and let them grow by themselves. This can be done by using membrane filters of just the right size to let the smaller cells—those which have just divided—through the filter and hold back the larger cells which are about ready to divide. Another method is to hold the cells at low temperature, for a considerable interval, which apparently slows down division faster than it slows down "growth." When these cells are returned to growth temperature, synchronous growth frequently occurs.

Synchronous growth studies permit us to study what happens between the time the cell has divided and before the time of another division. Such studies should reveal something about the mechanisms and stimuli responsible for cell division. But such studies are just beginning and we do not now know what will be found out.

Discussion of Exponents and Logarithms

Any student of science, even of the science of bacteriology, must be able to handle easily very large or very small numbers. There may be 100,000,000 bacteria per cubic centimeter of a sample or one may wish to say that one bacterium weighs 0.000,000,000,001 g. The reason for these numbers is the very small size of bacteria and the correspondingly enormous numbers of them which can exist in a small space.

Such numbers are awkward to handle and they are often expressed more conveniently by writing them as some simple number times ten raised to a power (the power is indicated by an exponent). Thus, 100,000,000 may be written as 1×10^9; the exponent 9 means 10 raised to the ninth power. 2,300,000 can be written 2.3×10^6 or 23×10^5, the latter meaning 23 with the decimal point five places to the right.

Similarly the number 0.000,000,000,001 may be written 1×10^{-12} which means that the decimal point must be moved twelve places to the left. Negative exponents may be written in two ways, thus the number above may be 1×10^{-12} or $1/10^{12}$.

To *multiply* numbers written in exponents, add the exponents; to *divide*, subtract the exponents. Since 1×10^{-12} g is the weight of one bacterium, the weight of a thousand bacteria is $1000 \quad (=10^3) \times (1 \times 10^{-12}) = 1 \times 10^{-9}$ g. The weight of one bacterium is 1×10^{-12} g. Since 1 g is 10^{-3} kg, one bacterium weighs 1×10^{-15} kg. Other factors in the indicated product or quotient are multiplied or divided as usual. A few simple examples follow.

Multiplication:

$$2^2 \times 2^3 \times 2^4 = 2^{(2+3+4)} = 2^9.$$
$$10^5 \times 10^{-2} = 10^3.$$
$$(2 \times 10^5)(4.37 \times 10^6) = 8.74 \times 10^{11}.$$

Division:

$$10^6/10^2 = 10^{(6-2)} = 10^4.$$
$$10^2/10^{-5} = 10^{[2-(-5)]} = 10^7.$$
$$(6 \times 10^2)/(2 \times 10^3) = 6/2 \times 10^{(2-3)} = 3 \times 10^{-1} \text{ (or } 0.3).$$

To obtain powers of exponential numbers, the exponent is multiplied by the power:

$$(10^2)^3 = 10^2 \times 10^2 \times 10^2 = 10^{(2\times3)} = 10^6.$$
$$(2 \times 10^3)^4 = (2)^4 \times 10^{(3\times4)} = 16 \times 10^{12}.$$

To obtain roots of exponential numbers, the exponent is divided by the root desired. In case the exponent cannot be divided evenly, it must be changed to a form in which it can be done.

$$\text{The square root of } 5^6 = (5^6)^{1/2} = 5^{6/2} = 5^3.$$
$$\text{The cube root of } 8 \times 10^{12} = (8 \times 10^{12})^{1/3} = 8^{1/3} \times 10^{12/3} = 2 \times 10^4.$$

$$\text{The square root of } 5 \times 10^{-7} = (5 \times 10^{-7})^{1/2} = (50 \times 10^{-8})^{1/2} = 50^{1/2} \times 10^{-8/2} = 7.1 \text{ (the square root of 50)} \times 10^{-4} = 7.1 \times 10^{-4}.$$

To *add* or *subtract* exponential numbers, they must be changed to the same power of 10:

$$10^2 + 10^3 = 1 \times 10^2 + 10 \times 10^2 = 11 \times 10^2 = 1.1 \times 10^3.$$
$$(6 \times 10^{-2}) - (2 \times 10^{-3}) = (6 \times 10^{-2}) - (0.2 \times 10^{-2}) = (6 - 0.2) \times 10^{-2} = 5.8 \times 10^{-2}.$$

But exponentials themselves, while not difficult to handle, may become cumbersome. Just as you can express a number as the number times an exponent, you can also express any number as an exponent. That is:

$$100 = 10^2.$$
$$1000 = 10^3.$$
$$850 = 8.5 \times 10^2 = 10^{.9294} \times 10^2 = 10^{2.9294}.$$

This exponent which represents the number is called the **logarithm.** Thus, 0.9294 is the logarithm of 8.5 and 2.9294 is the logarithm of 850, generally written log 850 = 2.9294. The logarithms of all numbers which are integral powers of 10 are whole numbers; log 1 = 0, log 10 = 1, log 100 = 2, log 1000 = 3, etc. The logarithm of the intervening numbers are not whole numbers but are composed of two parts:

The **characteristic,** which indicates the decimal point, and the **mantissa,** which indicates the number. Thus

$$\log 850 = \overbrace{2}^{\text{characteristic}}.\overbrace{9294}^{\text{mantissa}}$$

For a number composed of the same sequence of integers, the mantissa is the same—only the characteristic changes:

$$\log 85 = 1.9294,$$
$$\log 850 = 2.9294,$$
$$\log 8{,}500 = 3.9294,$$
$$\log 85{,}000 = 4.9294,$$

The mantissas for numbers are found in logarithm tables, and such a table gives only the mantissas; the characteristic is determined by where the decimal point is.

The great advantage of logarithms is that they are exponentials and they therefore follow the laws of exponentials—i.e., to multiply, add the exponents; to divide, subtract them. You can then reduce multiplication and division, two of the most laborious parts of arithmetic, to addition and subtraction, two of the easiest.

All of these operations are reasonably clear except for numbers less than one, for here the characteristic becomes a negative number while the mantissa remains positive.

$$\log 85 = 1.9294,$$
$$\log 8.5 = 0.9294,$$
$$\log 0.85 = \bar{1}.9294 \text{ or } 9.9294 - 10,$$
$$\log 0.085 = \bar{2}.9294 \text{ or } 8.9294 - 10, \ldots, \text{ etc.}$$

While there are a variety of ways of handling the negative characteristic, in our experience the use of the [9.9294 − 10] notation is the simplest.

Examples:

What is 3.76×0.0247?

$$\log 3.76 = 0.5752 \quad \text{(mantissas from}$$
$$+\log 0.0247 = \underline{8.3927 - 10} \quad \text{logarithm table)}$$
$$\log x = 8.9679 - 10.$$

Look up this mantissa (9679) in the log table; it corresponds to the number 9288. This could be 9.288 (or more) or 0.00009288 (or less); where the decimal point should be is shown by the characteristic, $= -2$, so $x = 0.09288$.

What is $3.76 \div 0.0247$?

$$\log 3.76 = 0.5752,$$
$$-\log 0.0247 = \underline{(8.3927 - 10)}.$$

Log 3.76 can be written 10.5752 − 10:

$$-(8.3927 - 10)$$
$$\log x = 2.1825 - 0,$$
$$x = 15.22.$$

A considerable help for at least rough calculations (and it is surprisingly accurate) is the slide rule, an instrument graduated in lengths proportional to the logarithm, but marked in digits. Hence to multiply, one adds the lengths; to divide, one subtracts.

This brief review of the mathematics of exponentials and logarithms is merely to refresh your memory and is not, nor should it be considered, a fundamental discussion of this subject. We assume that you know these matters, but that you may have forgotten them since you last used logarithms.

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QUESTIONS

1. Define or identify:

binary fission	lag phase
logarithm	growth curve
exponentials	first-order reaction rate
characteristic	denaturation
mantissa	physiological growth
growth phase	synchronous growth
log phase	thymine
stationary phase	unimolecular reaction
death phase	S curve
survival phase	log death phase
nutrient agar	crowding
generation time	assay
lyophilization	chemostat

2. Outline three theories of the lag phase. Under what conditions is each correct?

3. An organism growing on the log phase shows a plate count of 1.55×10^6 organisms per milliliter and 4 hours later, still in the log phase, it shows a count of 3.7×10^6 organisms per milliliter. What is the generation time? (Answer: 191 minutes.)

4. An organism freshly inoculated into a new medium shows the following turbidity readings. Each turbidity reading is equivalent to 3×10^6 bacterial cells. Plot this data and, from the log phase of the curve, calculate the generation time. Hour, turbidity: 0, 0: 6, 114: 8, 234: 10, 380: 12, 512: 24, 604.

5. Name a bacterium with a generation time typically of the order of 30 minutes; of more than 60 minutes.

6. At what stage of the growth curve is there likely to be the greatest variation in cell morphology? Why?

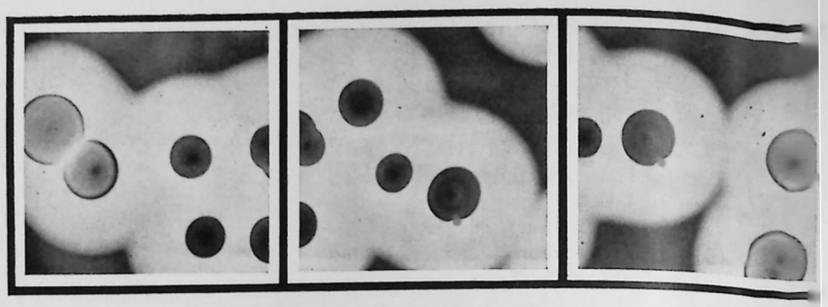
7. In an actively multiplying culture of bacteria, are there any young cells? any old cells? Why?

8. Does the term "growth" mean the same thing when applied to:
   (a) an individual bacterial cell?
   (b) a bacterial culture?
   (c) a plant or animal?

9. Sometimes growth data is expressed as "doublings per hour." How is this related to the generation time? If you had two doublings per hour, what would be the generation time? If the generation time was 45 minutes, how many doublings per hour would the culture show?

10. If you inoculated a broth medium, allowed it to reach half its maximum turbidity, and at this point removed half the medium and replaced it by fresh, uninoculated medium, would you have a log phase? If you allowed this to grow to half-maximum turbidity, again removed half the medium and replaced it with fresh, and kept this up every time the medium reached half-maximum, would it ever reach the stationary phase? Why?

Chapter 5



NUTRITION

"We saw how bodily life carries with it its own evidence that its origin is terrestrial. If we employ exotic to mean of nonterrestrial provenance, there is no ingredient of bodily life which is exotic. Its chemical elements are among those commonest on our planet. Its whole is redolent of Earth, whence it was dug."

SHERRINGTON, *Man on his Nature*

BACTERIA grow in nature. Milk sours because bacteria grow in it and produce acid. Foods spoil because bacteria grow in them. Bacteria grow on the body and in the intestinal tract. Bacteria decompose the tree fallen in the forest, they ripen cheese, they grow in a sulfur spring, in soil, in the gut of a termite. For all these creatures there is a place in the world; somewhere, somehow, nature will provide. Nature must provide the necessities of life: an environment suitable for growth and a supply of energy. Therefore, when we wish to cultivate bacteria, we first try to simulate the environment we may find in nature. Does the organism grow in the sea? Then perhaps it will grow in the laboratory if we give it sea water. Does an organism grow in the body? Then perhaps it will grow in the laboratory on media made from protein extracts (meat broth, perhaps).

But why is it that some microorganisms grow well on a medium composed of glucose, ammonium salts, and phosphates, while others will only grow if supplied with several amino acids, some vitamins, and even more exotic factors? Not all microorganisms are alike—even in their nutritional requirements. But why are they different? If one organism can build its cell from simple ingredients, then there *are* ways to do it; why can't all organisms do it?

Of course, the answer is that not all organisms have these synthetic systems. In general, the "higher" the organism, the less its synthetic ability and the more that is required in its diet. The same differences occur among higher organisms. The rat, for example, requires no vitamin C in its diet but the guinea pig does. The reason is that the rat has the metabolic mechanisms for synthesizing vitamin C in its own body, but the guinea pig does not. It has to use the vitamin C in its metabolism but it cannot make it. It therefore has to have an external source—usually in the diet.

All this really begs the more fundamental question: why do not all organisms have the necessary synthetic systems? Again, we can be ready with the rather glib answer that of course the genetic constitution of various organisms is different. If organism A does not have in its nuclear DNA the right "code" to tell the cell to make the system synthesizing substance B, then it will not, and B must be supplied before A can grow. And this would be right. Yet all of this leaves one enticing corner of the problem in the dark: how does it happen that these genetic differences occur? If we think that "survival of the fittest" is the keystone to progress, are not those organisms unable to synthesize B less fit than those able to? Why indeed should they survive at all? Whether or not we are able to answer this, they have indeed survived. We ought, then, to examine the kinds of microorganisms which do exist, looking at them from the viewpoint of their food requirement.

Nutritional Requirements of Microorganisms

A few bacteria (and most algae) are photosynthetic and can obtain their energy from light. But the majority of bacteria obtain energy from the food supplied. The food consumed is used for two purposes: one, as a source of energy and, two, as the actual material or the "building blocks" that make up the cell. The inorganic elements required for most bacteria are carbon, hydrogen, oxygen, nitrogen, phosphorus, sulfur (in decreasing order of amount required) with much smaller amounts of iron, calcium, magnesium, potassium, sodium, and chloride. Still smaller amounts of the following are necessary, frequently an amount so small as to make it difficult to demonstrate any requirement: manganese, zinc, copper, boron, molybdenum, iodine, and strontium. Molds, particularly, might generally require such "trace" elements. With most bacteria the requirement for these materials is so small as to be met by contamination from glassware or other constituents of the medium.

These elements are the actual "building blocks" that go into the cell structure. These are the elements from which are made all of the fats and proteins and carbohydrates of the protoplasm. From these are fashioned the cell wall, the cell membrane, and the nucleus and the cytoplasm. Under the best of conditions about one-third of the elements in the food appears in the newly grown cell; generally much less than one-third of the foodstuff appears in the cell, sometimes as little as one-hundredth. About two-thirds of the food is broken down and ripped apart to provide the energy to make all of the substances in the cell. Every living thing needs a source of energy, always and continually, as long as it lives.

Microorganisms may be separated into a few great groups based on what they can use for energy (Fig. 5-1). There are the **photosynthetic** organisms. They can absorb the energy of light and, using this, can build their cell substance from CO_2 and minerals. The photosynthetic bacteria do not possess exactly the same chlorophyll as plants, but the photosynthetic pigments are closely related. There are relatively few photosynthetic bacteria in nature, but of

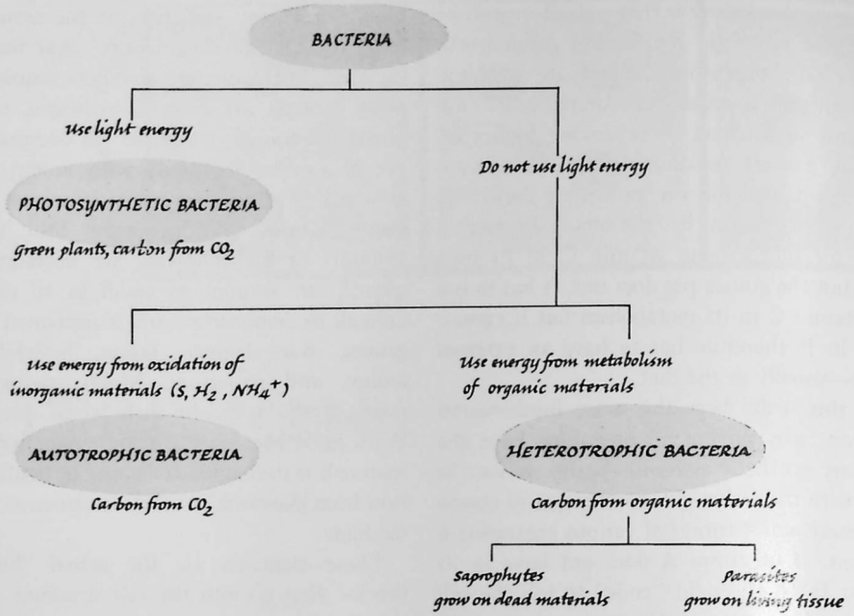


FIG. 5-1. *Types of energy supply employed by microorganisms.*

course green plants (and this includes algae) are photosynthetic and supply the organic matter which the rest of the creatures live upon. There are also the **autotrophic** bacteria, which derive their energy from the oxidation of inorganic materials, such as sulfur, hydrogen, or ammonia. They obtain their carbon from carbon dioxide, as do the photosynthetic organisms, and in some cases can use other organic materials for carbon as well. Few, if any, higher organisms are autotrophic. Finally, the majority of bacteria are **heterotrophic** organisms, which obtain their energy, and in most cases their carbon, from the breakdown of organic materials. Carbon dioxide may be used but it cannot serve as the sole source of carbon. Heterotrophic bacteria may be further divided into **saprophytes,** organisms which live on dead material, and **parasites,** organisms which grow upon living material. Saprophytes may exist with a wide range of requirements—some may live on very simple materials whereas others may require very complex media. Parasites can grow on living tissue and hence they can be cultivated on it. Two laboratory methods for doing this are chick embryo culture (p. 470) and tissue culture (p. 471), but in general, except for virus and Rickettsia, we can frequently grow parasites away from living tissue if we can find the right medium for them. The term parasite means, then, that the organism can grow on living tissue, not that it can grow only on living tissue.

Most heterotrophs are saprophytes and the laboratory media upon which they will grow reflect, to some extent, the environments in which they are found in nature. A commonly used medium is **nutrient broth** (with 1.5% agar added to solidify the medium it is called "nutrient agar") or sometimes "beef extract-peptone" broth (or agar). It is usually made by dissolving 3 g of beef extract (final concentration, 0.3%) and 5 g peptone (final concentration, 0.5%) per liter; sometimes "tryptone," a peptone fortified with tryptophan, is used. It was earlier the custom to add 0.3% NaCl, which gave a degree of osmotic protection, but this was found to be unnecessary in most cases. However, we mention it here because many of the older descriptions of microorganisms are based on media with the salt present and such descriptions differ slightly from those based on the medium without salt. The beef extract (or "meat extract") is a water extract of meat concentrated to a syrup and thus contains the water-soluble portions of the meat—sugars, amino acids, etc. The peptone is partially digested protein, i.e., protein (it may be casein) treated with pepsin or trypsin to break down the protein to peptides and amino acids. Such a medium contains many unknown constituents and varies from preparation to preparation, but it does contain a wide array of building blocks: amino acids, small amounts of sugar, phosphates, etc. Since it is a soluble meat extract it would resemble the food which microorganisms would encounter in the body. Indeed, its use dates from the early interest in pathogens (the first real medium in bacteriology was the eyeball of an ox, used by Koch to cultivate those organisms causing anthrax). The nutrient broth medium itself is low in sugar and low in buffering capacity and in fact it may readily be improved upon; but it has been in such general use for so many years that every bacteriologist is familiar with it. Simple improvements result in a medium of the following composition:

peptone (or tryptone)	0.5 g	
yeast extract	0.5 g	(to provide an array of minerals and vitamins)
glucose	0.1 g	(to provide a ready source of energy)
K_2HPO_4	0.5 g	(to provide buffering capacity to take care of acid formed during growth)
water	to 100 ml	

An unknown organism from a natural environment may grow well upon a nutrient broth medium or it may grow poorly, if at all. If it grows well, it is probably living in an "affluent society" and we may try to simplify the medium so that we may obtain media of known and constant composition upon which such organisms would grow. Experimentally, we find a wide gradation of requirements:

1. Growth on glucose and mineral salts.
2. No growth on above but growth upon the addition of one or more of the following:
   - (a) one amino acid
   - (b) several amino acids
   - (c) purines and/or pyrimidines
   - (d) vitamins
   - (e) in addition to some of the above, sometimes certain factors—heme, for example, a substance obtained from hemoglobin

A medium for an organism requiring only glucose and mineral salts (*Escherichia coli*) is given in Table 5-1; a more complex medium, for an organism requiring more additions, is given in Table 5-2.

TABLE 5-1. *Medium for an Organism Requiring Glucose and Mineral Salts*

Glucose	5.0 g
Dipotassium phosphate (K_2HPO_4)	7.0 g
Potassium dihydrogen phosphate (KH_2PO_4)	3.0 g
Sodium citrate ($.3H_2O$)	0.5 g
Magnesium sulfate ($.7H_2O$)	0.1 g
Ammonium sulfate	1.0 g
Water to 1 liter	

TABLE 5-2. *Medium for an Organism Requiring More Additions*

Acid hydrolized casein (vitamin free) (or mixture of amino acids)	5 g
Glucose	10 g
Salts A (10% each K_2HPO_4)	10 ml
Salts B ($FeSO_4$, $MnSO_4$, $MgSO_4$, NaCl)	5 ml
L-asparagine	250 mg
L-tryptophan	50 mg
L-cystin	100 mg
DL-methionine	100 mg
Cystein	100 mg
Ammonium citrate	2 g
Sodium acetate (anhydrous)	6 g
Adenine; guanine; xanthine; uracil each	10 mg
Riboflavin; thiamin; pantothenate; niacin; each	500 μg
Pyridoxamine	200 μg
Pyridoxal	100 μg
Pyridoxin	200 μg
Inositol and choline, each	10 mg
p-aminobenzoic acid	200 μg
Biotin	5 μg
Folic acid (synthetic)	3 μg
Make up to 1 liter with distilled water	

A few aspects of this kind of study may be remarked upon. In addition to providing media of known and thus controllable composition, the development of such media has permitted the use of microorganisms for the assay of vitamins, purines, pyrimidines, and amino acids. In many cases microbiological assays were the only way in which nutritive factors required for man or animal could be assayed, in other cases microbiological assays were much more economical. A typical assay situation was illustrated earlier (Figs. 4-14 and 4-15). Also, the simplest possible medium upon which an organism may grow may be useful in estimating its "self-reliance," but if we compare such organisms to those grown on richer media, which provide things other than the minimum needed for growth, we may obtain an estimate of the capabilities of an organism. In a world of scarcity, an organism may adjust its life to make do with the minimum; in a world of plenty, it may allow itself some luxuries. For example, many organisms may form pigments on rich media, but dispense with them on poor media.

An organism from a natural environment may not grow well or not grow at all upon nutrient broth. Under these circumstances we attempt to add to the medium likely constituents of the organism's environment. If, for example, we were trying to isolate an organism known to occur in blood, we might add blood to the medium. In other words, we try to simulate the natural environment in which the organism occurs. Once we have obtained growth, then we can begin to eliminate various constituents so that the minimum requirements can be determined.

It is possible to have too rich a medium; there are a few cases where organisms do not grow on complex media but grow well on very simple media. *Azotobacter*, for example, grows well on a mineral salts medium containing alcohol, but grows very poorly on nutrient agar. And an organism that lives in the ocean is likely to thrive on sea water with relatively little nutrient rather than to grow on beef-extract brain infusion. More generally, the problem with richer media is that so many different organisms grow, and some so well, that it is difficult to isolate from such a mixed population the specific one that we may be interested in. There are still a few organisms that it has been impossible to cultivate on artificial media, but these are relatively few; within the past decade most of the organisms—even active parasites—have been cultivated. With respect to the preparation of media, two further factors need to be considered. First, all bacteria live on food in solution. They do not, like some protozoa, engulf insoluble food particles nor do they have mouths or stomachs or intestines to digest the food. The food must get in through the cell wall and the cell membrane and it must therefore be dispersed into particles of molecular size, i.e., it must be soluble. As mentioned in Chapter 3, this process may be one of "mere" *diffusion* across a cell boundary, or it may be more complicated and require an *active transport* system, but to start into the cell the food must be in solution.

This necessity for soluble food becomes of importance in relation to the amount of growth one may obtain from a given amount of material. For example, 1 g of sugar dissolved in 100 ml of water will be used much more readily than the same gram of sugar dissolved in 100 liters or in a lake of water. There are many environments—natural waters, oceans, lakes—which contain in the aggregate a large amount of material on which bacteria can live but which support very few bacteria because the material is so dilute.

This effect sometimes results in peculiar observations. One of these is that most lake water if plated in a boat or immediately upon reaching land, shows very few bacteria; but the same sample of lake water held in a sterile (!) bottle for 24 hours may show many hundred bacteria. The reason is that organic materials on which bacteria could grow were so dilute in the lake that only a few organisms could manage. When such water is put in a sterile glass bottle, much of the organic materials it contains are adsorbed onto the glass; as such their concentration at the glass surface increases and at this surface many more bacteria can now grow. When these are shaken off preparatory to plating, they are measured as increased plate count. The amount of food materials has not increased, but their concentration has.

There is a second observation of considerable importance industrially, related to the concentration of food in solution. As illustrated in Fig. 5-2, right at the cell surface the cell has generally used up most if not all of the food. At any rate, its concentration is less than it is some distance away. The food at a distance must then diffuse across this concentration gradient. If we can make this layer thinner, we can frequently obtain better growth. One way to decrease the distance of diffusion is to stir the medium vigorously. Many industrial processes, particularly those which require air (or oxygen), are greatly benefited by actually violent stirring or shaking, particularly since oxygen is only slightly soluble in most solutions; even in its saturated solutions oxygen is relatively scarce (about 5 ml of oxygen per liter of water solution saturated with air).

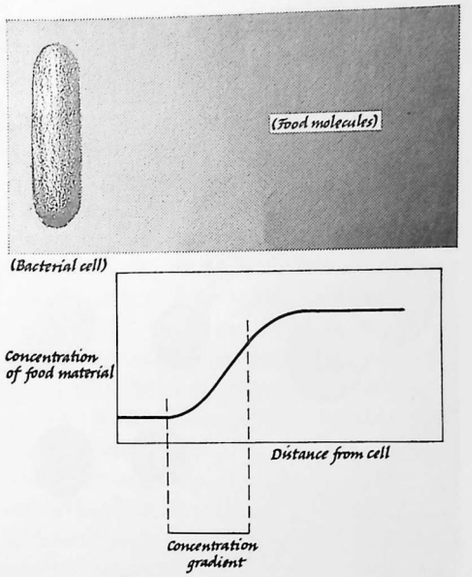


FIG. 5-2. *The concentration of food at various distances from the bacterial cell. Upper diagram uses the intensity of black to represent the food concentration. The lower diagram uses a graph to represent the same phenomenon.*

Some bacteria can attack insoluble things: starch, cellulose, paper, leather, even sulfur. How do they do it? The answer is that they render such materials soluble, generally by breaking them down. Starch, or cellulose, for example, is converted to sugar outside of the cell; the sugar then enters the cell and is metabolized.

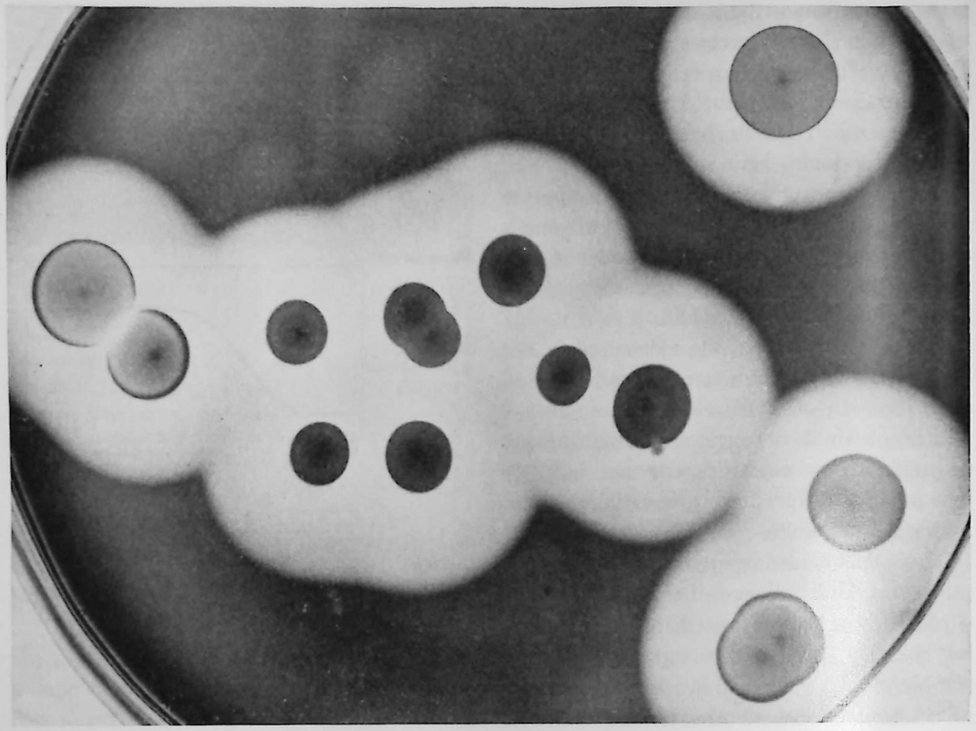


FIG. 5-3. *Organisms attacking insoluble food material. These are colonies of a microorganism which hydrolizes starch and agar. In this case it has been grown on an agar medium containing starch; after growth the plate is treated with I—KI, which colors the remaining starch blue. Note the clear zones from which the starch has been removed.* [*From VeldKamp*, J. Gen. Microbiol., **26**, 331 (1961). *Courtesy of Dr. VeldKamp.*]

But let us be perfectly clear; if a bacterial culture does not possess the means for rendering an insoluble substance soluble, then it cannot attack it. For example, a given organism can attack cellulose—say, filter paper. It does this by sending out an enzyme to hydrolyze it and uses the sugar so obtained (described on p. 338). But another organism, one that cannot attack cellulose, would starve to death in the presence of plenty of cellulose, although it would grow luxuriantly if that same cellulose were hydrolyzed to sugar by heating in acid, for example. To repeat, an organism can attack an insoluble food material only when it possesses a means for rendering it soluble. If it lacks this means, it cannot use the insoluble food.

The most common mechanism for attacking insoluble materials is for the organism to excrete a solubilizing agent (Fig. 5-3). This is generally an enzyme (defined on p. 149). The solubilizing enzyme converts the insoluble material to soluble material (as starch to sugars) which latter diffuses back into the cell where it can be used. Since there is both a diffusion out (of enzyme) and in (of soluble food), it is a great advantage for the organisms to be in close contact with the insoluble material. This indeed happens; the organisms attacking cellulose cling closely to the cellulose fiber. The formation of the extracellular enzymes may be demonstrated on plates as shown in Fig. 5-3, as well as in other ways. Starch hydrolysis, casein (or protein) hydrolysis, and "lipase" formation (fat hydrolysis) are common bacteriological tests.

For the purpose of general microbiology we may think of five large groups of extracellular hydrolytic enzymes:

amylases . . . convert starch to sugar (maltose)
lipases . . . convert fats to glycerol and fatty acids
proteinases . . . convert proteins to peptides
peptidases . . . convert peptides to amino acids
cellulases . . . convert cellulose to sugars.

As you can see, they are named (usually) after the material acted upon (the **substrate**) by adding the term ase to the end. A few have other names: amylase—rather than starchase, which is awkward to say—comes from the Latin term for starch; lipase—rather than fatase—after lipids, means fatlike materials.

The Growth Factors of Microorganisms

As we have mentioned, some microorganisms grow on a very simple medium: glucose and mineral salts, as described in Table 5-1. But others will not grow in such a simple medium. Sometimes the addition of one or more amino acids will permit growth. But more often, in addition to certain amino acids and sometimes purines and pyrimidines, there are other growth factors required, generally required in small amounts. In the interests of obtaining a chemically defined, but not necessarily simple, medium, efforts were made to isolate and chemically identify these growth factors. In many cases, it turned out that the microbial growth factor was also a growth factor required by man or animal; in the latter case it was generally called a vitamin. That is, a **vitamin** is a substance which must be present in the diet of man or animal in order to permit normal growth or function. It turned out, in short, that many microorganisms required the same vitamins as animals or man. This was of momentous importance for the investigation of vitamins. The vitamins could be measured much more rapidly by measuring microbial growth than by measuring the rate of animal growth or development; the isolation and chemical identification of the vitamins was thereby enormously accelerated. Table 5-3 shows the vitamins which are recognized today as requirements of animals or man. The isolation of most of these was accomplished by using microbiological assays. Also, because microbiological vitamin assays could be developed, it was possible to determine the vitamin con-

TABLE 5-3. *Vitamins*

Letter	Name	Microorganisms Used in Assay
A		None
B_1	Thiamin	*Streptococcus, Lactobacillus, Staphylococcus*
B_2	Riboflavin	*Strep. lactis, L. casei*
—	Niacin	*Lactobacillus, Streptococcus, Staphylococcus*
B_6	Pyridoxine	*Lactobacillus, Neurospora, Streptococcus*
B_{12}	Cyanocobalamin	*Lactobacillus*, protozoa
C	Ascorbic acid	none
D	Calciferol	none
—	Folic acid	*Lactobacillus, Streptococcus*
—	Pantothenic acid	*Lactobacillus, Leuconostoc Strep. lactis*
K		none
E		none
	Biotin*	*Lactobacillus*
	Para-aminobenzoic acid*	*Lactobacillus*

* Not required by man

tent of many kinds of foods, to study easily the effect of cooking, canning, etc. on the vitamin content; thus, by the use of this tool provided by the microorganisms, we have been able to vastly improve our nutrition. Not only were bacteria used for this purpose, but molds, yeasts, algae, protozoa—the whole gamut of the microbial world—provided organisms responding to one or more of the vitamins and usable for their assay.

The growth factors required by many microorganisms have proved in the long run to be the same as the vitamins required by man: niacin, thiamine (vitamin B_1), pyridoxine (vitamin B_6), riboflavin, folic acid, and vitamin B_{12}. Further, similar factors, such as biotin, para-aminobenzoic acid, inositol, pantothenic acid, were found to be required by certain microorganisms. These are not necessarily required by man, although (by manipulating the diets) many can be shown to be required by animals. In addition, there were some growth factors not required by animals. The important point is that these substances occur in virtually every kind of cell, and in each cell type they perform a particular function. Organisms which require them for growth do so because they are unable to synthesize the substance. They therefore must find it in the environment if they are to grow.

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QUESTIONS

1. Define or identify:

vitamin	simple media
autotrophic	defined media
heterotrophic	complex media
photosynthetic	"nutrient" medium
energy	peptone
trace elements	artificial media
saprophytes	amylase
parasites	lipase
cellulase	proteinase
growth factor	peptidase

2. A medium composed of a water extract of pond lily is prepared by a microbiologist in a laboratory isolated in the woods. What chance will he have to cultivate a microorganism which he suspects may be causing a disease of moose, common to the area, which feed on the water lily? What chance of cultivating an organism causing a disease of the water lily?
3. In producing streptomycin by the growth of an organism requiring large amounts of oxygen, it is a great advantage to have stirring paddles in the tanks and to stir vigorously. Why does this increase the yield?
4. While lake water is perfectly clear and one can find few, if any, bacteria in it, a clean glass slide suspended in the water for a day or so and then removed and stained shows hundreds of microorganisms. Why? Do you suppose that this method will give one a knowledge of the living population of the water?
5. An organism is found to attack cellophane. How would you look for an extracellular enzyme which it might form for this purpose? If you find one, what would you name it?

Chapter 6

THE CLIMATE OF GROWTH

"To accomplish anything, you need an interest, a motive, a center for your thought. You need a star to steer by, a cause, a need, an idea, a passionate attachment. Men have followed many guiding lights . . . they have pursued power, wealth, holiness . . . something must beckon you or nothing is done, something about which you ask no questions. Thought needs a fulcrum for its lever, effort demands an incentive or an aim.

"No one, I fancy, will deny that knowledge has been the aim of our day and generation, the pursuit of knowledge and the increase in knowledge. Never has it been sought with greater determination and perserverance, and never with greater success . . . and yet what a polished structure is this built for us by science, how admirably designed, how perfectly proportioned. But as regards ourselves the teaching of modern knowledge is easily summarized. It proclaims our complete unimportance . . . The more worthless the object, the more enduring . . . The stone outlives the flower, the oak of the forest, the man.

"There is something peculiar in this self-depreciation, in the unanimity with which science and philosophy combine to humiliate us. There is here, one thinks, more than is easily accounted for by the evidence for the thesis. We must remind ourselves that the concentration of modern inquiry into the nature of physical things inevitably exalts their interests and importance . . . Do not forget that this

priority is of our conferring, that it is we ourselves . . . who have clothed the material world in the imperial purple of its present rank and dignity. The material increase has given use to an infinitude of things, to all of which it is totally and equally indifferent, stars and systems, men and animals, morals, religions, philosophies, hopes, aspirations, ideals, aims, purposes, joys, sorrows, affections. . . . It is certainly remarkable, this cosmos as described by modern science. Its indifference to itself . . . is amazing."

DIXON, *The Human Situation*

AMONG THE COSMIC FORCES which shape man's life and control his destiny, certainly climate is one of the foremost. It is not the extremes, which may be endured for an interval, but the sameness, the steady drip of time, which molds our lives. The climate of a microbe is more restricted than that of man; landscape and soil, rainfall and wind have naught to do with it. For the microbe the main factors of climate are the condition and availability of the water, the temperature, the supply of oxygen, and the acidity. There are lesser conditions: the surface tension, the carbon dioxide supply, and the accumulation of wastes; but in the main, the microbe's climate is water, temperature, oxygen, and acidity. Water, temperature, and acidity are always there; it is not (except in extremes) their presence, but their degree which is of concern.

The factors of climate, large or small, have this in common; if there is an excess, or if there is a scarcity, the cell cannot grow. But within a range—neither too much nor too little—the cell grows and thrives. Adjustment to the nature of the world is a condition of all life. But the microorganism is limited in the degree of adjustment possible; it has no tool, like hand and brain, to master its climate. It cannot construct for the arctic a heated cabin nor air-condition its camp in the tropics; but it can, and does, as we shall see, wall itself off from a hostile world when this is possible within the range of its construction.

These components of climate—water, temperature, oxygen, and acidity, with surface tension, carbon dioxide, and waste as an addendum—can on occasion become lethal. We shall meet some of them again in Chapter 12, where we are concerned with killing microorganisms. However, here we wish to explore their necessity, the way in which they condition and control the living cell, and how some cells have responded to the opportunities of nature to adventure, to explore and go forth into all the world.

Water

Water is a most unusual substance. It dissolves more substances than any other solvent. It causes many dissolved substances to break down into fragments (ions). It changes temperature more slowly per unit of heat applied than most other materials. It expands when frozen, and has its greatest density above its freezing point. Since changes occur faster between dissolved molecules than between solids, and such changes occur even more rapidly between ions, water, in effect, is acting as a catalyst. In a real sense, water converts solids to the gaseous state at lower temperature. So unusual are its properties that if it did not exist it would have to be invented before life could go on.

Water occurs in nature in both a free and bound form, the latter being associated in some not necessarily clearly defined manner with other molecules and thus not free to act

like water not so associated. Such water is called **bound water.**

What this means is that such bound water cannot be used as a solvent. It can be measured in the following fashion. It is known that each mole per liter of a nonionizing solute, such as sucrose, for example, will depress the freezing point of water 1.86°. Suppose that we have a bacterial suspension whose water content we can measure by drying, and we dissolve a mole of sucrose in a liter of it; the freezing point depression will be more than 1.86°, i.e., some of this water, which can be driven off by heat and when condensed is real water, is not available for dissolving the sucrose. Hence a liter of such water contains less than a liter of water in which sucrose will dissolve. The water that does not act completely like water is the bound water. Presumably it consists of water molecules so close to other surfaces as to be attracted and so is not able to move freely, or that possibly caught in holes or pores of other molecules or structures.

What we do know is that living cells, while they have much bound water in them, cannot grow, or at least grow well, when bound water is too great a part of the climate. For example, agar itself binds water, and at 1.5–2% agar there is considerable bound water in the medium—but also adequate free water in most circumstances. If, however, one uses 10–15% agar, so much of the water is bound to the agar that growth is inhibited.

In addition to requiring free water and being unable to use bound water effectively, the cell is influenced by three further factors having to do with water: osmotic pressure, surface tension, and solute concentration.

Osmotic Pressure

Even the child of today "knows all about" **osmotic pressure;** every grade school has demonstrations of the thistle tube and a **semipermeable membrane.** This last means, by definition, that it allows one kind of substance—i.e., one molecular species—to pass, but not another. But the nature of semipermeability and what makes such a membrane semipermeable are still somewhat obscure. The membrane does not have to be alive or even come from living tissue. Suffice it for our purpose that the water will flow from the less concentrated solution into the more concentrated. If the more concentrated solution is held within an enclosed space, it will tend to burst out. Indeed, we had earlier indicated that one of the functions of the bacterial cell wall is to constitute just such an enclosed space and that if it is removed, the cell bursts; unless indeed we adjust the external solution to a "high enough" osmotic pressure to prevent this flow of water into the cell.

Measurements of such bursting show that the internal osmotic pressure of many gram-positive bacteria is of the order of 20 atmospheres; gram-negative bacteria of the common laboratory type have an internal osmotic pressure of 5–10 atmospheres. Fundamentally this means that in solutions containing dissolved substances of such a concentration, the osmotic pressure is higher than these internal pressures; water will flow out of the cell, and under these circumstances a cell cannot live. The membrane of the cell, of course, must be semipermeable to the dissolved material.

It is not at all clear as to just how the nutrient-carrying water, after being pulled into the cell by the high osmotic pressure, is pushed out again (if indeed it is) to keep a steady stream of water flowing through the cell. It is quite clear that if the water cannot enter the cell, the cell cannot grow. Therefore, in a general way, solutions of high osmotic pressure such as 15–20% salt or 20–50% sugar do not usually support bacterial growth or that of other microorganisms. But here is an environment—of high salt and of high sugar—which some organisms have succesfully invaded, as described below.

There are certain words in common use when the subject of osmotic pressure is discussed:

hypertonic (solution or condition) means that the osmotic pressure is higher

hypotonic (hypo = low) means that the osmotic pressure is lower

osmophilic—liking high osmotic pressure (philic = liking)

halophilic—liking high salt (halo = salt)

Honey, on occasion, is decomposed yeasts, which grow on 70–80% sugar. Some of these osmophilic yeasts, indeed, grow *only* in high sugar concentrations. They do not tolerate high salt, for example, or high osmotic pressure from any other source. How they manage to live under the circumstances is not precisely known.

There are other organisms (usually small gram-negative rods) which can live in very high salt (NaCl) concentrations. These halophilic organisms are of two principal types: moderate halophiles, which can get along on as little as 1–2% salt but will grow in up to 20% salt, and grow well in media containing 10% salt. Most nonhalophilic bacteria can do without anything but traces of salt and begin to be inhibited at 2–5% salt. The other type are the extreme halophiles, which *require* above 10–15% salt and can grow in as much as 31% salt. Why this salt is necessary and what the cell has done to explore this bizarre climate are matters still under study. For our purposes it suffices to know that such creatures exist.

The amount of available water required for growth varies with organisms, but in general molds require about 15% available water, bacteria about 18%, and yeasts 20%. If the moisture content is less than these values, the organisms cannot grow. This property is, of course, used in the preservation of food—grain, hay, dried milk, fish, jams and jellies, etc. Actually, in practice, the situation is a little more complicated, but what is important here is that when the available water goes below a given value, organisms cannot grow.

Surface Tension

One usually thinks of **surface tension** in terms of an interface between water and air in which, as illustrated in Fig. 6-1, the molecules in the surface of the liquid are subject to forces of attraction sideways and downward, but not upward, while those in the interior are subject

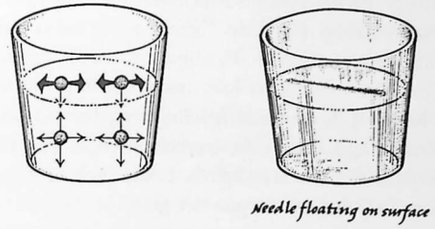

FIG. 6-1. *Representation of surface tension. A molecule beneath the surface is attracted and repelled by other molecules from all directions. At the surface the attraction to the air is less than to the fluid, so that the attraction is greater at the molecules sides (represented by heavier arrows). This extra attraction is strong enough to float a needle.*

to similar forces in all directions. The net result is that the surface acts like a skin; a drop of water remains a drop and does not flow out unless it is attracted to its contact material (unless it "wets"). Even more dramatically, one may show surface tension by carefully placing a needle on the surface of a dish of water. Although the needle is much heavier than the water and will rapidly sink once it has penetrated the surface at any point, careful horizontal positioning will enable the force of surface tension to keep it afloat. The surface tension of water, at 20°C, is 72.7 dynes/cm and very few materials are able to raise the surface tension. Many things—proteins, soaps, fats—tend to lower surface tension; they tend to concentrate at the surface and to weaken the attraction between adjacent water molecules.

What does this attraction between molecules

at a gas-liquid surface (the top of a tube of broth, for example) have to do with a bacterium growing in the medium? Very little indeed; but a similar surface tension (sometimes called **interfacial tension**) exists wherever there is a surface. Obviously there is such an interfacial tension between the cell and the medium. This, too, is of little importance, but those things which lower surface tension collect at such surfaces and thus they tend to collect at the surface of cells. If, therefore, surface tension of a medium is low, materials in solution which are responsible for lowering the surface tension will tend to be concentrated at the cell surface; if these are slightly toxic, such as soap, they may inhibit or prevent growth.

Temperature

Temperature, a measure of the kinetic energy of molecules, is a factor of life and a condition of it. Living activity is possible only within a narrow range of temperature, if all creatures are considered. For a single creature, this temperature range is narrower still—by a good deal indeed. All organisms become inactive when their temperatures drop to near 0°C. Most organisms do not live above 40°C, a few may live up to 70–75°C, but at 80°C life is no longer possible. But few individual species of plants, animals, or bacteria are active over even half this range; in fact, a 20°C range—between the lowest possible temperature at which the organism can grow and the highest which is possible for it—is more common. Bacteria, of course, are at the temperature of their environment and have no way to cool themselves or heat themselves, nor do they have temperature-regulating mechanisms.

Since all creatures have a temperature range in which they can live, this range has limits, with a minimum and a maximum at either end. Somewhere in the center there is an optimum temperature, that temperature at which they grow best. Of course, "best" may mean slightly different things at one time compared to another. Best for what?—for the most growth? for the fastest growth? These "best" temperatures are not necessarily exactly the same, but they are generally close.

While any species has a range of less than 25°C, there are general groups of bacteria, even though their temperature ranges may overlap a little:

Psychrophilic (cold-liking)
Range: 0°C → 25°C; optimum usually 20–25°C, characterized by growth (in 4–5 days) at refrigerator temperature (4–10°C)
Mesophilic (middle-liking)
Range 20°C → 45°C; optimum 30–37°C (37°C is human body temperature). Most organisms belong in this group
Thermophilic (heat-liking)
Range 45°C → 70°C; optimum 50–55°C

What is curious about these groups is that the thermophile will not grow at all at 37°C, nor will the mesophile grow in the refrigerator. While it is easy, perhaps, to understand how it is possible for an organism to adapt to the cold, it is more difficult to understand how the thermophile can stand the heat—heat which is frequently enough to coagulate the proteins of a mesophile. It appears that in adaptation to heat the thermophile has made a change in its proteins so that they do not coagulate except at much higher temperatures.

The point to be made here, however, is that in the course of evolution in nature, there has been adaptation both to cold and to heat. But this adaptation is not an external one, like the Eskimo's or the Hottentot's (Fig. 6-2), but lies in the fundamental nature of the cell itself.

Of all the factors of climate, temperature is unique. While it is always present, like acidity or water, it cannot be excluded from the cell, as can acidity. That is, it is not possible for an organism to extend its temperature range by excluding the excess factor. It cannot escape temperature; to extend its range it must itself change, for in a water environment it has no chance to be at any other temperature than that of its environment.

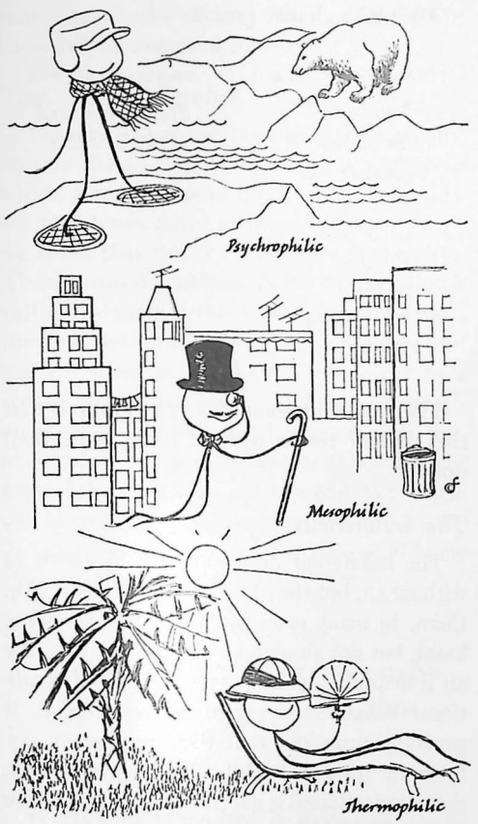

FIG. 6-2. *Illustrating the meaning of the words used to describe temperature ranges for microorganisms.*

Oxygen

The oxygen contained in air is of such great importance to life that many people suppose there is "no life without air." These people forget the bacteria, for among the bacteria there are forms—the anaerobes—in which air or the oxygen it contains is not only not required, but is actually toxic.

In relation to air there are four kinds of bacteria, which we shall call the strict aerobes, the facultatives, the indifferents, and the anaerobes. But before discussing them we should mention the use and importance of air, which is obviously used for the oxygen in it. Oxygen comprises 20% of the air; most of the remainder is inert nitrogen, but the traces of CO_2 and H_2 are of biological importance. The oxygen is used for respiration, a process by which the oxygen gas is used up to provide energy to the cell. But when bacteria were found which were capable of obtaining energy without using gaseous oxygen, some people felt that it was the energy-obtaining part of the definition of respiration which was important and not the use of gaseous oxygen. Hence, some people defined and used the term "respiration" to mean any reaction which released energy, whether it used oxygen or not. This naturally caused (and still does cause) some confusion. The situation got even worse when some oxygen uptakes were shown to yield no energy to the cell. In a situation such as this, it is necessary to define the terms one is going to use. Therefore we shall take the somewhat controversial words to mean the following:

Respiration will mean the uptake of oxygen gas, generally from air. Such respiration largely benefits the cell by providing energy, but there are respiratory systems—groups of chemical reactions in living cells resulting in oxygen uptake—which do not yield energy to the cell. So long as these systems take up oxygen, we shall call their process respiration, whether or not this respiration is of use to the cell. By "uptake of oxygen" we mean the actual disappearance of gaseous oxygen, it being converted into H_2O, H_2O_2, or other oxidized products. The nature of respiration and how it is brought about are

discussed in Chapter 9, but for the moment it suffices to note that respiration, as used here, means oxygen uptake.

For a metabolic reaction, or for a life process which goes on without the use of air, we shall use the term **fermentation** or, on occasion, **metabolism.**

The Strict Aerobes

The **aerobes** are organisms which must have air in order to live. If air is removed, or the oxygen in the air used up, they smother and die. Virtually all higher organisms belong to this group, but among the bacteria the strictly aerobic organisms are found mostly in the family Pseudomonadaceae; the following genera are regarded as typically aerobic:

GRAM-NEGATIVE	GRAM-POSITIVE
Nitrobacter	*Sarcina*
Nitrosomonas	some *Bacillus*
Pseudomonas	many *Micrococcus*
Acetobacter	(See Chapter 7 for
Azotobacter	for the meaning of
Rhizobium	these terms)

The Facultatives

The **facultative** organisms can live without air (by a fermentative metabolism) but can also live in air. However, the air "does them good," i.e., they are able to grow better in air (usually ten times better per unit foodstuff) than without it. Sometimes they have been called "facultative aerobes" because, some said, they are "really" aerobes but have the faculty of living anaerobically. However, others confused this by saying that they should be called "facultative anaerobes" because they have the faculty of living anaerobically. Hence the terms "facultative aerobe" and "facultative anaerobe" began to mean the same thing, so the term "facultative" is now adequate.

Typical facultative genera:

GRAM-NEGATIVE	GRAM-POSITIVE
Vibrio	*Staphylococcus*
Spirillum	many *Bacillus*
Escherichia	
Aerobacter	
Klebsiella	
Erwinia	
Serratia	
Salmonella	
Shigella	

The important point about this group is that they benefit by air without being dependent upon it.

The Indifferents

The **indifferent** organisms can live with or without air, but the presence of air doesn't help them. In many cases the air may be of some harm, but not absolutely. Or, alternatively, the air is helpful only under very specialized conditions. When these organisms are studied, it generally turns out that their respiratory systems are such that while the oxygen is used, the energy released is not in a form useful to the cell. Sometimes the end product of the oxygen uptake is hydrogen peroxide (H_2O_2) rather than the more usual water (H_2O), and the peroxide may actually harm them.

Typical indifferent genera:

GRAM-NEGATIVE	GRAM-POSITIVE
	Diplococcus
	Streptococcus
	Lactobacillus
	Propionibacterium

Sometimes these organisms do well with relatively small quantities of oxygen and there are **microaerophilic** organisms, which grow best with a very small amount of air. This type somewhat shades off into the anaerobes, where they will be discussed further.

The Strict Anaerobes

The **anaerobes** are organisms that can live only in the absence of air. There is a group in which a small amount of air is tolerated; they are sometimes called microaerophilic, although we think that this is a wrong use of the term. These forms do not grow *better* with a little air (although there is this kind of organism too), they are only able to grow in its presence and are not killed by air. This is in contrast to a second group, the strict anaerobes, for which air is toxic—they will die if exposed to air; but, also, even without total exposure, they will not grow if there is any air at all around. Why there can be such things as anaerobes, we shall discuss in Chapter 9; at this point it suffices to know that such organisms exist.

Typical anaerobes:

GRAM-NEGATIVE	GRAM-POSITIVE
Bacteroides	*Clostridium*

Acidity

It was early recognized, in studies on the climate surrounding bacteria, that acidity and acid content were not the same thing. Dexterous handling of these qualities by the early bacteriologists, unequipped with the concepts and methods we now possess, is one of the noteworthy achievements of intuitive science. That there was a fundamental difference between the "acid effect" of a normal solution of acetic acid and a normal solution of hydrochloric acid they surely recognized, and they differentiated to a nicety between the acidity of a solution (which they called its "reaction," meaning actually how it acted to litmus) and its actual concentration of acid. These early bacteriologists, up to about 1915, developed processes and did research with materials and methods requiring a delicate balance of *p*H without clearly recognizing just what was happening and with the hopelessly inadequate concept of "strong acids" and "weak acids." We believe today that the effective acidity of a solution is a function of its hydrogen ion activity—roughly, its hydrogen ion concentration. Since there is no life without water, and no water without hydrogen ions, these are an ever-present factor in any biological system. One measures the hydrogen ion concentration in terms of a *p*H scale. In case you have forgotten what *p*H is, how it is calculated and how it is determined, we refer you to the digression on *p*H at the end of this chapter. If you already understand *p*H you may skip it when you reach it. Only one warning. From here on, we will assume that you know *p*H and will no longer explain or paraphrase it.

The metabolic activity of the bacteria culture may produce from the components of the medium a variety of end products—usually acidic, more rarely basic as with NH_4^+ or the metal ion left after a salt such as sodium succinate is used up. Even if the medium were initially at *p*H 7.0, the release of such substances during growth may shift the *p*H above or below the outside limits for growth, and cell reproduction would become impossible. It is therefore necessary to include as a component of a culture medium a chemical which will act as a hydrogen ion buffer over the *p*H range at which the organism can grow. The most widely used buffers are K_2HPO_4 and KH_2PO_4, used individually or in combination. Frequently, the main purpose of phosphates in a medium is just this buffering capacity. The optimum *p*H for most microorganisms is generally within 0.5 *p*H unit of neutrality, i.e., 6.5 to 7.5; hence media are usually buffered at this point.

Because **buffering capacity** is frequently not understood, a brief explanation is given, mostly in practical terms. One may look at the dissociation constant of any acid and determine at what

*p*H it will be a good buffer. For example, the dissociation constant (K) of acetic acid is 1.86 $\times 10^{-5}$. This means that the acid is a buffer at *p*H 5 (10^{-5} = "*p*H 5"). Actually, the midpoint of its buffering range is $-\log K = -\log 1.86 \times 10^{-5} = 4.73$. (This is called *p*Ka, compared to *p*H.) The *p*Ka represents the *p*H at which the acid is half and half salt, i.e., its greatest buffering capacity. At *p*H 3.73 (1 *p*H unit lower), the acetic acid is 90% in the form of the acid and 10% in the form of its salt and its buffering capacity is about used up toward the acid side. At *p*H 5.73 the acetic acid is 90% salt, and its capacity to buffer is about used up at this side. Hence, acetic acid-sodium (or potassium, etc.) acetate mixtures are good buffers from *p*H 5.7 to 3.7, but best at 4.7. Some *p*Ka values for common buffers are given in Table 6-1.

While these tables are helpful (more extensive ones may be found in chemical data handbooks), it is useful to have some more practical notion of how much buffer is needed. Perhaps the easiest way to start is to examine a medium in which an organism is producing acid. Let us consider the following medium, inoculated with *Streptococcus lactis*, producing lactic acid.

Medium	Grams per 100 ml medium
Yeast extract	1.0
Tryptone	1.0
K_2HPO_4	1.0
Initial *p*H 7.5	

With no glucose the organism grows poorly and the *p*H rises to 7.7–7.8. With increasing glucose the growth and final *p*H found are as shown in Fig. 6-3; that is, for an organism producing only acid from glucose, 1 g of K_2HPO_4 per 100 ml is capable of holding the *p*H to a suitable level for all of the acid produced from 0.3–0.4 g of glucose. In this case the yeast extract and tryptone contribute some buffering capacity too. In a synthetic medium, with an organism producing just acid, more phosphate (or other buffer of *p*H 7 range) would be necessary to counteract the acid formed. With an organism producing less acid and more of neutral products (compare Fig. 6-3), less buffer is needed.

Virtually all bacteria are quite unable to grow below a *p*H of 4.5. Indeed, when we are able to find an occasional organism which can do so, it is regarded as a great curiosity. Bacteria do not grow above a *p*H of 9. The range, then, for bacteria is *p*H 4.5 to 9.0. But actually, most grow best at *p*H 6.5 to 7.5 and grow very much more slowly at a *p*H of 6 or below or 8 or above. Streptomyces have the same *p*H range as bacteria.

Molds and yeasts, on the other hand, grow well at *p*H as low as 3.5 and up to *p*H 7 to 8, rarely higher. In fact, they mostly prefer an acid environment and tend to grow best at *p*H 5–6. (There are exceptions, of course.)

Organisms can make enough acid during their metabolism to actually stop their growth (and sometimes enough to kill themselves off). But one should mention that many bacteria, while they do not grow at a *p*H below 4.5, are not killed by this *p*H and may tolerate, for varied intervals, a *p*H as low as 1 or even 0.1 without great harm.

TABLE 6-1. *Some p*Ka *Values for Common Buffers*

Best *p*H Range	Material	*p*Ka
2	H_3PO_4	1.959
3	phthalic acid	2.90
	tartaric acid (K_1)	2.96
	citric acid	3.1
4	lactic acid	3.86
	barbituric acid	3.98
	tartaric acid	4.16
5	acetic acid	4.7
	citric acid (K_2)	4.7
	propionic acid	4.9
	malic acid	5.1
	benzoic acid	5.2
	citric acid (K_3)	5.4
6	uric acid	5.8
	carbonic acid	6.5
	pyrophosphoric acid	6.5
7	phosphoric acid (K_2)	6.7
8	tris, glycyl glycine	—*
9	boric acid	9.1

* This is a base and one may develop a *p*Kb just as one did a *p*Ka but we have omitted this for simplicity.

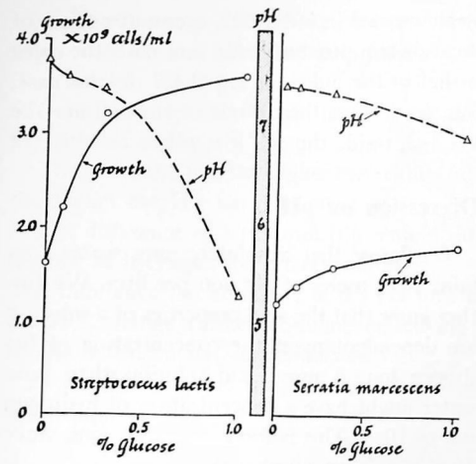

FIG. 6-3. *Growth and acid production related to the buffering capacity of the medium. Two organisms were grown in the yeast extract tryptone medium described, with 1% K_2HPO_4 as buffer and with different amounts of glucose. One* Streptococcus lactis *produces mostly lactic acid. At about 0.4% glucose the buffering capacity has been used up, the* pH *drops, and further growth is inhibited. The other organism,* Serratia marcescens, *produces much less acid even at 1% glucose; the* pH *has not dropped enough to be inhibitory. This organism also grows less than the* Streptococcus *on this medium.*

Other Climatic Factors

There are two other factors of the climate which affect bacterial growth. These are the waste products which the cell may have accumulated, and the CO_2. The effect of waste products, including acids when these are formed, is relatively easy to see; at best these wastes are inert and not usable to the cell; at worst they may be toxic to it. But the effect of CO_2, frequently described as CO_2 pressure or CO_2 tension, is somewhat more difficult to understand. Many pathogenic organisms when isolated from the body will not grow unless extra CO_2 (sometimes as much as 5% CO_2) is added to the air. Later, they may grow without added CO_2. Others may grow well in air without added CO_2 but will not grow if all the CO_2 is removed. Twenty years ago, this fact—that even nonphotosynthetic bacteria required CO_2 or at least could not grow without it—was a greater mystery than it is today. For then, everyone was firmly convinced that only photosynthetic and autotrophic organisms could fix or incorporate CO_2. Today we know that all cells fix CO_2, heterotrophs as well. Removing all of the CO_2 prevents the formation of some compounds essential to metabolism.

Extension of Climatic Range

Man ventures into the Arctic by taking his environment with him—heavy clothes, insulation from wind, even an igloo. Man ventures into the air by retaining the atmospheric pressure he has on earth. Man ventures beneath the sea in a submarine, walling off the hostile environment. The microorganism cannot, of course, build such machinery and the extension of its range of growth into a hostile climate (where, however, there are advantages of less competition) is based upon what it itself can do. Of the main factors of climate, three—moisture, oxygen, temperature—have been mastered by some organism. There are osmophilic yeasts and halophilic bacteria; there are aerobes and anaerobes; there are thermophiles and psychrophiles. In all of these cases, so far as they are understood, the change permitting pene-

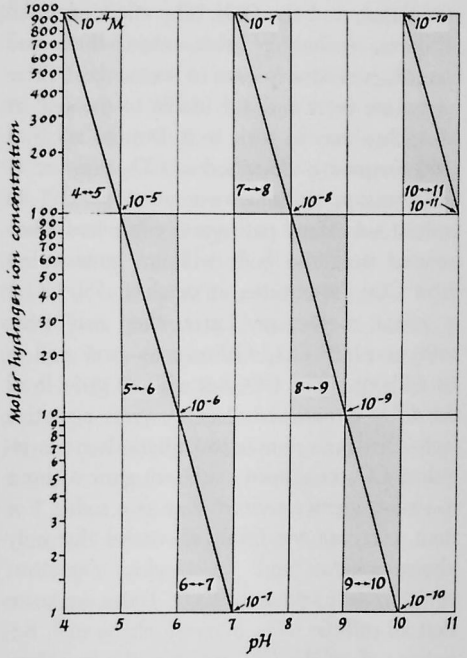

FIG. 6-4. *Relation between pH and the hydrogen ion concentration.*

tration of the otherwise hostile environment has occurred in the nature of the enzymes of the cells themselves.

But there is one case where the hostile environment is simply walled off, as man does in a submarine. Although there are few bacteria able to grow below a *p*H of 4.5, there is one that not only grows well at a *p*H down to 0.5 but actually will not grow at any *p*H greater than 6. This organism (called *Thiobacillus thiooxidans*) is further a most unusual one; it derives its energy from the oxidation of sulfur to sulfuric acid and all of its carbon from CO_2, and is a strict autotroph. When enzymes are isolated from it, however, they are the same enzymes that we find in heterotrophs. Even more striking, the enzymes operate at a neutral *p*H (characteristic of the enzyme), rather than being acid-tolerant. Further, when such cells are immersed in indicators, eventually some of the indicator penetrates and internally the color is that of the indicator at *p*H 6.7. In this case, therefore, while the outside of the cell may be *p*H 1–2, inside the cell it is *p*H 6–7.

Digression on *p*H

We know that absolutely pure water contains 10^{-7} moles of H^+ ion per liter. We further know that the acid properties of a solution are dependent upon the concentration of hydrogen ion. A more acid solution than pure water might have a concentration of hydrogen ion of 10^{-5}. This is *more* hydrogen ions, since 10^{-5} is a *larger* number than 10^{-7}.

Since water ionizes in the following fashion

$$H_2O \rightleftharpoons H^+ + OH^-$$

in pure water, the H^+ is 10^{-7} molar, the OH^- concentration is also 10^{-7} molar. (Why?)

At H^+ ion concentration of	*The OH^- ion concentration is*
10^{-7} molar	10^{-7} molar
10^{-5}	10^{-9}
10^{-2}	10^{-12}
. . .	. . .

(H^+ concentration)(OH^- concentration) = 10^{-14}

Pure water is neither acid nor alkaline; it is neutral. Therefore an H^+ ion concentration of 10^{-7} molar is neutral. Concentrations of H^+ ion less than this ($10^{-8} \ldots 10^{-14}$) are alkaline; concentrations more than this ($10^{-7} \ldots 10^{-1}$) are acid.

But it is difficult to use expressions such as 10^{-7} and 10^{-12}; hence a term *p*H ("potential hydrogen") was introduced as an expression of H^+ ion concentration. It uses the exponent of the old expression, i.e., H^+ ion concentration of 10^{-7} is *p*H 7; but it changes its sign. By actual definition,

$$pH = \text{logarithm of } \frac{1}{H^+} \text{ or}$$

$$pH = -\log H^+ = \log 1 - \log H^+.$$

(See logarithms, p. 108.) Thus we are able to express acidity and alkalinity on a *p*H scale, as illustrated in Fig. 6-4.

Certain things should be noted about this logarithmic scale:

The lower the *p*H, the higher the acidity, i.e., the higher the H^+ ion concentration.

The difference of 1 *p*H unit is a tenfold difference in hydrogen ion concentration.

A difference (or an error) of 0.3 *p*H unit is a 100% change (either doubling or halving) in the hydrogen ion concentration.

If all of this is too simple for you, try the following:

1. What is the *p*H of a 0.5 Molar HCl solution? (Assuming complete ionization.)

Answer: *p*H 0.301.

2. What is the *p*H of a normal solution of HCl which is 78% ionized? (Note: 1 normal solution would have 1 Mole HCl per liter.)

If it is 78% ionized, it would have 0.78 Mole H^+ ion per liter.

$pH = -\log H^+ = -\log 0.78$

$pH = -(\log 78 + \log 10^{-2})$

$pH = -(1.8921 - 2) = -(-0.1079)$

$pH = 0.1079$ (Answer).

3. What is the *p*H of a normal solution of acetic acid which is only 0.4% ionized?

Answer: *p*H = 2.4.

4. What is the hydrogen ion concentration of a solution which has a *p*H of 4.3?

Answer: 5×10^{-5}.

5. What *p*H corresponds to $H^+ = 10^{-7}$ *N*?

Answer: *p*H = 7.

6. What *p*H corresponds to $H^+ = 0.0002$ *N*?

Answer: *p*H 3.7.

7. What H^+ conc. corresponds to *p*H = 8.43?

Answer: 0.37×10^{-8} N.

8. Show that an error of 0.3 *p*H unit represents an error of 100% in the H^+ ion concentration regardless of the actual *p*H involved.

pH is measured by electrometric methods (the most common being the glass electrode) or by colorimetric methods. There are certain substances, called indicators, whose color depends on the *p*H; thus they serve as an index of the *p*H. Common indicators used in bacteriology are:

Indicator	Color at acid *p*H	Color at basic *p*H
Litmus	Red (*p*H 4.5)	Blue (*p*H 8.3)
Methyl red	Red (*p*H 4.2)	Yellow (*p*H 6)
Brom cresol green	Yellow (*p*H 3.8)	Blue (*p*H 5.4)
Brom thymol blue	Yellow (*p*H 6.0)	Blue (*p*H 7.6)
Brom cresol purple	Yellow (*p*H 5.2)	Purple (*p*H 6.8)
Phenolphthalein	Colorless (*p*H 8.2)	Red (*p*H 10)

While there are certain advantages to the *p*H scale, and these outweigh the disadvantages, you should be aware of the disadvantages. The *p*H scale represents degree of acidity and two solutions differing by one *p*H unit will differ 10 times in actual acidity. There is therefore this objection to the *p*H scale, that being logarithmic it unnecessarily complicates matters. Close to the neutral point, the *p*H changes rapidly with a small change in acid content [i.e., *p*H 6 ($H^+ = 10^{-6}$) to *p*H 7 ($H^+ = 10^{-7}$)]. There is a change of 0.000,000,9 (9×10^{-7}) moles per liter of acid content. But in a change of *p*H from 1 ($H^+ = 10^{-1}$) to *p*H 2 ($H^+ = 10^{-2}$), there is a change of 0.09 ($= 9 \times 10^{-2}$) moles per liter. This would be a very valid objection to the *p*H scale, since change in unit of scale bears a varying relation to change in hydrogen ion content, were it not for the fact that it so happens that the majority of processes in the living cell take place within a very narrow range of hydrogen ion concentration. It is therefore an enormous advantage to use a *p*H scale which changes rapidly where minute control is necessary.

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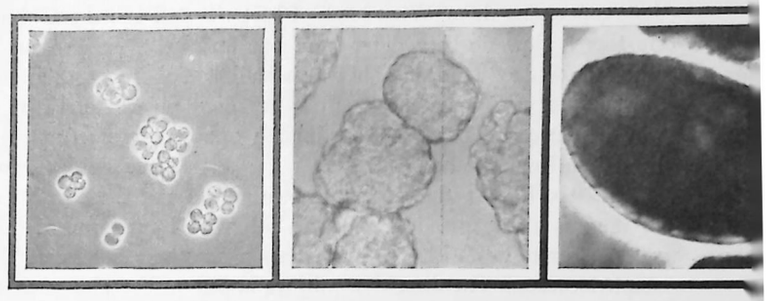
For background in function, see p. 491.

QUESTIONS

1. Define or identify:

surface tension	psychrophilic
bound water	mesophilic
osmotic pressure	thermophilic
semipermeable	anaerobe
hypertonic	aerobe
hypotonic	facultative
osmophilic	indifferent
halophilic	respiration
optimum	fermentation
microaerophilic	reaction
*p*H	buffer
*p*Ka	

2. It is known that plants which are resistant to freezing, and can thus survive in cold climates, have higher bound water than plants not so endowed. Why would this property tend to permit cold resistance?
3. A bacterial culture grows slowly in a medium composed of glucose, peptone, and 0.5% K_2HPO_4 (*p*H 8.3) at room temperature. What would you do to attempt to make it grow faster? How would you preserve this culture?
4. What concentration of sucrose would you need to obtain an osmotic pressure of 10 atmospheres? This answer is not in the textbook; where would you look to find it?
5. If we have a solution of 15% salt and 30% glucose, which has the highest osmotic pressure? Why?
6. What is the meaning of *p*Ka? If an acid has a *p*Ka of 4.62, over what *p*H range is it a good buffer? What would be its range if its *p*Ka was 6.35? 2.7?
7. Most bacteria do not grow below a *p*H of 4.5. What is the hydrogen ion concentration at this *p*H?
8. Show that a difference of 0.3 of a *p*H unit, at any part of the *p*H scale is a 100% change in the hydrogen ion concentration.
9. What is the *p*H of a normal solution of HCl (completely ionized)? What is the *p*H of a normal solution of another acid only 30% ionized?
10. Why is the concept of strong acid and weak acid not adequate to explain the biological effects of acids?



Chapter 7

THE KINDS OF BACTERIA

"To spend too much time in studies is sloth; to use them too much for ornament is affectation; to make judgment wholly by their rules is the humor of a scholar. They perfect nature, and are perfected by experience, for natural abilities are like natural plants, that need pruning by study; and studies themselves do give forth directions too much at large, except they be bounded in by experience. Crafty men contemn studies, simple men admire them, and wise men use them, for they teach not their own use; but that is a wisdom without them, and above them, won by observation."

FRANCIS BACON, *Of Studies* (1625)

WE HAVE PREVIOUSLY discussed various kinds of bacteria as they occur in nature and have grouped them upon the basis of their energy sources (photosynthetic, autotrophic, heterotrophic); on the basis of the nutrients required (simple to complex); with respect to their ability to grow in living tissue (saprophytic to parasitic); on their relation to temperature (psychrophilic, mesophilic, thermophilic); with respect to oxygen (aerobic to anaerobic); and several other properties. Any given bacterium may possess one or several of these properties; it may be a heterotrophic psychrophilic anaerobe or a mesophilic parasite.

Our discussion, further, has shown that organisms possess different forms, different resistance or sensitivity to harmful agents, different capacities to attack substances, different colony forms, and different "cultural characteristics." This almost infinite variety in the different kinds of bacteria naturally causes us some problems when we try to approach ques-

tions concerning them. What kinds of bacteria are there? How do they differ from one another? How may we determine which kind is in a culture? And sometimes you may wonder why it matters to be able to distinguish one bacterium from another.

It matters for several important reasons. One of these is a very practical reason, which we shall illustrate in detail with a "digression on staphylococcus." The digression will, we hope, illustrate the practical necessity of **taxonomy**, as this science and art of classifying and naming organisms is called, and also conveniently teach you something about *Staphylococcus*. Another reason is less practical but is nonetheless important. Taxonomy is more than a set of rules for distinguishing one organism from another; when successfully applied it should illustrate and illuminate relationships between organisms.

Digression on Staphylococcus

There exist a group of cocci which divide in more than one plane, resulting in clusters of gram-positive spheres arranged in a random fashion which suggested a cluster of grapes (in Greek, *staphylē*); hence the word "staphylococcus." These randomly arranged "cluster cocci" were more important; some caused disease, sometimes a fatal lung infection, at other times merely a boil, and at still other times a violent but general nonfatal intoxication, a food poisoning. One really had to be able to tell which was which, and hence they needed to be separated further.

At this point it is important to realize that the separations employed are not a matter of whim. That, indeed, really no one cares whether they do or do not ferment lactose or salicin or what not, but only as these are important to something else. What we do know is that every once in a while one finds a diseased condition in an animal or in man, and all one can find as the cause of the disease is a "cluster coccus." Yet we can find hundreds of these cocci; we can isolate them from almost anywhere. What we are interested in is how are we going to tell the difference between those which are pathogenic (i.e., cause disease) and those which are not? It happens that the importance of this group was its causing disease, but it could have been any other character —the ability to make beer or the ability to decompose rice. We should, then, use those characters for separation which delineate the properties which are important, and by important we mean important to us. It is further convenient to have a name for the "cluster cocci" that divide in two planes, but do not form tetrads—it is much easier to say "micrococci." These two-plane-dividing cocci are called *Micrococcus* or *Staphylococcus* (since they were thought to resemble a cluster of grapes; staphylo = grape). The strictly aerobic organisms are put into the genus *Micrococcus*, and we find few, if any, pathogenic forms among them. The facultative organisms are called *Staphylococcus* and contain both pathogens and nonpathogens. Hence we have a group of cocci with the character associated with *Staphylococcus*. What we really want to know is which of them cause disease? How might we go about determining this? We could, for example, obtain strains from diseased sources, and see how they compared with those from nondiseased sources. The results of this kind of study (Table 7-1) show that 96% (83 + 13) of the organisms from pathogenic sources have an orange pigment. Most of them bring about the clotting of citrated blood (**coagulase-positive**) and are hemolytic. If an organism has all three of these properties, it is highly likely to be pathogenic; if it has pigment, is coagulase-positive, but is not hemolytic, the chances are reversed; only 13% of the pathogenic strains lack hemolysis. Thus, various indexes of pathogenicity based on laboratory tests have different degrees of validity; some correlate well with what we really wish to determine (in this case the pathogenicity) and some do not. The kind of a test to use depends upon how well the test shows pathogenicity, and may be different from one group of organisms to another. For example, hemolytic activity is a secondary index of pathogenicity among the staphylococci, but it has no significance in distinguishing pathogens from nonpathogens among the gram-negatives.

TABLE 7-1. *Strains from Diseased Sources**

Strain	Coagulase: + / Hemolysis: + / Pigment: +	+ / − / +	+ / + / −	− / + / +
Staphylococcus strains isolated from diseased conditions where it was most probably the cause of the disease (90 strains)	83%	13%	1.4%	<1%
Staphylococcus strains isolated from food poisoning (49 strains)	85%	13%	<1%	<1%
Staphylococcus strains isolated from diseased conditions but where one is not certain that they are the cause of the disease (82 strains)	51%	6%	6%	
Staphylococcus strains isolated from milk and from body surfaces in the absence of disease	5%	0	1%	

* Modified from data of Chapman, Berens, Nilson and Curcio, *J. Bacteriology*, **35**, 311 (1938).

We find three species of *Staphylococcus* described in the literature: *Staph. aureus*, the orange-yellow pigmented species, was thought to contain most of the pathogenic strains and some nonpathogenic ones; *Staph. albus*, the white pigmented type, were considered rarely pathogenic; and *Staph. citreus*, the pale yellow pigmented strains. Many today believe that only the name *Staphylococcus aureus* should be used, that the rest are derived from it. If a *Staphylococcus*[1] strain has pigment, is hemolytic, possesses coagulase (and is the proper phage type—see p. 465), the chances are fairly good that it is pathogenic. Other types of tests also show some correlation: the ability to grow in the presence of crystal violet in the medium, the fermentation of lactose under alkaline conditions (in brom thymol blue agar), and the fermentation of mannitol. If all are positive, the strain is very likely pathogenic. The first criteria are more important than the last, and if the first leave some doubt, then the other tests may help one to decide pathogenicity.

The staphylococci possess two modes of disease production. First, they may grow in tissues, either actually in the blood stream and thus throughout the body, or in rather circumscribed areas, causing boils or "pimples"; in cattle they may grow in the udder and cause an inflammation or a "mastitis." Initially, most of these could be treated with penicillin with great success; however, recently the penicillin-resistant staphylococci have become of considerable importance and new antibiotics have been introduced specifically for these purposes.

Second, the staphylococci grow well in nonliving material and produce therein a **toxin**, a poisonous substance produced by the cells and causing staphylococcal food poisoning (see p. 362). These strains also show up in laboratory tests as pathogenic-positive. There seems at the moment to be no satisfactory way, on the basis of laboratory tests, of distinguishing between the pathogenic staphylococci which invade the

[1] The term *Staphylococcus* (capitalized and in italics) refers to the genus name. The uncapitalized term refers to this general kind of organism but not strictly only to this genus.

body and those that produce the food intoxications. Both are pathogenic since both cause disease.

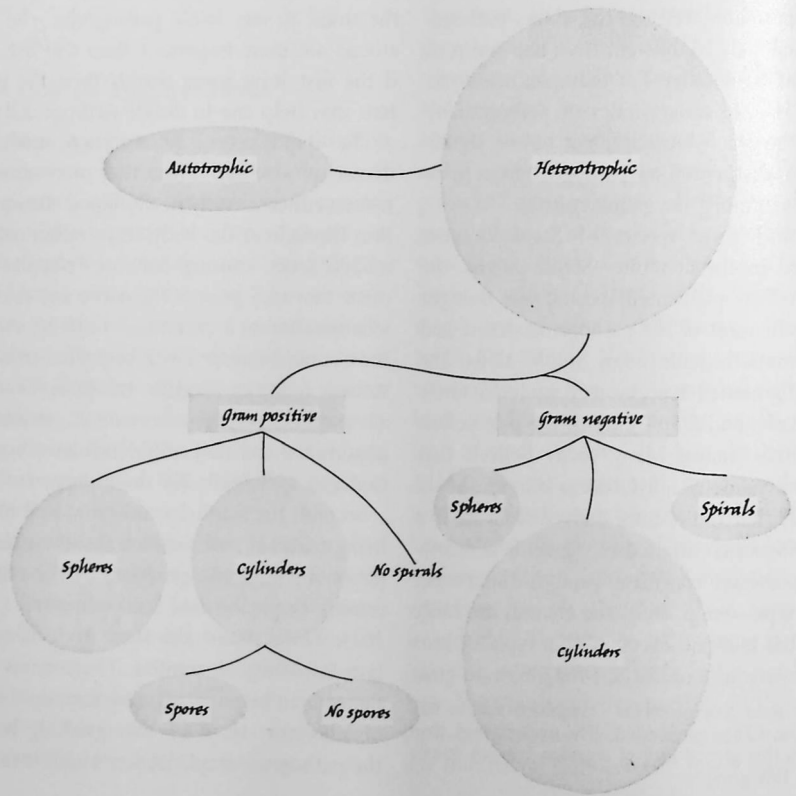
In the course of subsequent chapters we shall find it necessary to describe separations of other genera and even groups of bacteria, usually for a very practical purpose. In many cases, where no such purpose is served, we shall merely be content with the genera.

The Kinds of "True Bacteria"

We shall first confine our attention to the "true" bacteria, those organisms which conform exactly to our initial definition of bacteria. The essential words were single-celled, microscopic (plants), devoid of chlorophyll, and reproducing by binary fission. For our purpose, if an organism possesses anything else, for example, chlorophyll, or if it reproduces in some other fashion, but is otherwise related to the bacteria, we shall omit it now, and call it a "higher bacteria," which we shall discuss later (p. 274).

Microorganisms are named, like plants or animals. The name consists of two parts—the **genus** and **species.** In the bacterial name *Bacillus subtilis,* the *Bacillus* is the name of the genus and *subtilis* the name of the species. Each distinct kind of microorganism is called

FIG. 7-1. *The kinds of bacteria.*



a species. A difficulty arises sometimes in trying to decide whether two cultures are "distinctly" different enough from each other, but we shall not face these difficulties now. For our purpose, each species is a distinctly different kind of bacterium.

A genus is a group of related species. Groups of like genera are placed in a **family** (terms end in *aceae*), groups of related families in an **order** (terms end *ales*), related orders are placed in a **class,** then these into **divisions,** and **kingdoms** (plant and animal). The written name, however, includes only the genus and species.

The most ambitious attempt to classify bacteria and to name them is a collaborative effort by several bacteriologists known as *Bergey's Manual of Determinative Bacteriology,* which divides the bacteria into 10 orders, 47 families, and 190 genera. No microbiologist is expected to know all of the names nor is he obligated to adhere to this classification. However, it is convenient to have a system of terminology with which many, if not all, microbiologists agree, and so we shall (with few minor exceptions) use the names prepared in the latest edition (7th edition, 1957) of *Bergey's Manual.*

But before we begin to consider names, it is well to look at the kinds of true bacteria found in nature. These are roughly sketched in Fig. 7-1; the size of their background area is a rough index of their importance. Of course, you should ask, importance for what? So let us say, their importance to the microbiologist. These areas are a rough index of what he needs to know about them.

The first great division is between the autotrophic and heterotrophic mode of life, certainly an important distinction, earlier described (p. 114). We will discuss autotrophs elsewhere (p. 114) and will therefore devote the remainder of the discussion here to heterotrophs.

Heterotrophs

It will be noted that the kinds of heterotrophs are first delineated on the basis of their gram-staining character. This might seem to be, indeed, a rather trivial character, but the gram stain is so closely related to deep-seated characters of the cells that its use in this case is justifiable. We may therefore divide all heterotrophic bacteria into gram-negative (including gram-variable) and gram-positive bacteria, recognizing that associated with these staining properties are a group of physiological characteristics, somewhat as follows:

GRAM-NEGATIVE	GRAM-POSITIVE
More susceptible to	More susceptible to
proteolytic enzymes	acids (low *p*H)
high *p*H (alkali)	detergents
azides	alkyl sulfates
tellurides	organic solvents
oxidizing agents	iodine
lysis by complement	penicillin-type antibiotics

There are, in this respect, two general kinds of bacteria that stain "wrong," compared to their physiological properties. These are first the gram-negative cocci (two genera: *Neisseria* and *Veillonella*) which stain gram-negatively, but whose properties are more like the gram-positive organisms. Actually, these frequently appear to be "almost" positive. They are discussed in more detail in Chapter 25. The other group is the physiologically gram-variables. Of course, even gram-positive organisms may be gram-negative if the cultural conditions are not proper (see p. 27). But the gram-variable organisms are those which in normal cultural conditions may be distinctly positive when old, but negative when young. In real gram-positives, we usually find the opposite, i.e., positive when young, and negative when old—if indeed they become negative. The gram-variables

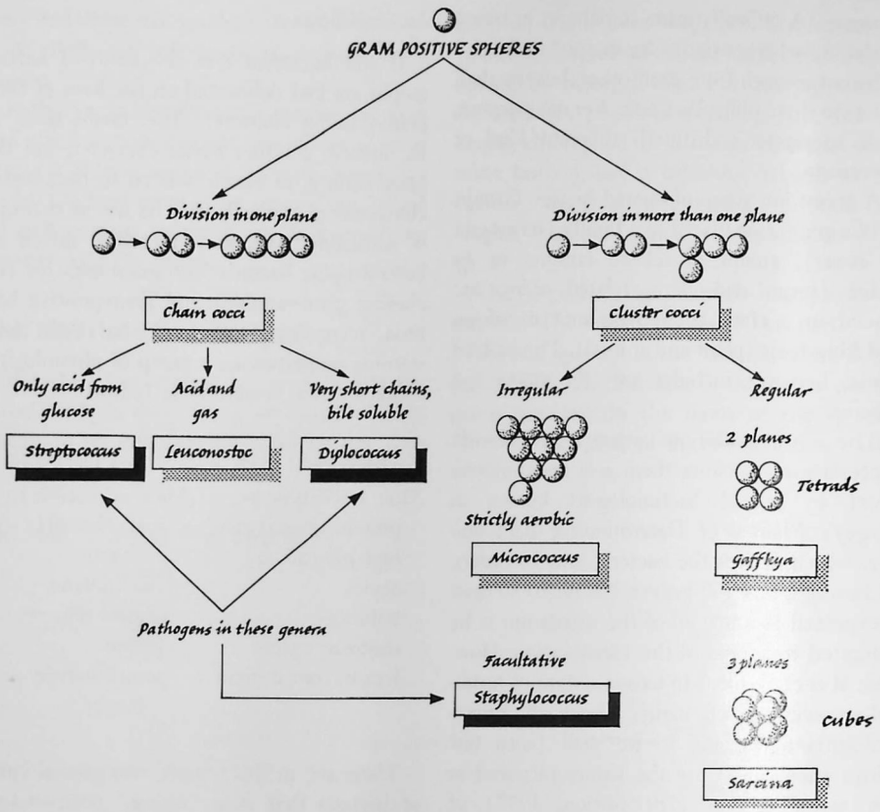


FIG. 7-2. *The relationships between the gram-positive spheres, the cocci.*

(negative young, positive old) are physiologically like the gram-negatives and have been placed with them in our subsequent discussion. We shall proceed by a discussion of each of the major groups shown in Fig. 7-1, with the important genera involved in each group. Note that one group is missing; there are no gram-positive spirals among the true bacteria.

Gram-Positive Spheres (Cocci)

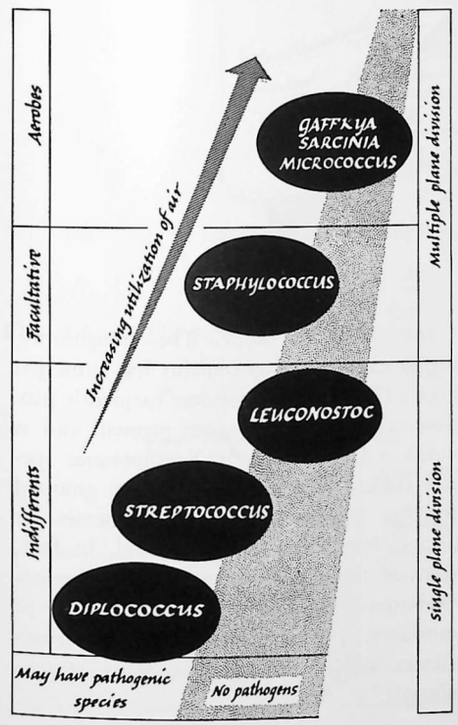
The gram-positive cocci are divisible into two large groups on the basis of their morphology (Fig. 7-2) and this division also reflects their physiological character. For example, their relation to air and their ability to utilize oxygen, as illustrated in Fig. 7-3, correlates well with their mode of division. We have discussed the *Staphylococcus* earlier in this chapter. The other three genera of the cluster cocci are all aerobic, grow on body surfaces and in air, are generally pigmented, but otherwise are of no special concern to us since they are not normally involved in disease causation nor do they play any distinct or characteristic role in any other phenomena in which we have an interest. While there are many species of each of these genera, in contrast to only three in *Staphylococcus*, yet under most circumstances we need not identify species and genus identification suffices.

The chain-forming cocci are of such importance, especially in their relation to disease, that we shall later devote an entire chapter (Chapter 25) to them as the principal agents of acute respiratory disease.

Gram-Positive Cylinders

There are two major kinds of these bacteria, distinctly different and easily separated upon the basis of spore formation as shown in Fig. 7-4. The *Lactobacillus* species are closely related to the *Streptococcus* in general, and while there are no pathogens within the group, they are important in the dairy industry; they are specifically discussed in further detail later (p. 369). The same may be said of *Propionibacterium*, except that these are quite different physiologically from the *Streptococcus* and resemble *Leuconostoc*. They characteristically produce propionic acid and are responsible for the flavor of Swiss cheese.

FIG. 7-3. *Some physiological properties of cocci; the relation between mode of cell division and oxygen-utilizing ability.*



From the *Lactobacillus* we can discern a chain of development leading to a large and important group of microorganisms, the *Streptomyces* (discussed later in Chapter 15). First, we find organisms whose cells appear to show "branching." There is the distinct possibility that these organisms divide by a process distinct from binary fission. As such, they are not quite true bacteria and hence we have two genera which have structures resembling branching: *Corynebacterium* (among which are species causing diphtheria) and *Mycobacterium* (among which are organisms causing tuberculosis and leprosy). These genera are discussed in more detail in Chapter 15.

From these groups, extending upward in a continuous series, are organisms with increasingly more and more complex morphological and reproductive structures, until actual filamentous forms are reached, the *Streptomyces*.

The spore-formers are divisible into two clearly differentiated groups—the aerobes and the anaerobes. The anaerobic forms are all placed in the genus *Clostridium* (their further separation is discussed on p. 364). The aerobic (and facultative) forms are placed in the genus *Bacillus*, which contains a variety of species but relatively few pathogens. In the main, however, the principal species are grouped as shown in Fig. 7-5.

Gram-Negative Cylinders

The gram-negative organisms, comprising the largest number of genera and the most varied kinds of bacteria, are divisible into three groups on the basis of their morphology: cylinders (rods), cocci, and curved rods or spirals. There are no spore-formers among gram-negative organisms. By far the largest group is the gram-

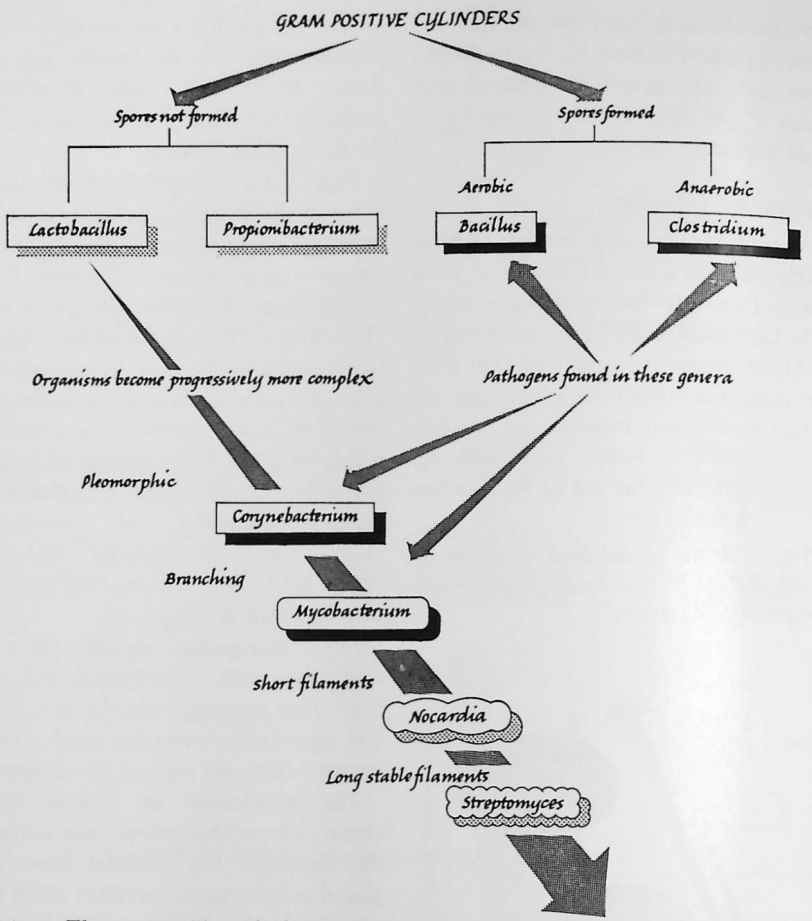


FIG. 7-4. *The gram-positive cylinders.*

negative cylinders and they are the most difficult to separate. Any kind of scheme is bound to have in it some contradictions; the one we propose is really no better than many others, but we have found it useful.

We think it sensible to regard the gram-negative cylinders as comprising a few main physiological groups. These are, as shown in Fig. 7-6, the highly oxidative form, the fermentative, the fastidious, and certain others; the size of the area devoted to them in Fig. 7-6 is a rough estimate of their importance to the microbiologist.

THE OXIDATIVE GROUP. The nongummy, highly oxidative group contains few principal genera (Fig. 7-7). *Pseudomonas* frequently produces a yellow-green to green pigment and is motile with polar flagella. *Xanthomonas* species (older name, *Phytomonas*) are grouped together because they produce diseases in plants. *Acetobacter* oxidizes alcohol. In fact, many of the organisms in these minor genera characteristically attack a particular kind of substance; for example, *Protaminobacterium* attacks alkylamines and *Mycoplana* attacks phenols.

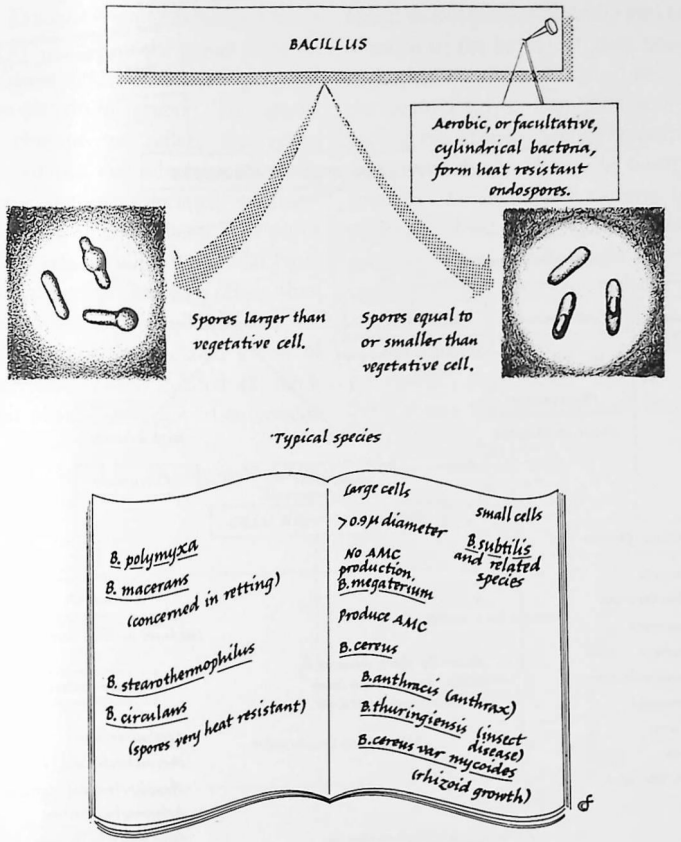
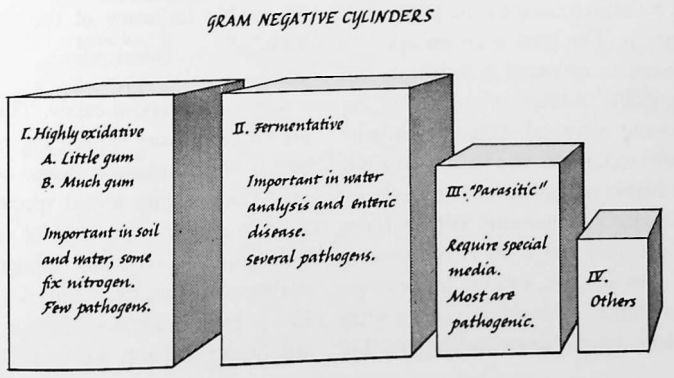


FIG. 7-5. *Important species in the genus* Bacillus.

FIG. 7-6. *The gram-negative rods.*



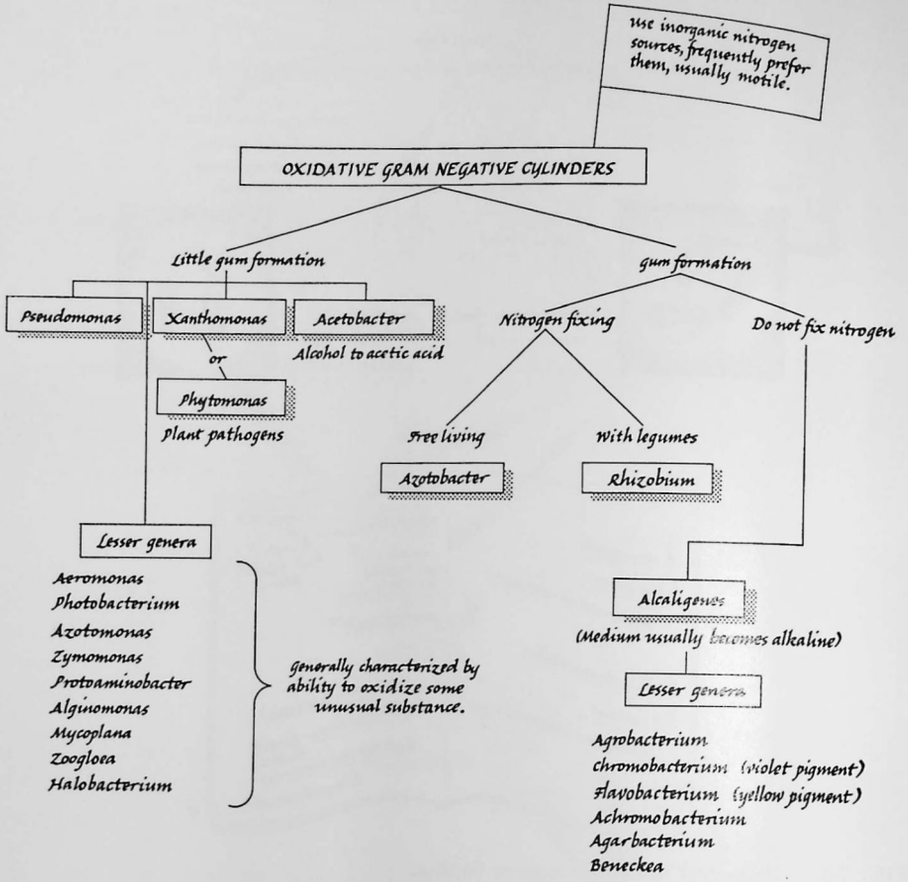


FIG. 7-7. *Important genera among the gram-negative oxidative rods.*

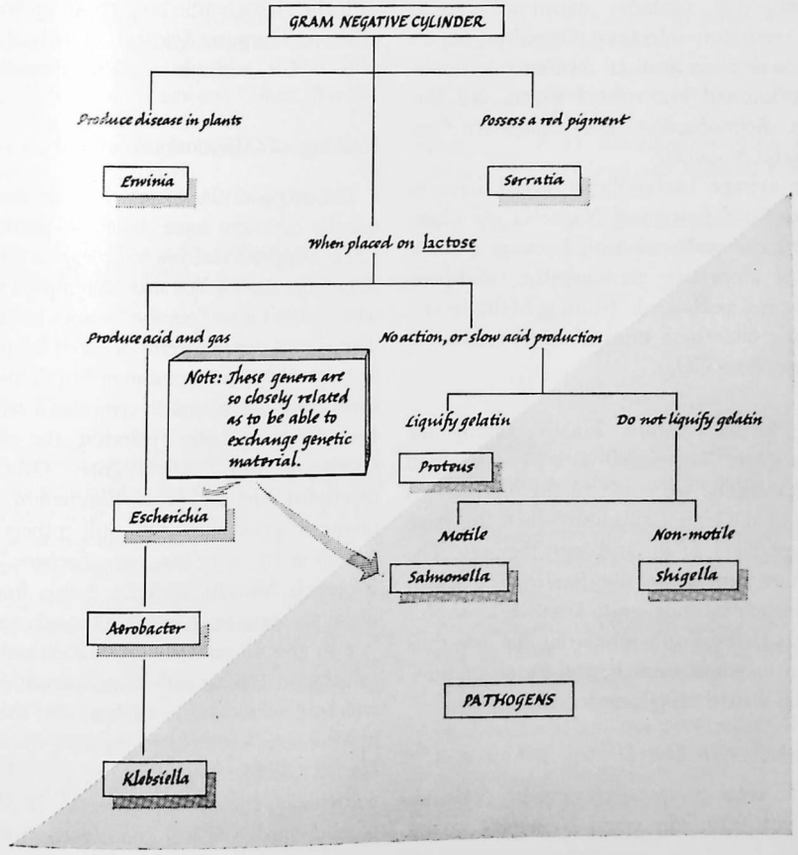
The gummy type of highly oxidative gram-negative rod is characterized by the presence of a gummy growth. The gum is an extracellular material, sometimes arranged in a definite capsule, but generally merely in masses of unorganized gummy material, generally carbohydrate, i.e., polysaccharides of a variety of kinds. Most of the highly oxidative rods not only use inorganic nitrogen (ammonium salts, nitrates, etc.), but frequently prefer it. *Azotobacter* is able to "fix" nitrogen, i.e., can use nitrogen gas as a nitrogen source. *Rhizobium* can fix nitrogen in symbiotic association with legumes. The others are of less importance. *Alcaligenes* is responsible for many of the cases of "ropy milk."

THE FERMENTATIVE GROUP. These organisms are facultative and ferment sugars producing a variety of products from them—acids, alcohols, etc. They contain several species pathogenic to man and it is therefore of importance to distinguish between the pathogens and non-pathogens within the group. A typical separation is given in Fig. 7-8. Those organisms causing blights and soft rots in plants are placed

in the genus *Erwinia*. Those producing a deep red pigment are placed in the genus *Serratia*. The remainder are separated for very practical reasons into six major genera. The genus *Escherichia* attempts to collect the gram-negative fermentative rods which are typically not pathogenic but are associated with the intestinal tract of man and animals. The genus *Aerobacter* attempts to collect the similar types associated with natural habitats other than animal, i.e., grapes, soil, etc. These are not pathogenic. A heavily encapsulated group of organisms, certainly closely related to *Aerobacter* in their physiological properties, are collected in the genus *Klebsiella* and are the cause of much of the bronchial pneumonia occurring in both animals and man. The remainder of the gram-negative fermentative rods contain pathogenic species. Some *Proteus* species are responsible for bladder infections. *Salmonella* contains the organisms causing typhoid and paratyphoid fevers. *Shigella* contains the organisms causing bacterial dysentery. We shall consider all of these in more detail later (Chapters 17 and 27).

One may ask why we regard these as "fermentative" organisms when the chart (Fig. 7-8), shows that they do not ferment lactose.

FIG. 7-8. *Important genera among the gram-negative fermentative rods.*



The answer is that they do ferment glucose and other sugars; lactose merely happens to be a useful diagnostic tool.

THE FASTIDIOUS GROUP. By "fastidious" we mean that these organisms usually require some special media or some special condition in order to grow on artificial media. Most are pathogenic. The important genera are as follows:

Pasteurella contains the organisms causing plague and tularemia.

Brucella contains organisms causing undulant fever and contagious abortion of cattle. When first isolated, strains require added CO_2 in the atmosphere.

Hemophilus contains organisms causing upper respiratory infections. Organisms require blood to grow on artificial medium.

Less unusual but related genera are *Bordetella, Actinobacillus, Calymmatobacterium, Moraxella, Noguchia.*

The average bacteriologist would have to look up the definition of *Noguchia granulosis,* but *Brucella melitensis* would convey a whole array of properties; gram-negative, nonspore-forming rod, pathogenic (causing Malta fever), requiring something unusual in its media (in this case, extra CO_2).

THE "OTHER" GROUP. Finally, among the gram-negative organisms, a truly large and complex family, we come to the orphans. As is true of any large community, there are some who just don't fit in, and here they are. The important genera are the *Bacteroides,* which are nonspore-forming, gram-negative, *anaerobic* rods, characteristically inhabiting the intestinal tract of man and animals, and *Fusobacterium,* which is a rod having pointed ends.

Gram-Negative Cocci

These cocci comprise two genera, *Neisseria* and *Veillonella.* The genus *Neisseria* contains the organisms causing gonorrhea and meningitis (epidemic cerebrospinal meningitis); see Chapter 25. The genus *Veillonella* collects the gram-negative cocci which are obligate anaerobes. There are no pathogens in this genus.

Gram-Negative Curved Cylinders and Spirals

The curved rods or spirals are collected into the main genera of *Vibrio* and *Spirillum.* Those called *Vibrio* are comma-shaped (sometimes S-shaped) cells, and most are motile. One is pathogenic to man, *Vibrio comma,* the cause of Asiatic cholera. Among the minor genera is *Cellvibrio,* which contains *Vibrio*-like organisms, somewhat longer than *Vibrio* and with the characteristic property of growing on cellulose. The genus *Spirillum* consists of coiled cells, motile, with characteristic shapes.

Naming of Microorganisms

The origin of the microbial names used frequently occasions some comment, particularly when it appears that you must learn a group of unfamiliar names. You may be tempted to ask, why couldn't more "sensible" names be chosen? The names were generally selected by persons first describing the organism or first delineating them from others, and so comprise a selection from Greek or Latin (reflecting the classical training of early bacteriologists). Others are descriptive names, as *Propionibacterium,* which produces propionic acid. Still others were named in honor of someone—*Escherichia* for Escherich, *Shigella* for Shiga. Some, however, make no sense to us either, although we hesitate to give an example since we do not want to get into trouble with other microbiologists who may see some sense to them. But this kind of taxonomic knowledge is the stock-in-trade of the microbiologist and should be learned. More particularly, you will find it useful. If *Shigella* is mentioned, you will know, even from just knowing a chart like that of Fig. 7-8, a great deal about the organisms involved and in what ways *Shigella* differs from *Staphylococcus,* for example.

So far we have been considering the names of the genera. Why is it necessary to divide organisms further into species? In some cases, it is admittedly of little importance to the average microbiologist. In other cases it is of great importance. From the point of view of the practical microbiologist, it depends upon what it is he wishes to know. This may be distinguishing pathogens from nonpathogens, or establishing the patent rights to a new process, or trying to trace evolutionary development. In each case, the degree of separation required varies. Sometimes knowing only the genera will suffice; in other cases, the species and even strains within a species must be characterized.

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QUESTIONS

1. You have discovered a short gram-negative rod always associated with a disease of rabbits, and you have good reason to believe that this is the cause of the disease. Why would you wish to name the organism?
2. Using the same situation as in question 1, how would you go about selecting the proper name for the organism?
3. You have isolated a gram-negative, facultative, mesophilic, rod-shaped organism, producing acid on sucrose and on lactose, which is highly proteolytic and rapidly liquefies gelatin. To what genus does it belong?
4. Professor Schlagenfurth reports in the *Journal of Zitunpunk* that *Salmonella putzerwhy* forms spores when placed on unsterilized carrot agar. Why would you not immediately add this information to the textbook of general bacteriology you are writing?
5. An organism, rod-shaped and gram-negative, can be observed in several forms, each interchangeable with the other. One form has short flagella, another long flagella, a third is capsulated, and a fourth has no flagella. Otherwise they are the same. Do you think it advisable to set up more than one species? Why?

Chapter 8

METABOLISM

"Man, in commerce with nature and with his fellows, looking out from himself as from an observation post, has gradually reached a markedly self-consistent scheme of all which is perceptible to him. This it has taken him long to do. Almost from his first beginnings as man he has been about it, originally without conscious aim, latterly with conscious aim. To some men at least the desire to obtain a unified conception of all which the manifold of the world presents was in itself a motive. But long before that, and aside from it, the need for further knowledge of the perceptible must have been thrust upon man and flogged into him from every direction of sense. There was an urge within him to know, but the external situation likewise drove him to try to know. If we may guess at evolutionary history the external drive had been a situation surrounding living things immeasurably long before man's coming. But the internal urge to "know" was a new position which surely had not existed before man. Out of that position arose gradually from scattered origins, and with chequered progress, at first almost insensibly slowly, a wider acquaintance with the perceptible world. Man, social animal as he has been called, was no less significantly a tool-user, a tool-maker, and a tool-designer. He is an exploiter of his planet as all life must be. But of him in that capacity his tool-using is an especially human trait. The tool lent his exploitation a special character. And his exploitation reacted on himself. He grew to see that to exploit the world more successfully he must understand the world."

SHERRINGTON, *Man on His Nature*

ANY DISCUSSION of the metabolism of bacteria is a somewhat pretentious undertaking, particularly since it involves a considerable amount of chemistry, some of which is exceedingly complex. However, there are certain principles and certain generalizations and certain general types of reactions which occur in living bacterial cells about which it is of some importance to know.

A cell carries out the main portions of its metabolism to obtain energy. Energy is the ability to do work. What kinds of work has the cell to do? First, there is the work involved in assimilation, the building of all those thousands of compounds which make up the living cell, absorbing the raw foodstuffs, absorbing the particular chemicals that it cannot synthesize, building up the storage reserves, building up the warp and woof of the cell itself. Second are the energy requirements for maintenance. So long as the cell is alive, even if it does not assimilate or do other synthetic work, it requires a continual stream of energy. Its low endogenous metabolism goes on always. "Endogenous" means the metabolism of the cell itself. If this energy of maintenance stops, the cell will die. This is a somewhat surprising thing —after all the cell is built up, why does it need energy, so long as it does not do extra work? The point is that even when the cell is resting and not growing, it is still working. We might use an analogy to a boat, which is apt: a boat will float without energy, but a leaky boat requires bailing. A living cell much resembles the leaky boat and it requires energy and bailing to keep the membrane intact, to keep the reactions from running backward and so forth, so that a stream of energy goes through it all the time. Some have supposed that this "leaky boat" is a sign of poor craftsmanship in the way that cells have been designed. Actually it is not, it is the price of flexibility and control. One can compare a bar magnet which does not require a continual stream of energy in order to work with an electromagnet which works only when the energy is there. A bar magnet has many uses, but it is not nearly so useful and cannot be nearly so well controlled as can the electromagnet. The cell machinery, like any other kind of machinery, needs energy to run and to do its work. But, in addition, unlike most man-made instruments, it also requires energy to maintain its delicately adjusted status quo. There is, then, in all living cells so long as they are alive, a requirement for maintenance.

Third, a cell expends some energy on minor uses such as expanding its surface. Bacteria which are motile expend energy on motion; some bacteria produce light, and there is certainly energy required in cell division.

These, then, are the requirements for energy of the cell: first, for the synthesis of the material that goes into the cell; second, for the maintenance of all the compounds and potentials and osmotic pressure differences which constitute the cell; and third, a small amount for expanding the surface, cell division, and other minor requirements.

Reactions inside a bacterial cell are not quite comparable to chemical reactions in a beaker or a flask. There are space separations within a cell. A reaction going on within one part of a cell may well be incompatible with another going on in a different part of a cell. Yet both of them can go on because both are separated from each other in space. And virtually all the reactions of the cell are catalyzed by agents called "enzymes." Before we consider metabolism, we should know something of the nature of the agents which make it possible—the enzymes.

Enzymes

An **enzyme** is an organic catalyst produced by the living cell. We may illustrate their action by a simple problem. Suppose we have a lump of sugar, and let us assume that this

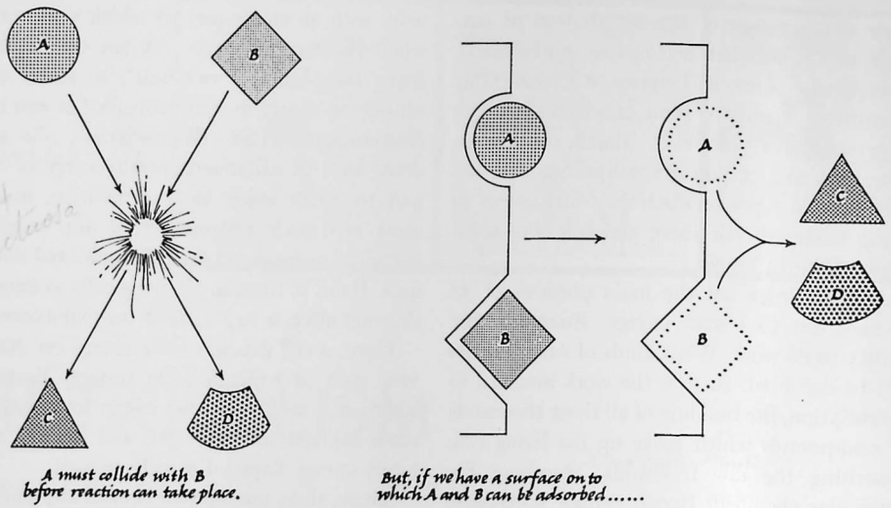


FIG. 8-1. *The effect of a specific surface on a reaction rate—surface catalysis.*

lump of sugar breaks down into glucose and fructose, its constituent parts, at the rate of a million molecules per second. How long would it take before a lump of sugar would show a detectable loss, assuming that we could measure one microgram? The molecular weight of sucrose is 342 so that 342 grams would contain 6.03×10^{23} molecules. One microgram is equal to 10^{-6} g. Hence, in one microgram we would have 2×10^{15} molecules. If, per second, we lose 10^6, that is, a million molecules per second, it would take 2×10^9 seconds before we would have lost a microgram of sucrose. In one year there are 3×10^8 seconds. It would, therefore, require six years before we could detect the loss of one microgram.

A lump of sugar weighs approximately 2 g. In the presence of an enzyme, sucrase—another name for it is invertase, since its product glucose and fructose is sometimes called invert sugar—the entire 2 g or 2 million micrograms could be decomposed in a few moments. The quantity of an enzyme required to do this would be less than a milligram. However, sucrase when it forms glucose and fructose, does not go all the way, but only to equilibrium and this is a reversible reaction.

The enzyme would not change that equilibrium. It would end up with exactly the same materials (mostly fructose and glucose with a trace of sucrose) as if it were catalyzed by any other kind of catalyst. These things illustrate the properties of enzymes which are similar to those of inorganic catalysts. They do not initiate a reaction, they merely change the rate of reaction already in progress. The quantity is very small compared to the quantity of substance changed. The best analogy that we could use at this point is that an enzyme is something like a steam shovel moving a mountain. The size of the steam shovel compared to the mountain is pretty small, but in the end it is capable of moving the entire mountain. Further, the action of an enzyme is generally reversible and its action does not change the equilibrium point.

The action of enzymes, however, differs from the action of inorganic catalysts in that enzyme action is very specific. The inorganic catalysts —for example, platinum, nickel, aluminum.

acid—catalyze many reactions. An enzyme catalyzes only one or very few. That is, for each reaction there is an enzyme. The enzymes are similar to living cells in that they are inactivated usually by high temperatures, they are slowed down by low temperatures, and they possess an optimum temperature range. They require certain salts for activity and they function only within a certain *p*H range. But the distinguishing character is their specificity. How do enzymes act as catalysts? Perhaps the best concept that we can use today as to how an enzyme is able to catalyze a reaction is taken from the statement by Michaelis, who said, "When each molecule of two reacting substances must collide in solution, the chance of change is small. But when they are held close together at appropriate orientation and juxtaposition with respect to each other, and when they remain in this spatial arrangement for a long enough time, a reaction occurs more readily." This process is pictured in Fig. 8-1.

Suppose a molecule A must react with a molecule B. The chances that A and B will collide and that the ends as marked will be close enough together to react when they do collide is very small. Hence, the reaction between A and B, however possible it may be, is unlikely to occur so long as it is dependent upon the collision of two molecules. However, suppose that these two molecules are adsorbed upon a surface, and that this adsorption brings the reactive parts of the molecule close together. They remain in this "appropriate orientation and juxtaposition" for a few moments, and now it is possible for them to react.

The catalytic activity of the enzymes thus depends upon the nature of the surface to which the reactants can be adsorbed. The materials whose reaction an enzyme catalyzes are called the substrate of that reaction. All known enzymes are proteins. Enzymes are inactivated at a high temperature and they are slowed down by a low temperature. The increase in rate of reaction for 10°C increase in temperature is about two; i.e., an enzyme will work at about twice as fast at 30°C as it does at 20°C. More frequently if one goes up to 40°, it should be twice as fast as 30°, but in most cases at 50° or more, the enzymes are inactivated and will not work at all. Enzymes further have a *p*H range and in this respect their *p*H range usually is very similar to that of the living cell from which the enzyme is obtained. Some 40 or 50 enzyme proteins have been crystallized. They have some degree of heat lability. Some enzymes are quite stable; some proteins are quite stable. Some are quite labile, just as some proteins are easily denatured. They have isoelectric points and at certain *p*H values are dispersable, and at other *p*H values they are precipitable. They tend to cocrystallize with other kinds of proteins so that it is not necessarily a criterion of purity that one is able to obtain an enzyme in the crystalline form. They tend to react with heavy metals such as mercury or silver and many times these substances destroy the activity of the enzyme.

The activity of the enzyme, since it is a protein, depends on the protein surface, which in turn depends on the chemical composition of the proteins: the numbers, the kinds, and the arrangement of the amino acid in the protein, about which little is understood at the present time. As for the specificity of enzymes, we might almost say that there is one protein for each reaction. For instance (outlined in Fig. 8-2), there is a substrate such as acetaldehyde whose reduction to alcohol is catalyzed by an enzyme. This is carried out by protein A. Pyruvic acid can be reduced to lactic acid. This is carried out by protein B. Oxalacetic acid can be reduced to malic acid. This is carried out by protein C. Protein A does not react with pyruvic or oxalacetic acid, protein B does not react with acetaldehyde or oxalacetic acid, protein C does not react with acetalde-

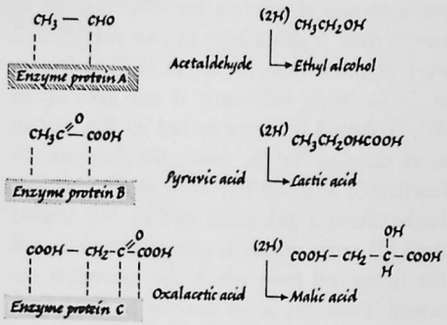


FIG. 8-2. *Specificity of enzyme proteins.*

hyde or pyruvate. You may notice in Fig. 8-2 that these substances are very much alike chemically, and that the specificity of these reactions can only be accounted for by an adsorption of each to a different protein. If we make the assumption that there is one protein to each reaction, or to each rather narrow range of reactions, then the question arises of whether there is room enough in the small bacterial cell to accommodate all the enzymes that are necessary.

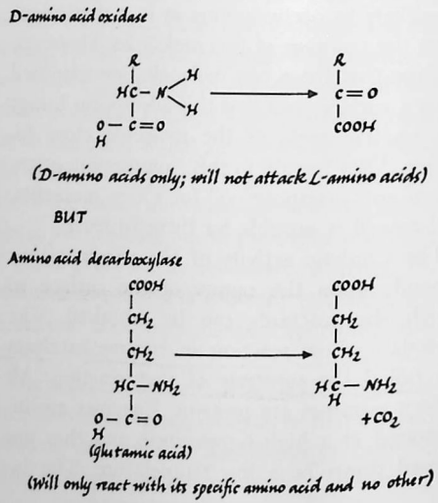
A brief calculation, assuming that perhaps a hundred or even a thousand molecules of a particular protein are necessary before a given reaction may proceed at a measurable rate, and assuming that a cell can carry out perhaps 100,000 reactions, possibly many more, then there is still room within the size of the smallest bacterial cell to accommodate many more proteins than are required by these assumptions.

However, the situation actually is that it is not always just one protein to just one reaction. For example, there is an enzyme called D-amino acid oxidase. This will oxidize an entire group of D-amino acids. The enzyme is specific, not for the entire molecule but for the one end of the molecule. This is illustrated in Fig. 8-3. However, this kind of generalized enzyme activity does not hold for most other enzymes, even those acting on amino acids.

Enzyme Nomenclature

In naming an enzyme, there are several methods in simultaneous use, which generates a considerable amount of confusion. It is generally agreed that the best way to name an enzyme is to name the substance acted upon, the "substrate," and add an "ase" to its name. For example, if an enzyme acts on sucrose, then the enzyme would be termed "sucrase." But we get into difficulty when we have an enzyme that acts, say, on lactic acid. We cannot call it "lactic acidase" or "lacticase" or "lactase"; lactase would sound like an enzyme that acts on lactose, not lactic acid. Hence, frequently the ending "ase" is used for the type of reaction: hydrolase, oxidase, reductase, dehydrogenase. We could speak of lactic dehydrogenase or lactic oxidase, but we could not speak of lactic deaminase or lactic hydrolase because these reactions cannot occur with lactic acid. But those reactions which can occur can be named after the substance acted upon plus the nature of the reaction, the latter word ending in "ase."

FIG. 8-3. *The "group specificity" of the enzyme oxidizing D-amino acids, D-amino acid oxidase.*



Of course, before these two name systems were employed, enzymes had been known for years, and sometimes were given trivial names, like pepsin or renin. So all three mixtures of nomenclature exist together and they generally cause some confusion. However, after a time you will get used to them and then you will be able to understand what is going on. For now, remember that the reactions of the living cells are carried out by enzymes. We have described what kinds of things enzymes are. The reason the cell carries out the metabolism is to obtain energy, and to obtain the "building blocks" that it needs for its own substance.

We may now turn to some general principles of metabolism. These are principles with which every organism must work. They are, in a sense, basic laws of nature which must be obeyed by all creatures. The first of these is that energy cannot be obtained by a cell from a reaction which occurs outside of itself. The reactions which yield energy to the cell—which give this cell a thing it needs to live and to grow—must occur within the cell. There are a few cases where a cell may absorb radiant energy. But what it really does is absorb the energy within the cell by a specific photosynthetic type system and then is able to use the energy once it is inside.

In order to obtain energy for its growth, a cell must take its food materials inside of itself. Normally, materials which are soluble diffuse into the cell or are carried in by more active mechanisms and inside they are torn apart and oxidized and used for whatever purpose the cell wishes. But for materials which are insoluble, the mechanism of utilization seems to be very much as follows. The organism prepares and sends out an enzyme which diffuses out of the cell. This hydrolyzes the insoluble material to a soluble substance which then diffuses back into the cell and can be utilized.

The second general principle of energy or of the metabolism of organisms is that while the reactions which yield energy are varied, there is not too great a divergence from one organism to another. All seem to employ somewhat similar types of reactions or similar types of reaction series. The metabolism that one finds in a bacterium is frequently not very much different from the metabolism that one may find in a mouse or an elephant or man. The differences that do exist are, of course, important, but there are certain basic mechanisms that seem to be so widely distributed in nature that they seem to be of basic importance. It is these that we intend to examine in some further detail.

A third principle of metabolism is that there are, in such metabolic pathways as we shall discuss, certain major crossroads of metabolism. Most cells live on glucose. Glucose is broken down in a variety of ways to an end product which seems to be common to most organisms as an intermediate. This compound is pyruvate. Pyruvate is further metabolized in a variety of different ways by different organisms but these "cross-points" of glucose and pyruvate seem to be common to many kinds of organisms. To approach the problem of metabolism in a broad sense is to be confused by the very mass of apparently unrelated information: one organism does this, the second organism does not, and a third does something different still. However, it is possible to find some unity, and with it some understanding, by separating the subject into more or less separate compartments, and to discuss broad general procedures of metabolism with the understanding that there may well be differences from one organism to another. For our purposes, we shall divide metabolism into several main reaction series. The first of these is a series of reactions which go from glucose to pyruvic acid. It is known as a Meyerhof-Embden system. The series of reactions has been known for many years and occurs in a wide variety of organisms; it is discussed on pp. 154–160. Another series of reactions involves an alternate and somewhat

more complicated pathway between glucose and pyruvic acid, and is known as the "monophosphate shunt." Its history will be developed and its queer name explained on pp. 160–163. A third block of reactions has to do with the further metabolism of pyruvic acid; these will be discussed on p. 171. A final block of reactions are those that are concerned with the contact which an organism possesses to the oxygen in the air; they are discussed on p. 176.

The First Path Between Glucose and Pyruvate: The Meyerhof-Embden System

Most organisms live on glucose and it is therefore of some importance to understand glucose metabolism. When you are first confronted with the array of reactions outlined later in this chapter, especially if you see them for the first time, you may feel like reading something else. It may look too difficult. But actually it just *looks* too difficult—it really is not.

It is best for you to start with something simpler, or at least something that looks simpler. And this is, in our opinion, a discussion of how it was determined that these are the "pathways" of glucose. This history, which is of interest in itself, cannot unfortunately be told directly nor is it itself a simple tale; for it went by fits and starts, and there were false leads and sincere but mistaken prophets. And, to make the story readable and short, we shall have to paraphrase here and there.

Our story can begin, strangely enough, with Pasteur, whom we would call a bacteriologist although he was trained as a chemist, and with Liebig, who was a chemist—a biochemist, perhaps the first one. And the story can begin—not with bacteria—with beer. Pasteur was studying it and why it spoiled; in fact, he was not studying bacteria but yeasts. He had watched them grow and concluded, rightly, that they were alive. He found also that in the "sick" beer vats where beer was not good, there were bacteria rather than yeasts, and they were making lactic acid. Both the alcohol from the yeasts and the lactic acid from the bacteria were coming from glucose. Since they were both alive, he concluded that it is living things which cause acid formation and alcohol. But Liebig's theory of fermentation claimed that it was not the yeast, but a vibration of the sugar molecules caused by albuminous materials which made the alcohol in beer; if there were yeasts, their function was merely to make the "albuminous material." In a sense, he too was right. Pasteur seemed to have settled it in his favor by showing that yeasts could grow and make alcohol from sugar without the "albuminous material," i.e., in a sugar-mineral-salts medium with ammonium sulfate. Neither man was likely to back down, and they argued and insulted each other publicly. The controversy (1860–1875) led Pasteur into the discovery of anaerobes and to the study of spontaneous generation.

The study of metabolism was conducted on yeasts rather than on bacteria for a rather simple reason—yeasts were available from the brewing and distilling industries. One could obtain pounds of yeast cells easily, but large quantities of bacterial cells were much harder to obtain. After the routes of metabolism were worked out in yeasts, they were applied to bacteria, but it was first done with yeasts. Without defining yeasts further (see Chapter 16 for a discussion), we shall begin to trace the studies leading to knowledge of metabolism, not necessarily in the order in which they were discovered, but so arranged as to provide a clear picture of why we know that this pathway happens.

At the time of Pasteur and Liebig it was established that yeast, in the main produced alcohol and CO_2 from glucose; for every molecule of glucose there were two molecules of alcohol and two molecules of CO_2 produced:

$$C_6H_{12}O_6 \longrightarrow 2CH_3CH_2OH + 2CO_2$$

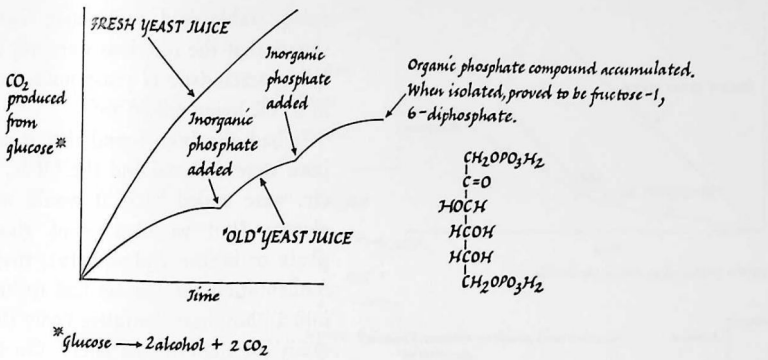


FIG. 8-4. *The chemical structure of fructose-1-6-diphosphate and the fermentation of sugar by fresh yeast juice and by the same yeast juice several days later.*

After their time, a man by the name of Buchner was able in 1897 to make an "albuminous substance" from live yeasts by crushing them. This preparation, obtained from the yeast itself, was capable of fermenting glucose and producing CO_2 and alcohol. In fact, such a "yeast juice" (which contained no living yeast) proved to be the cell juice itself. It was called zymase (from the Greek "zymē," to leaven), i.e. the enzymes from yeast. Originally zymase was no doubt thought to be one enzyme, but in time it proved to be a mixture (or perhaps one should say an organization) of several. What this demonstrated was that Liebig was right; fermentation could occur in the presence of chemical substances in the absence of life. Pasteur was also right, in that these chemical substances could only be made by living beings.

When such yeast juices were freshly made, they converted 1 glucose to $2CO_2$ and 2 alcohol just as did the live yeast, but this continued for perhaps only an hour. In 1905 Harden and Young found that, in a yeast juice in which the rate of CO_2 formation had decreased, this rate could be restored by the addition of inorganic phosphate and that in the process of alcohol and CO_2 formation by such yeast juices the inorganic phosphate was used up and converted into organic phosphate. This organic phosphate material was isolated and identified as hexose diphosphate or fructose-1, 6-diphosphate (Fig. 8-4), still known today as the Harden-Young ester. For the yeast juice, except under the very freshest conditions,

$$2 \text{ glucose} + 2 \text{ inorganic phosphate} \longrightarrow 2 \text{ alcohol} + 2CO_2 + \text{hexose diphosphate}$$

$$2C_6H_{12}O_6 + 2KH_2PO_4 \longrightarrow 2C_2H_5OH + 2CO_2 + C_6H_{12}O_6 \cdot 2(PO_3HK)$$

What hexose diphosphate had to do with fermentation was not known; some people supposed that it was an intermediate product and some that it was a by-product.

In 1906 Harden and Young found also that if they dialyzed the yeast juice it lost its activity; it could not be restored by the addition of phosphate or magnesium (or both), but it could be reactivated by the addition of the concentrated dialysate. That is, dialysis had removed an *organic* material necessary for fermentation. It took about twenty years before this material was isolated and its nature and function determined. It was called cozymase, a term sometimes still used today although we know it as coenzyme I or more commonly as DPN, after its structure, diphosphopyridine

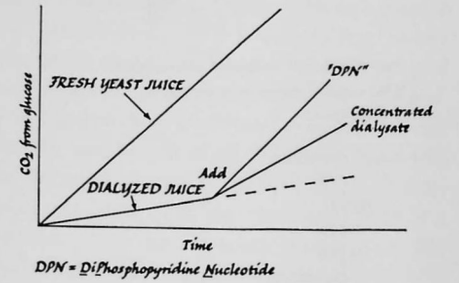


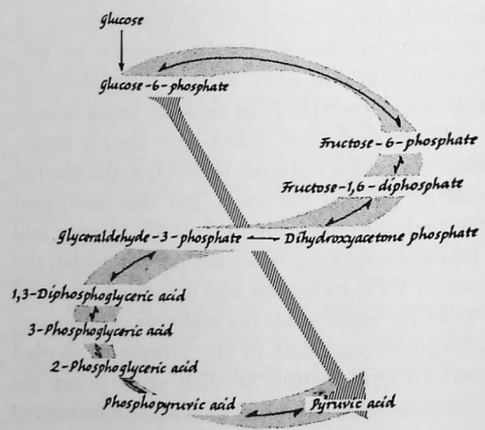
FIG. 8-5. *The fermentation of glucose by fresh and dialyzed yeast juice and the effect of coenzyme addition upon the latter. The structure of the coenzyme, called DPN (diphosphopyridine nucleotide), is also shown to contain the vitamin, niacin.*

nucleotide (Fig. 8-5). This proved to be a derivative of the vitamin niacin (nicotinic acid) and provides an explanation as to why this vitamin was necessary for living cells.

For the next twenty-five years a variety of compounds were isolated from such yeast juices treated in various ways: glucose-6-phosphate, fructose-6-phosphate, methylglyoxal, pyruvic acid, ATP, adenylic acid, acetaldehyde, etc. But in the early 1930's enough information was accumulated that the data began to fit together. In 1933 two groups of investigators—Meyerhof and Embden, Deiticke and Kraft—independently proposed a reaction series, today called the Meyerhof-Embden "system," which would explain the transformations of glucose to alcohol as found in yeast extracts (Fig. 8-6). In this system phosphate played an essential role; it not only explained how the phosphate esters accumulated in the yeast juices, but also why they were of importance. By the early 1940's each one of the necessary steps had been proved to occur and by the 1950's tracer experiments, using stable and radioactive isotopes, had shown that the reactions were not only a curious characteristic of yeast juice, but occurred in whole living cells as well.

It had also been found that if such a yeast juice were dialyzed and the DPN, phosphate, etc. were added back, it would not ferment glucose (but would ferment glucose-6-phosphate or hexose diphosphate); that is, before fermentation the glucose had to be converted into a phosphate derivative (why this was necessary we shall discuss later). On this basis a reaction was looked for and found in which glucose was converted to glucose-6-phosphate (Fig. 8-7). Basically, this is the only reaction that the glucose undergoes. Hereafter, virtually all of the reactions are concerned with the phosphorylated intermediates. Glucose-6-phosphate can be metabolized in several fashions. The first path is its conversion into fructose-6-phosphate, which is the one we are interested in here. But it may also be oxidized, as shown in Fig. 8-7, to the phosphogluconic acid, or,

FIG. 8-6. *The Meyerhof-Embden system of reactions. Note that, except where they cross the arrow, all 6-carbon substances are above the arrow; all 3-carbon substances below it.* [*Redrawn from Umbreit,* Metabolic Maps II, *Burgess, Minneapolis, 1960.*]



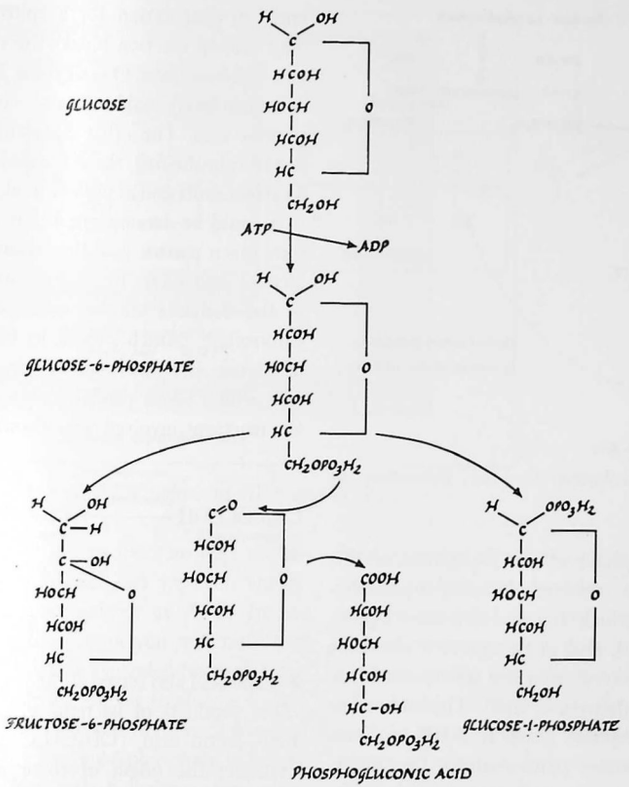


FIG. 8-7. *Alternative pathways of glucose-6-phosphate metabolism. The reaction, glucose plus* ATP *to form glucose-6-phosphate, is carried out by an enzyme called hexokinase.*

further, it may be converted into glucose-1-phosphate. But at the moment we are interested in its conversion to fructose-6-phosphate. This substance can be converted further by a reaction with another molecule of ATP to form fructose-1,6-diphosphate. This substance may divide under the influence of an enzyme to form dihydroxyacetone phosphate and glyceraldehyde phosphate. Although these are produced in equal amounts, there is an enzyme which converts one into the other, and since the glyceraldehyde phosphate is the least stable, the equilibrium is toward the dihydroxyacetone phosphate side. One may note at this point how glycerol may be formed from the system and also how glycerol may enter the system of metabolism (see Fig. 8-8). But the next enzyme that we are interested in is one which will oxidize the glyceraldehyde phosphate in the presence of inorganic phosphate and a hydrogen carrying coenzyme (DPN)[1] to form 1,3-diphosphoglyceric acid. This is a curious step and results in the conversion of inorganic phosphate into organic phosphorus (Fig. 8-9). At the next

[1] For purposes of convenience, we have chosen to write the reduced form of DPN as $DPNH_2$ although in reality it should be written $DPNH + H^+$. See Chapter 9 for a detailed description of the mechanisms by which DPN can carry hydrogen.

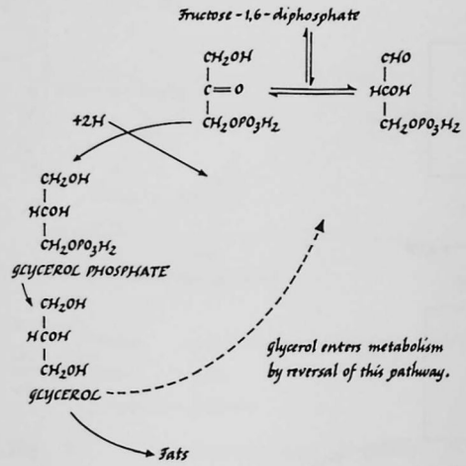


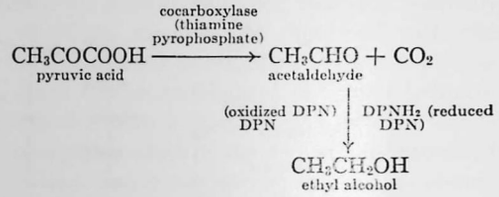
FIG. 8-8. *Pathways for the formation of glycerol and fat.*

step, the phosphate at the 1 position of this glyceric acid is removed, forming 3-phosphoglyceric acid, which is converted into 2-phosphoglyceric acid, and at this point a molecule of water is removed from the substance, forming enol phosphopyruvic acid. This substance gives up a phosphate group to ADP (to form ATP) and becomes pyruvic acid.[2] The hydrogen, which a moment ago we removed on DPN, can now react with the pyruvic acid to form lactic acid or the pyruvic acid may be decarboxylated to acetaldehyde and this reduced to alcohol by DPN. Since every glucose contains six carbons and each pyruvic acid contains only three carbons, glucose would yield two pyruvic acids and thus two lactic acids or two alcohols and $2CO_2$.

There are several very interesting things about this reaction series, and one might paraphrase the whole system as follows. First of all, the glucose reacts with a phosphate group and then, after some internal transformation, reacts with another phosphate group, apparently in preparation for a splitting reaction. This second reaction breaks the molecule into two 3-carbon units. One of these 3-carbon units is immediately oxidized and converted into pyruvic acid. The other 3-carbon unit is converted into the first, then oxidized, so that both 3-carbon units end as pyruvic acid.

[2] Throughout we refer to such substances as the free acids, but in tissues they occur as salts of potassium, sodium, ammonium, and other metals.

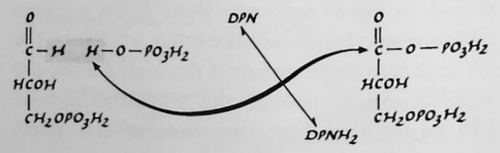
It could be demonstrated that if yeast juices were given pyruvic acid they would make ethyl alcohol and CO_2. By dialysis and restoration of the dialysate another coenzyme called cocarboxylase (which proved to be a derivative of vitamin B_1) was found and by adding materials which could combine with acetaldehyde, the reactions involved were shown to be:



Pyruvic acid also served as the precursor of most other products of bacterial glucose fermentation—lactic acid ($CH_3CH_2OHCOOH$), for example; the origin of these products from pyruvic acid is discussed in Chapter 9. What we are now considering is the nature and variety of pathways between glucose and pyruvic acid.

ENERGY. Since the purpose of this entire process is to provide energy to the cell, we

FIG. 8-9. *The oxidation of glyceraldehyde phosphate. The enzyme is called 3-glyceraldehyde phosphate dehydrogenase or, since these are 3-carbon sugarlike substances (trioses), it is also called triose phosphate dehydrogenase.*



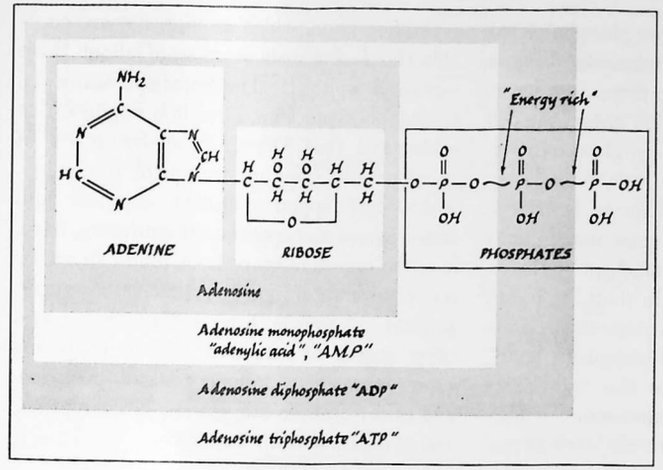


FIG. 8-10. *The chemical nature of ADP and ATP.*

should point out at this juncture that the energy carriers within the cell are such energy-rich phosphate compounds as ATP. In the Meyerhof-Embden system you will note that one such phosphate is expended for each 6-carbon when converting glucose to glucose-6-phosphate, and another phosphate for each 6-carbon when converting fructose-6-phosphate to hexose diphosphate. For each 6 carbons, two phosphates are obtained when diphosphoglyceric acid is converted to 3-phosphoglyceric acid and two more phosphates are obtained for each 6-carbon unit when phosphopyruvic acid forms pyruvic acid. For the first 6 carbons then, two phosphates are expended, and four phosphates regained; hence, this system generates organic phosphate from inorganic phosphate by converting one glucose molecule into two pyruvic acids forming two organic phosphates. Why?

Further, there are two substances, ADP and ATP, which seem to be darting in and out of these reactions. We must remember that a living cell is not like a steam engine. Heat, as such, is not of use to it—it needs energy that can be used. It is a chemical machine, it needs chemical energy. So it releases such energy, small portions at a time, in the form of energy-rich phosphate compounds. These compounds may be regarded as energy carriers. They are formed when energy is released by a chemical reaction within the cell, and then are used up when energy is required by the cell. That is, in order to convert glucose to glucose-6-phosphate, energy is required. This energy is provided by ATP. When phosphopyruvic acid goes to pyruvic acid, it releases energy, and this energy is recaptured by resynthesizing ATP. The chemical structures of the ADP and ATP are shown in Fig. 8-10, and the energy-rich bonds are indicated by this small symbol—$\sim PO_4$.

Energy is the ability to do work. But it is difficult to understand at times just what constitutes work for a bacterial cell. The simplest answer to this question is that any reaction which proceeds within a cell is doing work; that is, it is releasing energy. A reaction which requires energy will not go "unless energy is put into it" and the usual means of "putting energy into it" is to use phosphate derivatives.

This seems to be, indeed, a basic mechanism involved in most of the reactions of the living cell. Reactions which normally will not proceed

between molecules that are not phosphorylated may proceed when such molecules have a phosphate group attached to them. In a sense, then, in the Meyerhof-Embden system the cell uses up some energy to convert glucose to glucose-6-phosphate and then a little more energy to convert fructose-6-phosphate to fructose-1, 6-diphosphate. But it then gains more energy than it has used by the metabolism of the resulting two 3-carbon units. In short, by means of the Meyerhof-Embden system the cell obtains what may be called "phosphate bond" energy and it can then use this "phosphate bond" energy to run any other series of reactions which it might want to use, such as synthesizing nucleic acid, manufacturing protein, building cell wall or flagella, or even for pumping excess water out of its cell.

Thus the changes in the phosphate are part of a double mechanism which the cell utilizes to trap the energy released in a particular chemical reaction and store it, in order to use it later (sometimes in other parts of the cell) in reactions which require energy. The energy in the phosphate bonds of ATP, the ones labeled "energy-rich phosphate" in Fig. 8-10, are in fact the currency of the cell. It is able, without the use of wires or heat or any of the mechanical devices employed in cruder systems, to transfer energy from one reaction to another by the actual physical transfer of the substance which takes part in one reaction ("releasing energy") and also in the other ("utilizing energy"). The curious thing about it is that it is the same substance in both reactions. We will not go into further detail as to the exact nature of the energy-rich phosphate bond, or ask whether other similar bonds of this type might not occur. (They do, but they are somewhat limited and are generally convertible to phosphate bonds when necessary.) Suffice it for our purpose to know that the cell uses the phosphate bond as a means of transferring energy from one type of reaction which releases it to another type of reaction, which requires it; very probably this is the main purpose of all of these various phosphorylated intermediates employed in metabolic processes. Now it is important to understand that Meyerhof-Embden series of reactions and those in subsequent discussion, which may appear unusually complex and whose names may appear quite confusing, have, over the course of years, been found in an essentially identical form in a wide variety of organisms: in muscle, liver, kidney, brain, and other organs of man and animal; in plants (corn, wheat, oats, legumes), algae, protozoa, and earthworms; in fish, insects, birds, reptiles, and of course in many bacteria.

The Second Pathway Between Glucose and Pyruvic Acid: The Monophosphate Shunt

The complete Meyerhof-Embden system may be absent in some organisms. But even though particular parts may be missing, generally portions, and very large portions, of the Meyerhof-Embden reaction system will be found in every body. All of the intermediate steps have been carefully identified. The enzymes carrying out each of the stepwise reactions have been isolated and in most cases crystallized. The evidence for the Meyerhof-Embden system is consistent with the distribution of isotopes given to many bacterial forms and will account for a great deal of the intermediary metabolism which occurs in bacteria as well as in higher forms.

Thus for many years the Meyerhof-Embden system was thought to be the only pattern of glucose metabolism. It was known that glucose-6-phosphate could be oxidized by some organisms to phosphogluconic acid; that is, they would oxidize the carbon on the glucose at the end opposite to that containing the phosphate. However, it was almost impossible to find any further reactions for phosphogluconic acid. This seemed to be a side reaction, and it was

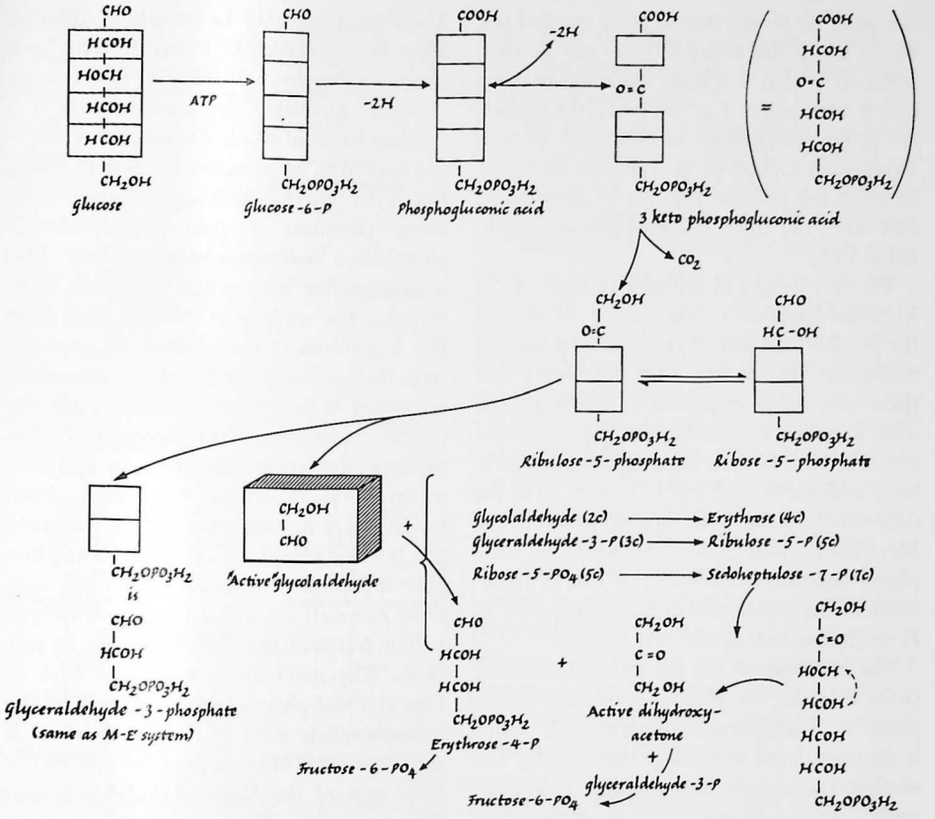


FIG. 8-11. *A diagrammatic illustration of the principal alternative to the Meyerhof-Embden system—the monophosphate shunt.*

called the monophosphate shunt because it shunted off a certain amount of glucose from the main pathway of metabolism. Today we know that there are two other pathways of glucose metabolism beginning with glucose-6-phosphate after oxidizing it to phosphogluconic acid. Although they are main pathways and are not mere shunts, the name has remained. The monophosphate shunt is a process involving phosphogluconic acid and its subsequent oxidation to 3-keto-phosphogluconic acid. It is our second pathway, in addition to the Meyerhof-Embden system, between glucose and pyruvic acid.

The details of this pathway are shown in Fig. 8-11. It is indeed somewhat complicated, but we may trace it simply as follows. Glucose is converted into glucose-6-phosphate, which is oxidized by way of a gulano-lactone phosphate to phosphogluconic acid. This is then oxidized on carbon 3, enzymatically, of course, to form 3-ketophosphogluconic acid. This compound is decarboxylated; that is, it loses a CO_2 enzymatically to become ribulose-5-phosphate. At

this point we should stop to point out that one way in which this entire pathway may be characterized is that a glucose molecule entering into it loses carbon 1 at this point; the remainder of the reactions are involved with carbons 2 through 6. Hence, we can estimate the extent to which this pathway is in use by determining how much of carbon 1 labeled glucose is emitted as CO_2.

The very weight of evidence in favor of the Meyerhof-Embden system tended to obscure the possible existence of an alternative reaction mechanism. It was only with the finding that there were some organisms in which glucose with a radioactive carbon in the 1-position would be fermented in some organisms to form lactic acid, acetic acid, and CO_2, with all of the radioactivity in the CO_2. After all, by way of the Meyerhof-Embden system, it would be completely impossible to obtain this kind of result. Some of the radioactive carbon would *have to be* in the lactic acid that was formed.[1]

The first steps of the monophosphate shunt pathway, then, are glucose to glucose-6-phosphate to 3-ketophosphogluconic acid, which is decarboxylated to ribulose-5-phosphate. The ribulose-5-phosphate is something of a crossroads compound; just as glucose-6-phosphate and pyruvic acid may be metabolized in a variety of different ways, ribulose-5-phosphate may be converted first to ribose phosphate, or it may be split into active glycol aldehyde and glyceraldehyde-3-phosphate. Or it may react with ATP to form ribulose diphosphate, whose further metabolism we will examine in a moment.

Glyceraldehyde-3-phosphate we have already recognized as part of the triose phosphate of the Meyerhof-Embden system. The active glycolaldehyde is labeled "active" because it carries out the reactions listed, but added glycolaldehyde does not carry out these same reactions. Therefore, there must be something different about the glycolaldehyde formed directly from ribulose-5-phosphate so we call it active or "nascent" glycolaldehyde; presumably it is a 2-carbon fragment attached to either an enzyme or a coenzyme, whose nature is still not known. But active glycolaldehyde can combine with ribose-5-phosphate to form sedoheptulose-7-phosphate, a 7-carbon sugar intermediate. This is an important intermediate because it is involved in the synthesis of shikimic acid; since this is involved in the synthesis of aromatic rings, the compound sedoheptulose-7-phosphate is involved in the synthesis of amino acids like tyrosine, tryptophan, phenylalanine, and other aromatic ring compounds. But, in addition, sedoheptulose-7-phosphate may break down enzymatically to form active dihydroxyacetone and tetrose-4-phosphate; i.e., a 4-carbon phosphorylated sugar. This 4-carbon sugar may condense enzymatically with active glycolaldehyde to form fructose-6-phosphate which can, by way of the Meyerhof-Embden system, go back to form glucose-6-phosphate and thus re-enter the monophosphate shunt at the top again, or it may be fermented or oxidized by way of the lower part of the Meyerhof-Embden system down to pyruvate. The active dihydroxyacetone may condense with glyceraldehyde-3-phosphate to form fructose-6-phosphate, which may start again. If we trace the pathways of given molecules through this monophosphate shunt system, we see that, using a single glucose-6-phosphate molecule, we end up with either the ribulose or ribose phosphate plus one CO_2. If we use two glucose-6-phosphate molecules, we end up with a C-4 intermediate, tetrose phosphate, and a glucose-6-phosphate back again. The net result has been to convert one glucose-6-phosphate to 2 carbon dioxides and a tetrose. If we use three glucose-6-phosphate molecules, we end up with 3 CO_2 and one C-3 intermediate, triose phosphate, part of the Meyerhof-Embden system. The monophosphate shunt,

[1] You can trace this out for yourself and see that this is true, using the data of Fig. 8-6.

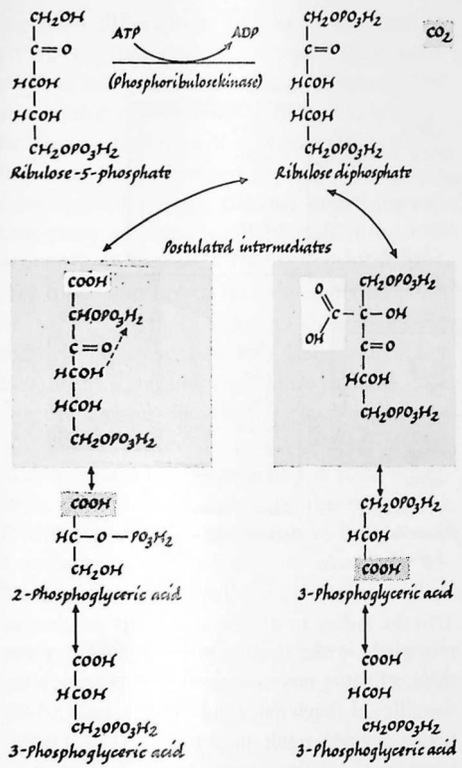


FIG. 8-12. *The fixation of CO_2 in photosynthesis involves ribulose-5-phosphate.* [*Redrawn from Umbreit,* Metabolic Maps II, *Burgess, Minneapolis, 1960.*]

then, is a process whereby CO_2 may be released from glucose essentially by taking 1 carbon at a time off the carbon chain.

This pathway is, of course, a little bit complicated, but it is nonetheless true and real; the enzymes carrying out each of these steps have been isolated, many of them in a pure state, and the process is known in considerable detail. There are, as you can see, 5-carbon intermediates, 4-carbon intermediates, 3-carbon intermediates, 7-carbon intermediates, and an almost bewildering array of internal condensations and isomerization. Nevertheless, the evidence is perfectly clear that there are some bacteria in which the monophosphate shunt is the major pathway of metabolism.

What we have in this reaction series is an alternative pathway to form pyruvic acid, so the cell is not strictly dependent upon the Meyerhof-Embden system to obtain pyruvic acid from glucose. Further, this reaction series furnishes an entire array of new carbon skeletons which the cell may use to synthesize a variety of different substances which it desires. In Fig. 8-12 we have shown the probable pathway of photosynthesis which occurs in photosynthetic bacteria, as well as in the green plants, and some of these steps, particularly the addition of CO_2 to the ribulose diphosphate, occur in the autotrophic bacteria as well; this last step seems to be the one by which they are able to fix CO_2. This reaction series similarly involves an array of phosphorylated intermediates and continuous exchange of the phosphate between ATP and the organic molecules. The purpose of the phosphate is precisely the same in the Meyerhof-Embden system: it is a carrier and a receiver of energy.

The Third Pathway Between Glucose and Pyruvic Acid: The Upside-Down Pyruvic

If we were to follow the pathway of a glucose molecule isotopically labeled in the 1 position, and if this molecule were fermented by way of the Meyerhof-Embden system, all of the carbon 1 of glucose would appear in the methyl group of the pyruvic acid formed.[1] If we were to follow a pathway to the monophosphate shunt, all of the carbon 1 would appear as CO_2.[1] But what would we say if pyruvic acid was isolated from a fermentation mixture in which all of the carbon of carbon 1 of the glucose was in the carboxyl group of the pyruvic acid? This was "the case of the upside-down pyruvic." It

[1] You can trace these by marking the appropriate compounds in Figs. 8-6 or 8-11.

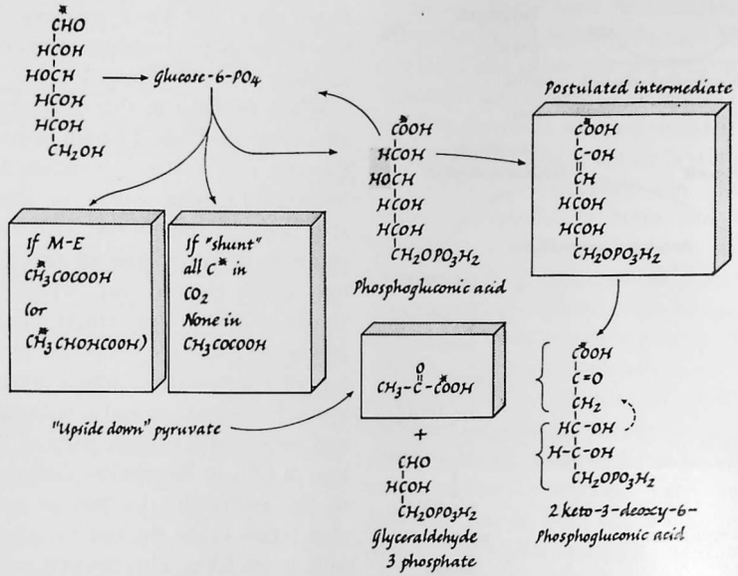


FIG. 8-13. *The case of the upside-down pyruvic acid.*

was necessary, under these circumstances, to look for and to find another pathway of glucose metabolism. This was found and an intermediate obtained as shown in Fig. 8-13; glucose plus ATP forms glucose-6-phosphate, which is oxidized to phosphogluconic acid. But instead of being oxidized at the 3 position, as it is in the monophosphate shunt, the next step is the formation of 2-keto-3-deoxy-6-phosphogluconic acid, which splits to form pyruvic acid with the carbon 1 of the glucose in the carboxyl group of the pyruvic acid and glyceraldehyde-3-phosphate, the familiar triose phosphate intermediate of the Meyerhof-Embden system.

The Glucose-6-Phosphate Crossroads and the Formation of Polysaccharides

We have so far mentioned three pathways between glucose-6-phosphate and pyruvic acid. There may be still other paths leading from glucose to pyruvic acid, but as of the moment we have no evidence for them.

But as if this were not enough, glucose-6-phosphate is the starting point for other reactions, of some very considerable importance in the life of bacterial (and other) cells, but which do not result in pyruvate. The "most important" of these were shown in Fig. 8-7. They show that glucose-6-phosphate is responsible for the formation of polysaccharides; one route, via glucose-1-phosphate, leads to starches, glycogen, and related polysaccharides. A branch of this route leads to UDP-glucose and thence to the uronic acid polysaccharides. Another main route starts with glucose-6-phosphate and aminates it to glucosamine derivatives, which appear in bacterial cell walls as well as in other structures.

Metabolic Perspectives

Our main subject in this chapter has been the nature of the three known pathways between glucose and pyruvic acid. We may legitimately inquire as to whether these occur together or separately; whether, indeed, one

organism differs from another in the kind of pathway it possesses. If we regard these pathways as being not only a source of energy, but as a source of intermediates from which essential materials of the cell must be built (as ribose into nucleic acid or sedoheptulose phosphate into aromatic rings), then we might suppose that every organism would have all of them. It is one thing, however, to suppose such a matter, and quite another to have experimental proof of it. Particularly in this case, where it is most difficult to prove that an organism does not have even a trace of a particular pathway. We can not answer the question experimentally at this point, but we can point out that, in general, one pathway predominates in some organisms; predominates, indeed, to the extent that it virtually excludes (but what we can not tell experimentally is whether it *absolutely* excludes) the other pathways. In many other organisms there is evidence of either of two things. First, there may be a mixture of pathways, some of the glucose reaching pyruvic acid via Meyerhof-Embden, some via shunt. There is at least one case known in which growth conditions influence the pathway present. If *Bacillus subtilis* is grown without air, the glucose-pyruvic acid pathway is predominantly the Meyerhof-Embden path. If the same strain is grown with high aeration (on the same media), the glucose-pyruvic acid pathway is predominantly the monophosphate shunt.

Second, in some cases studied, where one pathway seems to be absent, it is only one (or a few) step(s) in the path that is (are) missing. For example, in *Leuconostoc mesenteroides* glucose isotopically labeled in the one position with radioactive carbon is fermented to yield 1 lactic, ½ acetic, ½ ethanol, and 1 CO_2. All of the radioactivity is in the CO_2. By way of the Meyerhof-Embden system, all of the radioactivity would have to be in the lactic, acetic, and ethanol, and none in the CO_2. Clearly, the Meyerhof-Embden system is missing. But when the organism is studied, it is found that only one (or perhaps two) steps(s) is really absent. This organism cannot apparently convert fructose-6-phosphate to triose phosphate. But the remainder of the Meyerhof-Embden reactions are present.

We may, at this point, suggest that in most organisms one or another of the pathways usually predominates, but that even in such cases most of the reactions of the other pathways are present, generally in a considerably reduced amount. We think that this is the situation, not because we have as yet proof in enough different organisms, but rather because of the present conceptions of the function of these pathways. One function is to provide energy and any of the three routes can provide energy. The other function is to provide "building blocks," the intermediates from which the cell can manufacture its own constituents. In this function, each pathway is unique and each provides different intermediate substances. We therefore suspect that every organism has the major portions of each of the paths, in order to provide these building blocks. Where the path is missing, then the structures derivable from the intermediates must either be made from some other route or be supplied preformed from the outside.

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For background information, see p. 492.

QUESTIONS

1. Define or identify:

energy	Meyerhof-Embden system
maintenance	monophosphate shunt
endogenous	hexose diphosphate
enzyme	coenzyme cozymase
catalyst	diphosphopyridine nucleotide (DPN)
hydrolase	glucose-6-phosphate
dehydrogenase	fructose-6-phosphate
oxidase	3-glyceraldehyde phosphate
reductase	glycerol
dihydroxyacetone phosphate	pyruvic acid
ATP, ADP	triose phosphate
phosphoglyceric acids	"active" glycolaldehyde
cocarboxylase	UDP-glucose
energy-rich phosphate	

2. Trace one of the two major pathways between glucose and pyruvate.

3. You are studying the process of fermentation of mung beans. You find that this is done by a bacterium which is capable of fermenting glucose and fructose, but cannot seem to manage ribose. What possible reasons might there be for this kind of result?

4. An organism produces, from glucose, only lactic acid. If you gave it glucose traced with radioactive carbon in the *one* position of the glucose, would you find this carbon in the lactic acid?

5. Three organisms each ferment glucose. One produces ethyl alcohol and carbon dioxide, one only lactic acid, and the third produces lactic acid, carbon dioxide, and ethanol. Whenever carbon dioxide is produced, there is an equimolar amount of ethyl alcohol. It is claimed that all three organisms have the same pathway from glucose to pyruvic acid.
   (a) Would this seem possible to you?
   (b) How would you set about to prove or disprove this concept?

Chapter 9

THE SEARCH FOR ENERGY

"The width of applicability of this concept 'energy' bears witness to its analytic depth. It unites all sensible structure and brings it into a form of doing. By it the atom, the rose we cultivate, and the dog our companion are alike describable. Within the descriptive competence of this unification comes our whole perceptible world, what it is and what it does. The sailing cloud, the bird below it, the setting sun, the coast and sea, the ship and harbour, the lighted window, the flock and the grass down, the voice of the shepherd, it unites these all into one consistent existence whose identical underlying nature becomes through it in so far intelligible to us. Their seemingly endless variety gains thus for man the interest of a concerted system, and, making the interest more poignant, himself is one of them. A unified world is thus offered a sense, but it is split into fragments when we ask about final causes."

SHERRINGTON, *Man on His Nature*

THE LAST CHAPTER described the course of metabolism from glucose to pyruvate. And this process did indeed yield energy to the cell. But there was still a great deal of energy left in pyruvate (or lactate) formed. If one mole of glucose is converted into lactate (= pyruvate + 2H), about 22,500 calories can be available to the cell. But if glucose can be converted to CO_2 and water, 674,000 calories can become available. (The actual amounts vary a little, depending on the conditions under which the reactions take place.)

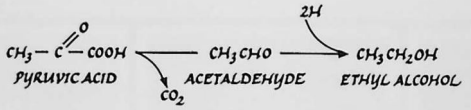


FIG. 9-1. *Yeast pyruvic acid decarboxylase and alcohol dehydrogenase. The decarboxylase is the first step and forms acetaldehyde from pyruvic acid. The second step reduces this acetaldehyde to ethyl alcohol. The enzyme alcohol dehydrogenase can remove 2H from alcohol, forming acetaldehyde, or can put 2H on to acetaldehyde, forming ethyl alcohol.*

Nature therefore devised various ways in which a microorganism could get at the energy remaining in these molecules. It is possible for us to arrange microorganisms in a series, each having a more and more complex, and frequently a more and more efficient, system of obtaining energy. Perhaps the greatest advance came when mechanisms for using the oxygen of the air were devised. It is, of course, tempting to think that such an array of organisms as we may set up, ranging from simpler to more complex energy-yielding mechanisms, may actually represent the evolutionary process by which the more highly efficient oxidative cells, which comprise most of the cells of higher organisms, came into being. But, alas, more sober consideration shows that such an array means merely that such organisms exist today, and tells us nothing about how they came to exist. But to set up such an array is still useful, whether or not it represents an evolutionary path, since from it we may begin to grasp the essential nature of the energy-releasing process.

Fermentation

Many organisms have solved at least part of the problem of how to get energy from the pyruvate, by transforming the pyruvate into other substances in the absence of air. This process of transforming materials in the absence of air or with no reaction with air, even though it be present, is called fermentation.

As outlined in Chapter 8, the product of glucose, as it passed down the Meyerhof-Embden system, was 2 pyruvates plus 2 (2H) per mole of glucose. Some organisms, mostly among the *Streptococcus* and *Lactobacillus*, do not, under the usual circumstances, do much more with the pyruvic acid than reduce it to lactic acid. The summary of the reactions involved is given by the equation

$$\text{glucose} \longrightarrow \underset{\text{pyruvic acid}}{2CH_3COCOOH} + \underset{\text{metabolic hydrogen}}{2\,(2H)} + \underset{\text{energy-rich phosphate available energy}}{2 \sim PO_4}$$

$$\downarrow$$

$$\underset{\text{lactic acid}}{2CH_3CHOHCOOH}$$

Organisms such as the streptococci and lactobacilli, when grown on glucose, produce large quantities of lactic acid as their major end product of metabolism. As much as 95% of the glucose used appears as lactic acid.

But the search for energy could not leave such a rich energy source as pyruvate unattacked for long, and so organisms devised other ways to extract the energy within it. One process was to decarboxylate it, and one group of microorganisms, notably the yeasts, did just this (shown in Fig. 9-1), reducing the resulting

acetaldehyde to ethyl alcohol. As such, they derived no available energy from the reaction, but at least they replaced a highly acid end product (lactic acid) with a neutral one. Other microorganisms devised a better way of "decarboxylating" pyruvic acid. If metabolic hydrogen were removed at the same time, then a new substance, having energy-rich properties, became available. The reaction is illustrated in Fig. 9-2; we will return to it on p. 171.

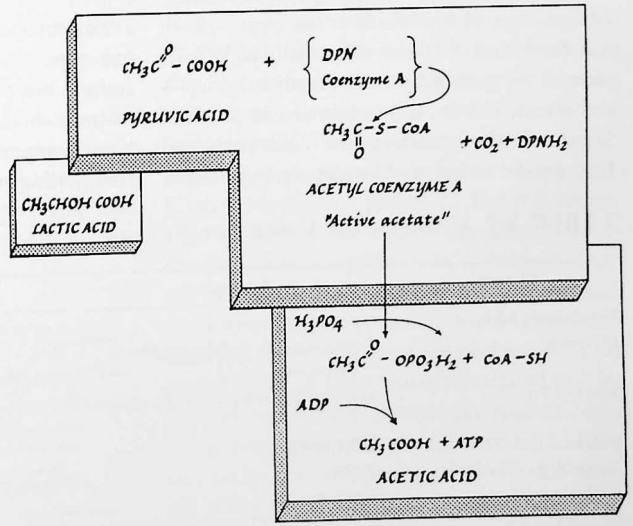
Digression on Coenzymes

We may define **coenzymes** as organic substances of relatively low molecular weight which are necessary before particular enzymatic reactions can proceed. We have earlier left our definitions and discussions somewhat vague. It is now necessary to be more precise. There appear to be four classes of coenzymes. One type seems to be adsorbed onto the enzyme molecule, and its function appears to be that of providing the proper chemical configuration on the enzyme surface. Coenzymes of this type are listed in Table 9-1 together with some notion, where it can be discerned, of their overall function. For example, pyridoxal phosphate appears to be involved in many different kinds of enzyme-catalyzed reactions having little relation to one another. But mostly these reactions involve substances having a free amino group and there is evidence that pyridoxal phosphate combines with free amino groups. We therefore suppose that the pyridoxal phos-

TABLE 9-1. *Surface Enzyme Activation*

Vitamin	Coenzyme	Function
Vitamin B_6	Pyridoxal phosphate	Amino acid decarboxylation, transamination, most reactions involving amino acids
Vitamin B_1 Thiamin	Thiamin pyrophosphate	Decarboxylation of α-keto acids whether or not these involve a simultaneous dehydrogenation
Biotin	Unknown	Carboxylation reactions, details obscure

FIG. 9-2. *The formation of acetyl-coenzyme* A.



phate—tightly adsorbed and bound to a spot on the catalytic protein, the enzyme—is capable of combining with amino groups of the reactants, the substrate, which is able to hold it in the "appropriate orientation and juxtaposition," thus enabling the enzyme to catalyze the reaction.

A second type of coenzyme has the function of carrying **metabolic hydrogen** from one enzyme to another. We have used the term "metabolic hydrogen" earlier without defining it, hoping that it would be understood in the context used. But we should here point out that the removal of hydrogen from metabolic intermediates in the living cell is done in a curious and unique manner. No hydrogen gas is released; no hydrogen ion plus an electron is involved (although this may appear later in the reaction chain *after* the hydrogen has been removed from the carbon containing substrates). Instead, the hydrogen is removed "2H" packets at a time and transferred to one of three **hydrogen-carrier** coenzymes, shown in Fig. 9-3. DPN and TPN are the most common, the latter having one more phosphate; but flavins are also sometimes involved in the direct removal of 2H from organic substrates. The position of lipoic acid is less clear.

A third kind of coenzyme actually picks up a piece of the substrate—a fragment or group—and transfers it from one reaction to another. These are the **group-carrier** coenzymes and their general nature and function are illustrated

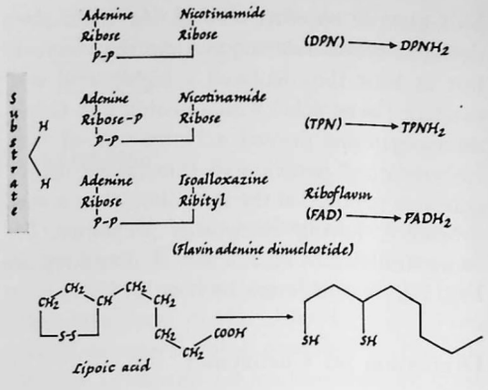


FIG. 9-3. *Carriers of metabolic hydrogen.*

in Table 9-2. We shall see many examples of group transfer reactions in considering microbial metabolism.

A fourth kind of "coenzyme" is not quite the same as the others in that it is sometimes difficult to clearly separate the energy aspects of the reaction from the mechanisms employed. Many reactions which require ATP for their operation do so because the ATP actually acts as a group carrier. Examples of such reactions, sometimes called **substrate activation,** are shown in Fig. 9-4.

To summarize the coenzymes, then, there is one type concerned with activation of the surface, two types concerned with transporting material from one enzyme to another—one type transporting hydrogen, the other type transporting groups of atoms—and still another

TABLE 9-2. *Group-Carrier Coenzymes*

Vitamin	Coenzyme	Function
Pantothenic acid	Coenzyme A	Carrier of acyl groups
Folic acid	Tetrahydrofolic or folic-glutamic derivatives	Carrier of one carbon and formyl in various oxidation states
Vitamin B_{12}	Adenine, B_{12}, unknown sugar complex	Glutamic-mesaconate metabolism, methionine, protein synthesis, and DNA synthesis
Niacin	DPN, TPN	Carry hydrogen, i.e., are somewhat different from group-carrier types
Vitamin B_2 (riboflavin)	FAD	
α-Lipoic acid	Unknown	

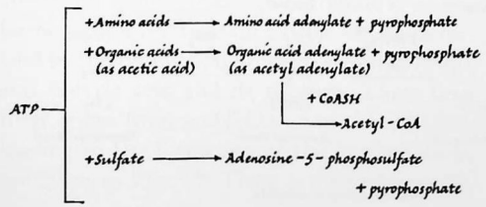
type involved in substrate activation which may or may not involve transportation from one site to another. A given reaction may involve more than one such coenzyme, and indeed may involve more than one type. It happens that the reaction we wish to discuss, the decarboxylation of pyruvate with the simultaneous removal of hydrogen, is just such a multicoenzyme reaction, which is why this digression was necessary.

The Decarboxylation of Pyruvate and the Removal of Hydrogen

The reaction, earlier pictured in Fig. 9-2, is actually one of the most important in metabolism, since it is by this reaction that entry is afforded into an array of alternative possibilities. Pyruvic acid in the presence of DPN (to accept the hydrogen) and reduced coenzyme A or CoA (i.e., in its -SH form to accept the acetyl group) and in the presence of an enzyme which contains thiamine pyrophosphate (cocarboxylase) and lipoic acid, as enzyme surface activators, is converted to acetyl-CoA + $DPNH_2$ + CO_2. The acetyl-CoA, in the presence of another enzyme, can be converted to acetyl phosphate, which can form ATP. Hence each pyruvate can now yield an energy-rich phosphate plus a 2H on the DPN and if a way can be found to use this 2H and extract the energy that is in it, the organism can benefit.

But the problem becomes how to use up the $DPNH_2$, preferably in the most profitable way. Indeed, organisms may be arranged in a series

FIG. 9-4. *Substrate activation.*



as to how efficiently they dispose of the 2H on $DPNH_2$. After all, there is only a little DPN in the cell and it must be used over and over again.

In the case considered in Fig. 9-2, so long as we start with pyruvic acid, we could use the $DPNH_2$ to reduce another pyruvic acid to lactic acid. Hence, in this case,

$$2 \text{ pyruvic} \longrightarrow 1 \text{ acetic} + 1 \text{ lactic} + 1CO_2$$

But if we start from glucose, the possibility of this reaction is not of much help since the product of glucose breakdown is not 2 pyruvic acid, but 2 pyruvic acid + 2 $DPNH_2$ (see Meyerhof-Embden system, p. 154). There is already too much "hydrogen pressure" and so even organisms which could carry out the reactions outlined in Fig. 9-2, and do carry out such reactions when given pyruvate, do not do so when given glucose. They have no place to put the hydrogen; they have no way, other than by lactic acid formation, to remove the hydrogen from $DPNH_2$.

Of course, it is possible to reduce acetyl-CoA to ethanol, and this requires 2(2H) or 2 $DPNH_2$. As such, for glucose we might form 2 ethanol and $2CO_2$ just as yeast does, but by forming acetyl-CoA rather than acetaldehyde. However, there would be no energy available to the cell. The 2H must be put somewhere else. We do find organisms among the *Streptococcus* and *Lactobacillus* which conduct a "mixed lactic" fermentation and whose major products of glucose metabolism are lactic acid, acetic acid, ethanol, and CO_2. But it is impossible to have acetic acid under these circumstances unless something else is reduced. Usually the reduced product found is glycerol and the amount of acetic acid formed is one-half, mole for mole, of the glycerol formed. This comes about in the manner illustrated in Fig. 9-5. Of the 3-triose phosphates formed from 1½ glucose molecules, one is converted to CO_2 and acetic, and the hydrogen so released (one 2H at the glyceraldehyde phosphate stage, an-

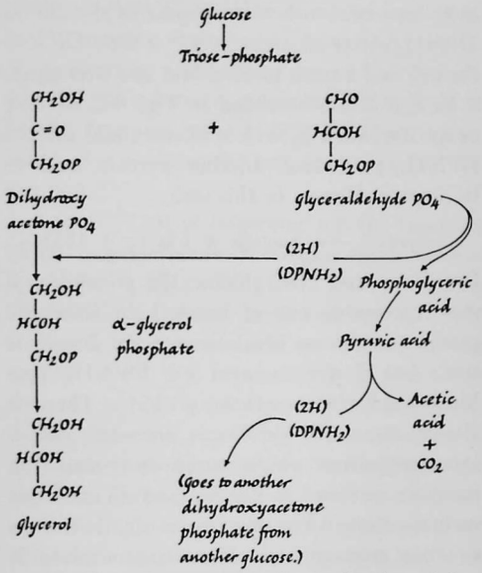


FIG. 9-5. *Pathways for the production of glycerol during fermentation.*

other 2H at the pyruvate to acetic stage) is used to reduce 2 molecules of dihydroxyacetone phosphate to glycerol.

At other times the reduced product can be something else. On fructose the reduced product is mannitol. But still the problem is what to do with the $DPNH_2$. One way to solve this

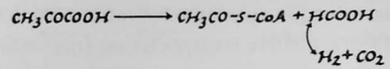


FIG. 9-6. *Pathway for the production of formic acid and hydrogen gas.*

problem has been devised mostly by the gram-negative fermentative rods (*Escherichia coli, Aerobacter aerogenes*, etc.). This process, shown in Fig. 9-6, consists of breaking the hydrogen off with the CO_2 (and thus form H_2CO_2 = HCOOH = formic acid) and later, in some cases, even releasing molecular hydrogen. As such, one finds fermentations like *Salmonella typhosa* yielding lactic acid, acetic acid, ethyl alcohol, acetic acid, and formic acid from glucose; and other like *E. coli* are essentially similar except that instead of formic acid there is now $H_2 + CO_2$.

There are other organisms which have solved the problem of the search for energy in still other ways. Typical fermentations are given in Table 9-3. In these cases there is the appearance of 4-carbon compounds. Since, after all, their origin is in pyruvic acid, a 3-carbon compound, the mechanism of these transformations is of some interest. The origin of succinic acid and propionic acid from glucose is illustrated in Fig. 9-7.

FIG. 9-7. *The origin of succinic and propionic acids.*

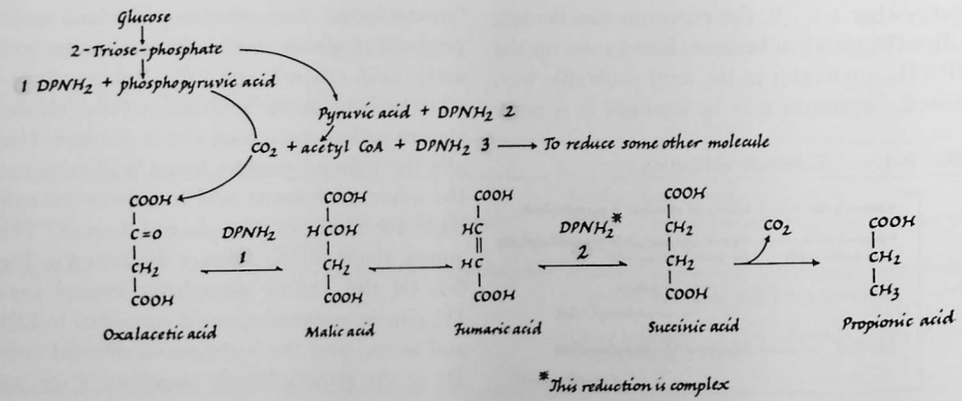


TABLE 9-3. *Typical Fermentations*

(mM per 100 mM GLUCOSE)

NUMBER OF CARBONS	SUBSTANCE	GRAM-POSITIVE NO SPORES			GRAM-NEGATIVE			SPORE-FORMERS AIR	SPORE-FORMERS ANAEROBIC			YEAST
0	H_2	—	—	—	—	38.6	48.9	51.5	233	166	72.7	—
1	HCOOH	—	—	—	39.3	0.9	21.2	—	—	—	—	—
	CO_2	—	87	63.6	—	50.1	178.1	166.2	195	220	197	195
2	Acetic	—	17.5	17.0	25.6	28.7	—	4.6	42.6	24.8	21.2	—
	Ethanol	—	75.5	—	25.4	41.6	66.8	52.0	—	—	—	195
3	Lactic	192	73.5	—	121.7	109.2	—	23.3	—	—	—	—
	Propionic	—	—	139	—	—	—	—	—	—	—	—
	Neutral Glycerol	—	39.5	—	—	—	—	—	—	—	—	—
	Neutral Isopropanol	—	—	—	—	—	—	—	—	—	151	—
	Neutral Acetone	—	—	—	—	—	—	—	—	22.3	—	—
4	Butyric	—	—	—	—	—	—	—	75.3	7.1	6.1	—
	Butanol	—	—	—	—	—	—	—	—	47.4	66.6	—
	AMC[1]	—	—	—	—	—	16.4	1.7	—	5.7	—	—
	Buglycol[2]	—	—	—	—	—	62.9	54.7	—	—	—	—
	Succinic	—	—	13.6	10.8	15.9	—	3.6	—	4.9	—	—
TYPICAL ORGANISM		Strep. lactis L. casei	L. arabinosus	P. Shermanii	Sal. typhosa	E. coli	Aerob. aerogenes	Bac. polymyxa	Cl. butyricum	Cl. acetobutylicum		Sach. cerevisiae
FERMENTATION TYPE		Simple lactic	Mixed lactic	Propionic	C—D—T Group			Glycol	Butyric	Solvent	Isopropyl	Alcoholic

[1] Acetyl methyl carbinol
[2] Butylene glycol

There is further a group of organisms, the anaerobic spore-formers (*Clostridium*), whose fermentation reactions on glucose are characterized by the production of large quantities of gas and butyric acid and its relatives. These take their origin from acetyl-CoA and the reactions leading to the formation of the final products are given in Fig. 9-8. There is a 4-carbon product, acetylmethyl carbinol, and its relatives still to be accounted for. It happens that in the formation of these materials, coenzyme A is not involved. There are indeed two separate pathways—one characteristic of bacteria, one characteristic of yeast. Both are illustrated in Fig. 9-9.

With these reaction series, we have ac-

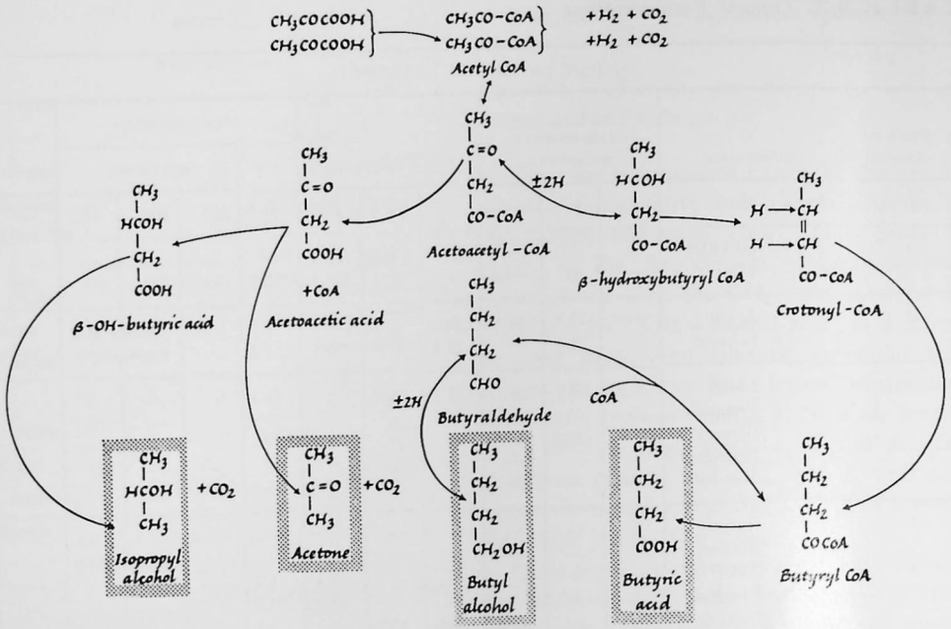


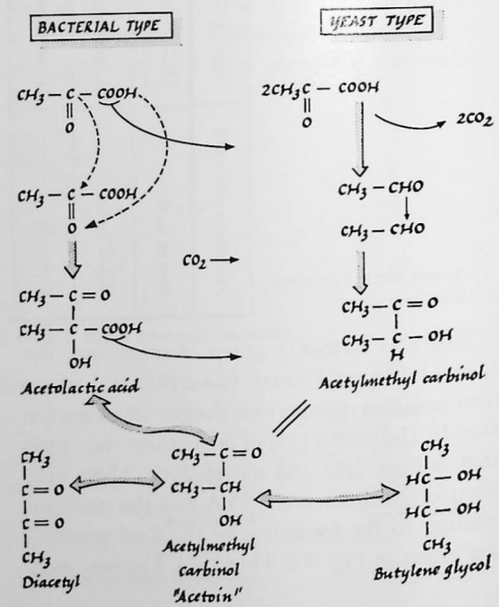
FIG. 9-8. *The origin of the products formed by various* Clostridium *species.*

counted for the major products of the breakdown of glucose by various organisms under anaerobic conditions, or under conditions in which air could not be used. All these products are made because the organism could get energy from the process.

The Citric Acid Cycle

All the metabolic reactions so far described would still not be capable of taking the glucose to CO_2 and H_2O, even if some way were found to remove the 2H. Some organisms have therefore developed a reaction series, called the citric acid cycle after one of its striking intermediates, which can, in fact, so maneuver the carbon in glucose and pyruvate that it is all convertible into CO_2. The cycle is usually involved in the aerobic oxidation of pyruvate, since it is possible in air to remove all of the accumulated 2H, but it will occur under anaerobic conditions. That is, there is nothing about the citric acid cycle that requires air; it is only that air is the most convenient way of utilizing the 2H released.

FIG. 9-9. *Acetylmethyl carbinol and its relatives.*



The reaction series comprising the citric acid cycle, shown in Fig. 9-10, begins with a condensation of acetyl-CoA and oxalacetic acid to form citric acid. This is progressively reacted until by one complete turn of the cycle the acetyl-CoA has been converted to CO_2 and 2H. Acetyl CoA, or for our purpose acetic acid (CH_3COOH), for complete oxidation would require the removal of 2 CO_2 (which occurs in reactions E and F) and the removal of 4 (2H) (which occurs in reactions D, F, H, and J). We end up with oxalacetic acid, the substance with which the cycle was started. Parts of this cycle are found in fermentative organisms as well as in aerobes, and we have earlier seen (Fig. 9-7) that the lower portion of it serves as a source of succinic acid and propionic acid. Further, as indicated in Fig. 9-10, there are other reactions, leading from and leading into the citric acid cycle, which provide paths for obtaining energy from these other substances or routes of synthesis of them when they are needed by the cell. Many molds, particularly, produce materials like citric acid, itaconic acid, etc., as end

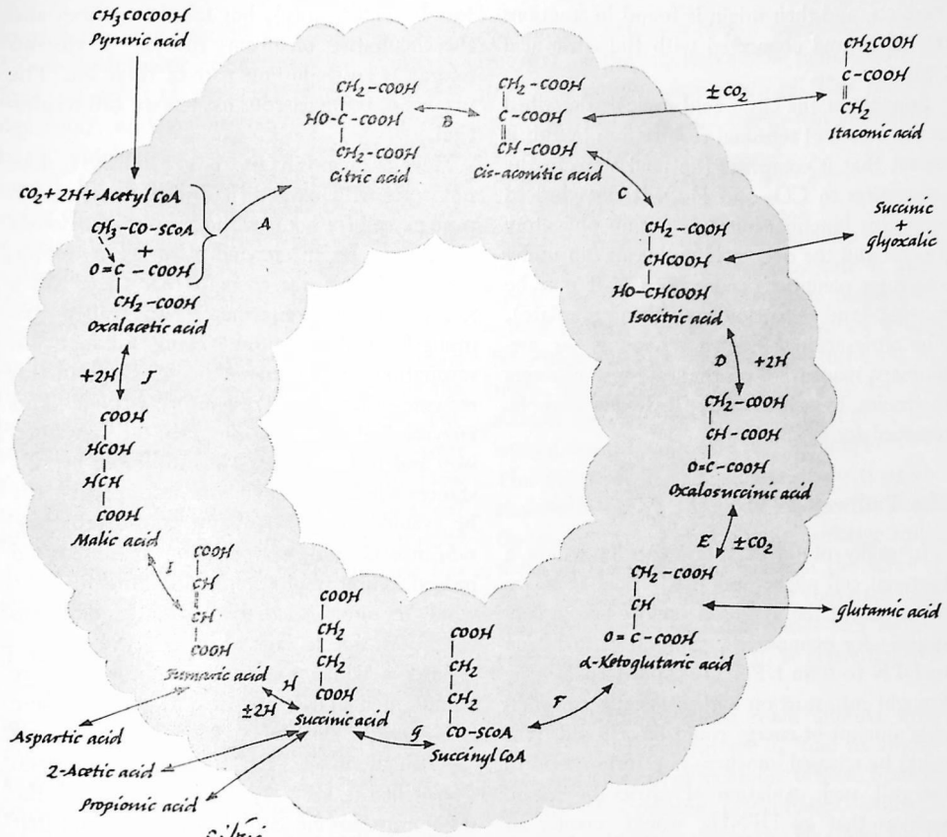


FIG. 9-10. *The citric acid cycle.*

products, and their origin is found in reactions related to and connected with the citric acid cycle.

Sometimes the citric acid cycle is described as the means of **terminal respiration;** by this is meant that it comprises the final steps in the conversion to CO_2 and H_2O. It does indeed serve this function, but it is not an obligatory process and the citric acid cycle can run under anaerobic conditions or portions of it may be missing (and thus products will accumulate). The citric acid cycle itself represents the preliminary maneuvers of the carbon-containing molecules by which their hydrogen may be removed for respiration.

The Pathway to Oxygen: Respiration

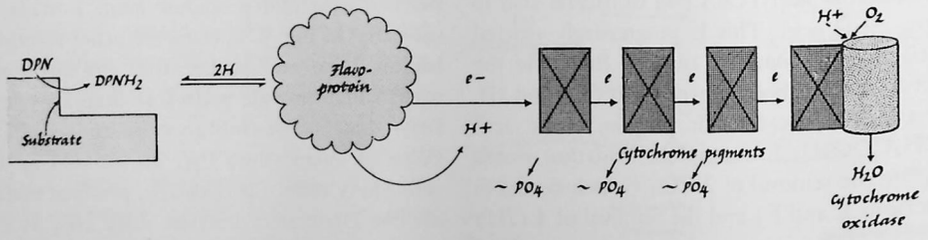
In many of the environments in which a bacterial cell might find itself, there is access to oxygen. If the hydrogen removed from substrates—for example, the removal of hydrogen to DPN to form $DPNH_2$—could somehow be brought into reaction with oxygen, a considerable amount of energy could be released. If it could be released somehow in a form useful to the cell, such oxidation of carrier hydrogen, such as that on $DPNH_2$, would provide an important source of energy for the cell.

Of course, if an organism has not developed systems for effectively utilizing the oxygen of the air, its presence may be toxic (as it is for the anaerobes) or at least a menace (as it is for the indifferents), but for the aerobes and the facultative organisms the use of gaseous oxygen is an important part of their life. The process of using gaseous oxygen we call respiration.

How do organisms use oxygen? $DPNH_2$ does not react with oxygen; it can be shaken with pure oxygen for hours without effect. Obviously there must be an enzyme involved in respiration. At first it was thought that there was a single respiratory enzyme. It was called "atmungsferment," *atmungs* being German for respiration and *ferment* being German for enzyme—that is, *the respiratory enzyme.* This enzyme had a curious property—it contained iron and in consequence was inhibited by substances which combined with iron, particularly by cyanide. And this was of considerable significance. Cyanide was poisonous because it did indeed inhibit respiration in the animal. (The word "cyanotic," i.e., like cyanide, describes conditions due to lack of oxygen.)

After a while, the situation became more complicated and it appeared that there were two enzymes; the second was promptly named "Zwischenferment," i.e., the enzyme between ("zwischen") $DPNH_2$ (or $TPNH_2$) and the respiratory enzyme. Still later it was found that there was really a chain of reactions between $DPNH_2$ and oxygen and that the older enzymes were part of this chain. The chain itself is illustrated in Fig. 9-11. It consists of four sections, the substrate coenzymes ($DPNH_2$), the carrier

FIG. 9-11. *The main respiratory chain. None of the oxygen enters the substrate molecule.*



flavin containing proteins called flavoproteins ("Zwischenferment" turned out to be one of these), a series of pigments (the cytochrome pigments), and an enzyme (cytochrome oxidase) which reacts with oxygen (this later proved to be the "atmungsferment" of the earlier workers). What happens along the chain is that hydrogen removed from the substrate is first converted into hydrogen ions, then into electrons, and the electrons are carried out to oxygen. The reason that all of this apparently complex machinery is necessary is that energy-rich phosphate bonds are generated during the process, rather than heat or electricity. The various steps, therefore, are needed to convert the energy released into these bonds. How this is done is not precisely known, but about three energy-rich phosphate bonds are formed during the passage of each 2H through this reaction series.

The Hydrogen-carrying Coenzymes

There are two of these coenzymes—DPN and TPN—directly concerned with the removal of hydrogen from substrates. Their action was illustrated in Fig. 9-3. Their ability to carry hydrogen resides in the peculiar properties of the nitrogen in the nicotinamide structure; because it can change from a valence of 5 to 3, the internal structure of the molecule can alter.

There is a further hydrogen-carrying system in the **flavoproteins.** There are actually several of these, having slightly different functions; the flavoprotein that reacts with $DPNH_2$ is not the same one that reacts with $TPNH_2$, for example. But all the flavoproteins have flavin bound to them in one form or another. The flavin is **riboflavin** (formerly known as vitamin B_2). Flavin can absorb two hydrogens and this reduced flavin can rearrange itself into a "free radical" state which enables it to move one electron, and then a second electron. Thus, the flavoprotein accepts two hydrogens and gives off two electrons, one at a time, and two hydrogen ions. It acts as a sort of transformer since the rest of the reaction chain cannot handle hydrogen atoms—only electrons. This process is capable of forming an energy-rich phosphate bond.

The Cytochromes

The **cytochromes** are cell pigments (hence the name) whose chemical structure is very much like that of the heme in blood and which contain iron in a state in which it can be oxidized to Fe^{+++} or reduced to Fe^{++}. As such, they can carry electrons. The Fe^{+++} accepts an electron and becomes Fe^{++}; the latter gives up an electron and becomes Fe^{+++}. The pigments can be identified by their absorption spectra. Each of these pigments poises the iron a little higher on the oxidative scale, so as an electron passes through the series it gives up energy which can be converted into two energy-rich phosphate bonds—for each 2 electrons moved, 2 ADP can be converted to 2 ATP.

Finally, the electrons reach another cytochrome, called cytochrome a_3 from its absorption spectrum, or cytochrome oxidase from its activity of oxidizing cytochromes, which is capable of uniting electrons, H^+, and O_2 to form water. It is the enzyme inhibited by cyanide—the "atmungsferment" indeed.

This cytochrome pathway accounts for most of the respiration of animal tissues and it or some slightly modified system accounts for the respiration of many bacteria. The cytochrome system is sometimes called the "electron-transporting" mechanism.

Other Respiratory Systems

There are several other pathways to oxygen of somewhat lesser importance as energy sources. The cytochrome oxidase system is a 4-electron system, i.e., for every O_2 it uses 4 electrons + $4H^+$ and forms $2H_2O$. There are

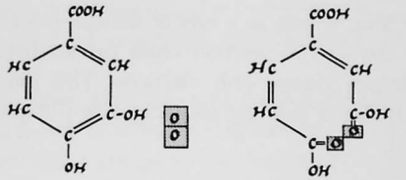


FIG. 9-12. *Both atoms of oxygen enter the substrate molecule.*

other similar kinds of electron-carrying systems (usually they are modified flavoproteins) which use only 2 electrons; for every O_2 these use 2 electrons + $2H^+$ and form $1H_2O_2$. In these two cases the oxygen used from the air appears not in the oxidized product, but in the H_2O or the H_2O_2 formed. The H^+ used, of course, comes from the environment and is not necessarily the same H^+ as was released when 2H was converted to 2 electrons + $2H^+$.

There are, further, two other kinds of oxidation systems, in both of which the oxygen actually enters the oxidized molecules. In the first of these, both atoms of O_2 appear in the oxidized molecule. An example of such a reaction is the oxidation of many aromatic compounds to break the ring structure; see Fig. 9-12 for an example.

The other kind of oxidation is one in which only one-half of the oxygen molecule appears in the oxidized molecule and the remainder in H_2O or H_2O_2. The oxidized molecule is usually hydroxylated, i.e., now contains a hydroxyl (—OH) group. An example is the hydroxylation of imidazole acetic acid as shown in Fig. 9-13. This system, which occurs in some *Pseudomonas fluorescens* strains, requires O_2 and simultaneously $DPNH_2$, mole for mole, and one-half of the oxygen used is found in the product, the other half in the water.

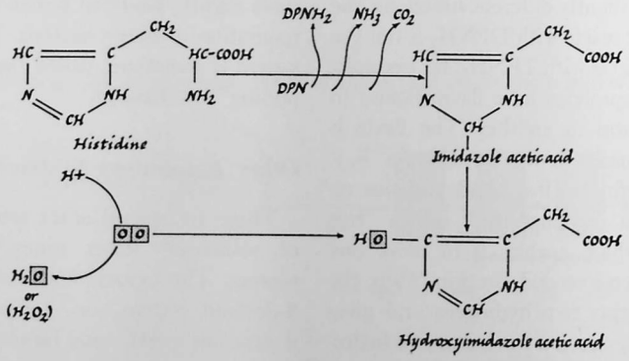
Of all of these systems of contact with oxygen, however, by far the most important as an energy source is the cytochrome-type pathway to oxygen. Our concern with the others at this point is only to note that they exist.

Energy from Oxidations

The one main reason for the development of pathways to oxygen, and one which benefits those organisms (the aerobes and the facultatives) which possess them, was to obtain energy from the oxidation. How much energy is so obtained? This may be approached in at least two ways.

First, we may calculate the energy available. If we take a simple case, such as conversion to lactic acid, the energy derived from converting 1 glucose to 2 lactic acids would be 2 energy-rich phosphate bonds. Suppose that such an organism had a citric acid cycle and an aerobic

FIG. 9-13. *One atom of oxygen enters the substrate molecule while the other does not.*



cytochromelike system yielding, on the average, 3 energy-rich phosphate bonds per 2H carried out to oxygen, starting with pyruvate.

We might make a supposition as to how this would add up, or we could calculate from the overall changes. It would not matter; we would get the same energy, as follows:

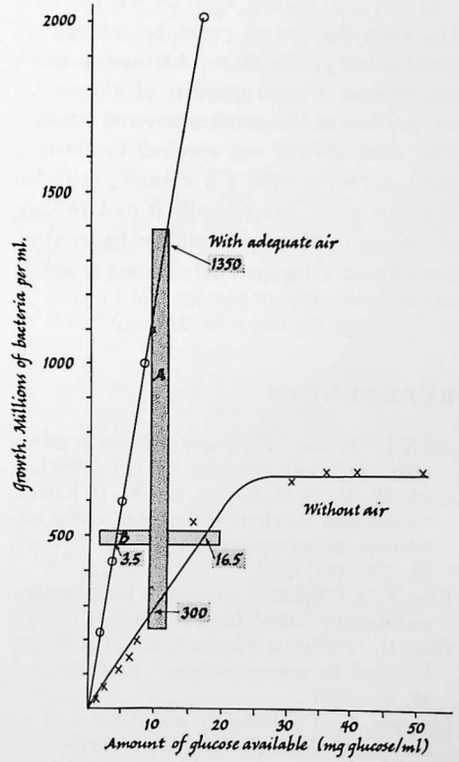
1. 1 glucose to 2 pyruvic acid	$2 \sim PO_4$
2. 2 (2H) from above path to O_2	$6 \sim PO_4$
3. 2 pyruvic acid to 2 acetyl-CoA 2 (2H) from above to O_2	$6 \sim PO_4$
4. 2 acetyl-CoA to 2 citric to 2 oxalosuccinic acid (using 2 oxalacetic acid) 2 (2H) from above to O_2	$6 \sim PO_4$
5. 2 oxalosuccinic acid to 2α-ketoglutaric acid to 2 succinic acid 2 (2H) from above to O_2	$6 \sim PO_4$
6. 2 succinic acid to 2 fumaric acid 2 (2H) from above to O_2	$4 \sim PO_4$[1]
7. 2 fumaric acid to 2 malic acid to 2 oxalacetic acid (replacing those used in step 4) 2 (2H) from above to O_2	$6 \sim PO_4$
	$36 \sim PO_4$

[1] There is evidence that this enters the cytochrome chain at a higher level than the others; hence less energy is liberated in the form of energy-rich phosphate.

Thus, by the complete oxidation of the glucose to CO_2 and H_2O, accomplished by the Meyerhof-Embden system and the citric acid cycle, 36 energy-rich phosphates are released, compared to 2 released when the same amount of glucose is converted anaerobically to lactic acid. Thus the aerobic processes have yielded 18 times the energy that the anaerobic process did, and we have calculated only that energy which we know is available to the cell in the form of energy-rich phosphate bonds. Actually, this is about half the energy actually available, for Glucose to 2 lactic acid liberates 22.5 kg cal/*M*. Glucose to $6CO_2 + 6H_2O$ liberates 674 kg cal/*M*. 674/22.5 = 29.8 times as much energy by aerobic oxidation.

We may experimentally determine the effect of oxygen upon growth, for example, as has been done in the experiments illustrated in Fig. 9-14. This figure represents the growth of a facultative organism, *Aerobacter aerogenes*, grown under conditions of rapid aeration, compared to growth under conditions where air is completely absent, in a synthetic medium in which the only source of energy is glucose. Up

FIG. 9-14. *The influence of oxygen upon the growth of* Aerobacter aerogenes. [*Redrawn from the data of Dagley, Dawes, and Morrison,* J. Bacteriol., **61**, *433 (1951). See text for explanation of bars A and B.*]



to a range of about 20 mg glucose/ml (20 gm/l or 0.2%), the amount of growth at the stationary phase, which is what is recorded in Fig. 9-14, is dependent upon the amount of glucose—twice as much glucose, twice as much growth. After this point, something else becomes limiting without air (anaerobic conditions) and the further addition of glucose does not give further growth. It is probable that acid is accumulating anaerobically in a sufficient concentration to inhibt growth at levels of 20 mg/ml of glucose and above.

We can at least compare the growth aerobically and anaerobically in that phase of the curves in which glucose is the limiting factor. For example, if we compare the growth at 10 mg of glucose/ml (bar A on graph), there were 300 million bacteria/ml when grown anaerobically and 1350 million when air was provided; i.e., when the glucose could be oxidized we could obtain (1350/300 =) 4.4 times as much growth from a given quantity of glucose. Or we may look at this another way and calculate how much glucose was required to obtain a given number of cells. For example, as in bar B on the graph, anaerobically it took 16.5 mg of glucose to obtain 500 million bacteria/ml, but with air it required only 3.5 mg to obtain the same growth, i.e., growth in air was (16.5/3.5 =) 4.7 times as efficient. In short, under the circumstances, this particular organism can grow almost five times better with air than without. In some circumstances the effect of air may be as much as a tenfold increase in growth.

This type of response gives either (1) better growth (3 to 10 times) per unit glucose used (bar A, Fig. 9-14), or (2) less substrate used per unit growth (bar B, Fig. 9-14). This response is called the **Pasteur effect** since it was originally described by him. Such a response is only possible with facultative organisms. The strict aerobes do not grow without air; the Pasteur effect is infinity, i.e., the concept does not apply. The indifferents and the anaerobes do not show increased growth in air, and so do not possess a Pasteur effect.

Since growth in air is increased 3 to 10 times in the facultatives, obviously they are obtaining more energy in air. All such organisms possess a cytochrome-type system but the cytochrome path may differ a little from one organism to another. So far as we now know, the cytochrome system is the only pathway to oxygen capable of yielding these amounts of energy to the cell.

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For background information, see p. 492.

QUESTIONS

1. Define or identify:

fermentation	mannitol
respiration	acetylmethylcarbinol
decarboxylation	acetoin
surface activity coenzyme	acetone
hydrogen-carrier coenzyme	citric acid cycle
group-carrier coenzyme	terminal respiration
activation coenzyme	"atmungsferment"
coenzyme A	"zwischenferment"
cocarboxylase	cytochrome system
acetyl-CoA	cytochrome
acetyl phosphate	cytochrome oxidase
hydrogen pressure	flavoprotein
	electron transport
	hydrogen transport
	Pasteur effect

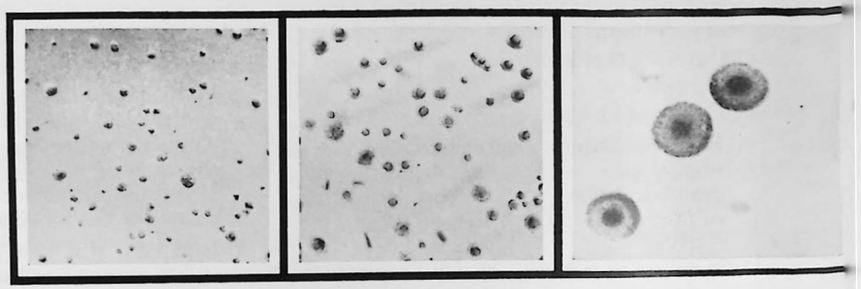
2. You are in charge of the development laboratory of the Poor Man's Fermentation Company and you are asked to develop a process for making the compound Jumping Acid. It is known that while this substance is produced under anaerobic conditions, it is only formed after the cells have grown. The cells can be grown with air without harming their subsequent ability to make Jumping Acid from glucose. Design a process which will give you the greatest quantity of Jumping Acid at the least cost.

3. Professor Doubtful Data reports that a certain mold isolated by him oxidizes a variety of substances. The oxidations are all completely inhibited by sodium cyanide. But using isotopic oxygen in air, no isotopic oxygen can be found in the oxidized products. What kind of a respiratory system would you suspect? Why?

4. It happens that it is important for practical reasons to make a distinction between *Aerobacter* and *Escherichia*. *Aerobacter* is capable of producing acetylmethylcarbinol but *Escherichia* is not, hence one can actually test for the presence of this substance. But without such a test for acetylmethylcarbinol, you find that *Escherichia* species produce a great deal more acid from glucose, somewhat less CO_2, and more hydrogen, and you devise a differential test based on this. It happens that these tests really work, but on your final examination you are asked to explain why *Escherichia* becomes more acid and has more H_2 in its gases than *Aerobacter*. How would you explain this?

5. At the start of World War I, airplanes were made of wooden frames covered with a thin canvas. This canvas was varnished with a rapidly drying mixture called "airplane dope" which contained large quantities of acetone. You have an organism, which, though it doesn't fly, makes a little acetone from glucose; but you suspect that you could get it to make much more if you could add the right intermediates. What intermediates would you try?

Chapter 10



THE PURPOSE OF METABOLISM–SYNTHESIS

"The main business of natural philosophy is to argue from phenomena without feigning hypotheses, and to deduce causes from effects, till we come to the very first cause, which certainly is not mechanical; and not only to unfold the mechanism of the world, but chiefly to resolve these and such like questions: What is there in places almost empty of matter, and whence is it that the sun and planets gravitate towards one another, without dense matter between them? Whence is it that nature doth nothing in vain; and whence arises all that order and beauty which we see in the world?"

NEWTON, *Opticks* (1704)

OUR DISCUSSIONS of carbohydrate metabolism and of energy have left one enticing corner in the dark: What do cells *do* with the metabolism and the energy? They build more cells; they synthesize all the complex and interrelated substances of the cell. Some microorganisms can start with carbon dioxide and mineral salts and energy and synthesize all of the substances needed: fats, proteins, nucleic acids, complex ring structures—the substances that make membranes and nuclei and cells. Other organisms are less capable of such synthesis and require certain substances preformed before they can grow—for the most part because they cannot synthesize them. The process of **synthesis,** therefore, is worth our attention.

We have so far considered only the breakdown of carbohydrates, but it must be understood that microorganisms can break down amino acids, proteins, nucleic acids, fats, etc., and that each of these breakdown pathways may have distinctive characters, depending upon the substance broken down. Further, the ability to attack a given material and the metabolic pathways which may be followed in their breakdown may vary from one organism to another. Some of these distinctions are useful in bacteriology because they may assist us in recognizing certain microorganisms. Sometimes these differences in metabolic pathways permit us to distinguish between otherwise closely similar organisms. Sometimes the distinctive substances produced are of importance to man, as in the production of antibiotics; other microorganisms form breakdown products which are poisonous. Except for certain outstanding cases where the products are of a distinct and characteristic value to microbiology, we shall not comment further upon metabolic breakdown processes, and shall leave this knowledge for your further study in the future. For now we wish to approach, inadequately prepared as we may be, the problem of synthesis; the building up of cells rather than the tearing down of external substances.

Initial Steps of Synthesis

There are microorganisms that can live on carbon dioxide, mineral salts, and energy. Sometimes this energy may come from simple substances, such as acetate or formate, or even from inorganic substances, such as sulfur or iron; sometimes, too, the energy may come from light. It makes little difference precisely what the source of energy is; the process of synthesis is much the same and it must start from simple materials—carbon dioxide, ammonia, phosphate. Of the elements which go to make the substance of a cell, carbon, hydrogen, oxygen, nitrogen, phosphorus, and sulfur are converted into organic substances, while potassium, magnesuim, and others exist mostly as inorganic materials inside the cell. The first problem of synthesis, then, is how the inorganic substances are converted into organic substances.

The ultimate source of hydrogen is hydrogen ion. It requires an electron to become [H]. The source of such electrons is the atom; either its disintegration on earth or in the sun, and the conversion of the sun's energy to electrons by photosynthesis. For a given organism growing on glucose, for example, the [2H] is already present in an organic form, having been put there by the energy of the sun when the glucose was formed in a plant. For a photosynthetic organism the [2H] is formed from electrons generated by the light. For a bacterium growing on sulfur the [2H] is derived from hydrogen ion using the electrons removed from the sulfur. Oxygen appearing in organic substances normally originates in water. Oxygen taken up in respiration usually appears in the water, but sometimes it may appear in the oxidized product. This discussion of the conversion of H and O to organic forms may seem to be unnecessarily complex. But the deeper we go into the matter the more we uncover complexities and uncertainties having to do with the fundamental nature of atoms—somewhat beyond the scope of this book and certainly beyond our knowledge of the nature of atoms. But it will suffice for our purpose to remember that there is a ready transfer of atoms of H and O between organic and inorganic forms, that we are sometimes unable to know precisely whether such atoms are organic or inorganic.

With CO_2 the matter is clearer. Biologically CO_2 may serve as an oxidizing agent, although a few organisms can reduce it to methane (CH_4). But more commonly CO_2 is converted into organic carbon by *addition* to particular carbon

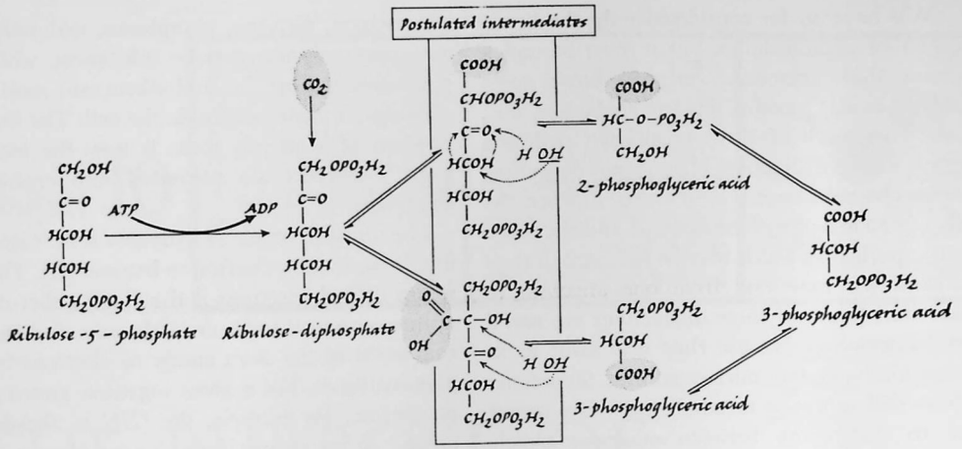


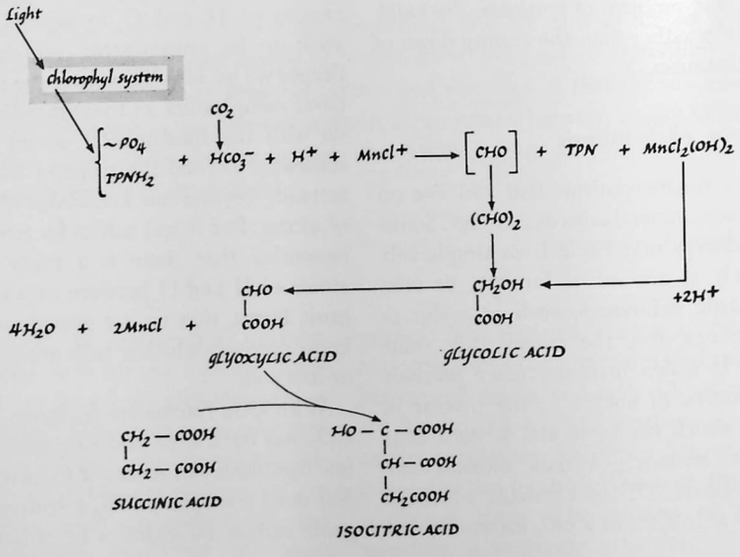
FIG. 10-1. *The ribulose diphosphate pathway of carbon dioxide fixation in photosynthesis.*

compounds. There are, first, a variety of decarboxylations, in which CO_2 is released, usually from a carboxyl (—COOH) group. Some of these are readily reversible as with

$$CO_2 + H_2 \rightleftharpoons HCOOH$$

But while such reactions might serve as sources of carbon compound synthesis, when such syntheses are traced it is found that there appear to be two main pathways by which CO_2 enters organic substances to form carbohydrates

FIG. 10-2. *A second pathway of photosynthesis.*



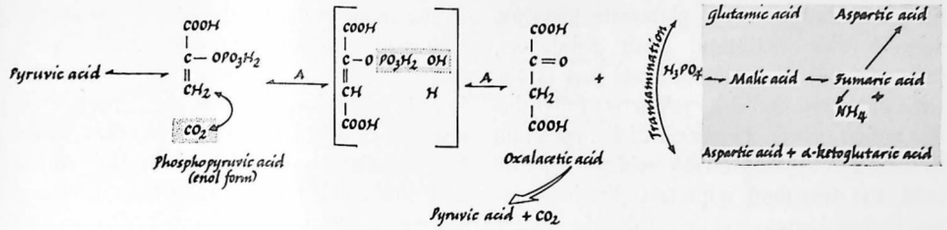


FIG. 10-3. *Oxalacetic acid decarboxylase and subsequent reactions.*

and a third pathway leading largely to amino acids.

CO_2 to Carbohydrates

The first path of CO_2 conversion to carbohydrate is that shown in Fig. 10-1. This has previously been discussed (p. 163) as a major pathway of carbon in photosynthesis. A second path of CO_2 fixation into carbohydrate is that shown in Fig. 10-2. This too occurs in some types of photosynthesis. Both of these pathways provide important intermediates from which cell constituents may be synthesized.

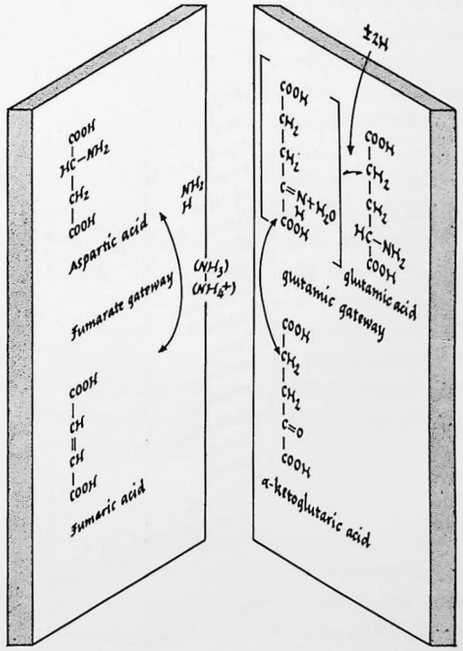
CO_2 to Amino Acids

There is a decarboxylation reaction which is, under suitable conditions, frequent in cells and readily reversible. The catalyst is "oxalacetate decarboxylase." There seem to be several of these enzymes but the one about which most is known carries out reaction A shown in Fig. 10-3. Oxalacetate itself is, of course, part of the citric acid cycle (see p. 174) and the fixation reaction will serve to replace compounds drawn off the cycle for other purposes. But oxalacetate is also a part of an active transamination system (see below) and is closely related in other ways to one of the primary mechanisms of nitrogen incorporation. As such, the oxalacetate formed by this process of CO_2 synthesis usually ends up as amino acids, as shown in the shaded part of Fig. 10-3.

Incorporation of Nitrogen

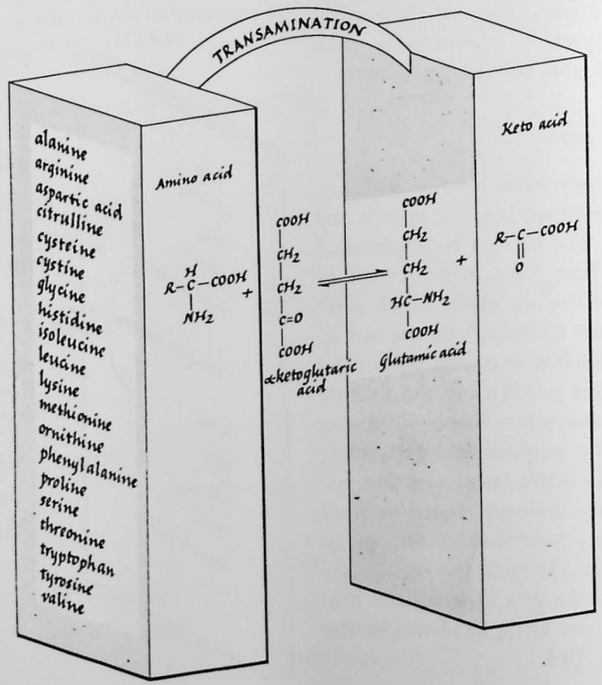
The form in which nitrogen is converted into organic forms is ammonia, in the form of its salts as it exists in cells. There are two gateways whereby ammonia is converted into amino groups and thus to amino acids and to proteins; they are illustrated in Fig. 10-4. One, the **fumarate gateway,** does not involve a reduc-

FIG. 10-4. *The gateways for the conversion of ammonia to amino groups.*



tion, but the other, the **glutamate gateway,** does involve reduction. Both substances, fumarate and α-ketoglutarate, are part of the citric acid cycle and this cycle serves to synthesize amino groups. The result of the operation of these gateways is **glutamic acid** and **aspartic acid,** the two most important dicarboxy (= two carboxyl groups) amino acids (the third is diaminopimelic acid of the cell wall). They differ from other amino acids in several ways and occupy a unique place in amino acid metabolism. We shall encounter them in other connections, but for the moment we may explain that they are the primary source of amino groups for all the amino acids. The general process by which an amino group on the dicarboxy amino acids may be transferred to form other amino acids is called **transamination.** As shown in Fig. 10-5, it consists of the exchange of the amino group for the keto group of the keto acid corresponding to the amino acid. Such keto acids are frequently parts of carbohydrate metabolism. For example, pyruvic acid, a part of carbohydrate metabolism, thus serves to synthesize alanine. Sometimes the amino acid synthesis is more complex than this and the amino group is introduced earlier in the reaction chain, but under most circumstances its ultimate origin is aspartic or glutamic acids. Since these acids can take their origin from ammonia, the introduction of nitrogen, consisting of its conversion from ammonia to amino groups, is achieved.

FIG. 10-5. *Transamination—the bridge between amino acids and keto-acids.*



Introduction of Sulfur

There are two amino acids—cystine and its reduced form, cysteine, and methionine—which contain organic sulfur in a reduced state; there

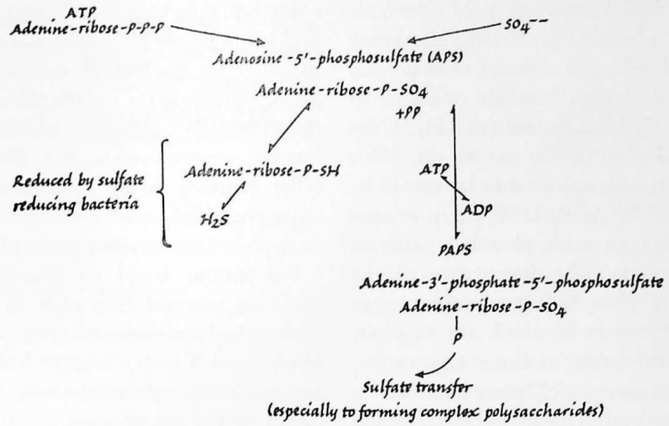


FIG. 10-6. *Pathways for the conversion of inorganic sulfate to organic sulfur.*

are also organic substances which contain the sulfate group. The pathway of sulfate (—SO_4) conversion to sulfhydryl (—SH) has only recently been clarified. We are assuming too that in the microorganisms it is closely similar to the pathway known in higher organisms. It is a particularly instructive pathway because it introduces a metabolic principle which applies to many kinds of synthetic pathways: in many cases a direct synthetic pathway is not used; rather, the substances involved are activated by being combined to carriers and reaction among the carriers accomplishes the synthesis. Sometimes these carriers are adenosine nucleotides (ATP, ADP, etc.), sometimes they are uridine or guanidine derivatives, sometimes they are vitamin derivatives, as coenzyme A; no doubt there are others still unknown. We shall find this **carrier synthesis** frequently enough to consider that it is a fundamental synthetic device. In sulfate conversion we see it quite clearly. It is therefore worth while to spend a little extra time on this reaction series—perhaps more than is warranted by the overall importance of sulfate reduction as such.

The first step is a reaction between inorganic sulfate and ATP to form an adenosine phosphosulfate (Fig. 10-6, where this is called APS). But this substance is not involved in the sulfonation of substances for example, it is not involved in forming the carbohydrate sulfate esters which occur especially in higher cells. To activate sulfate transfer, the cell reacts the APS with another ATP to form PAPS (phosphoadenosine phosphosulfate), which is capable of forming phenol sulfates, steroid sulfates, and chondroitin sulfate, a complex carbohydrate sulfate occurring in animal cells.

But APS can itself be reduced to APSH—equivalent to $SO_4^{--} \longrightarrow H_2S$; this reaction occurs in certain bacteria, which are capable of reducing SO_4^{--} to H_2S. Just how sulfate appears in cysteine is not clear at present, whether APS is reduced to PAPSH, which then reacts with pyruvate and ammonia (or possibly serine or alanine), or whether APS is converted to PAPS, and serine is sulfonated and then reduced is not known.

Phosphate

Organic phosphorus compounds found in living cells are all phosphates, organically esterified, of course. But there is never the reduction that one finds in —SO_4 to —SH, for ex-

ample. The initial conversion of inorganic phosphate to phosphate esters occurs during oxidation, either by the removal of hydrogen, as in 3-glyceraldehyde phosphate oxidation in the Meyerhof-Embden system (p. 154), or by the passage of electrons to oxygen (p. 176). Once formed the phosphate may be carried by nucleotides (ATP, ADP, UTP, etc.) or may be converted to an ester phosphate such as glucose-6-phosphate. The importance of the organic form of phosphate is twofold; one, as the actual substances involved, for example, RNA, DNA, the other, as the energy carrier, the function of energy-rich phosphate.

More Complex Synthesis

The cell has a variety of substances within it, all related and interrelated and all under some degree of harmonious control. The cell must make certain amounts of cell wall, certain amounts of membrane substances and nuclear materials, interlaced with fats, proteins, complex carbohydrates, not indiscriminately mixed but controlled, organized, and directed. The number and kinds of synthetic pathways are many and they are by no means all similar even in principle. For our purpose we shall take three examples of synthetic pathways, partly because their underlying nature is somewhat different, partly because their products are unique and important, and partly because the method employed to study these pathways are different and can be instructive to us. For illustration, we shall choose the formation of aromatic compounds from carbohydrate, the synthesis of RNA and DNA, and the synthesis of fatty acids. DNA and protein synthesis are discussed later, in Chapter 11.

Aromatic Ring Synthesis

There are three commonly occurring amino acids which contain **aromatic ring** structures: phenylalanine, tyrosine, tryptophan. There is a further ring compound, para-aminobenzoic acid. In the early 1950's Dr. B. D. Davis began to study mutants of *E. coli* which required these substances for growth. He obtained mutants which required phenylalanine, or required tyrosine, or tryptophan, etc. He then found other mutants which required tyrosine and tryptophan and some mutants requiring tyrosine, phenylalanine, and tryptophan.

For reasons based on genetic analysis, it could be assumed that each of the mutants studied had only one synthetic deficiency (a **block**), and if such a mutant had two requirements, as phenylalanine and tyrosine, this meant that a precursor common to both was missing. As shown in Fig. 10-7, we have the

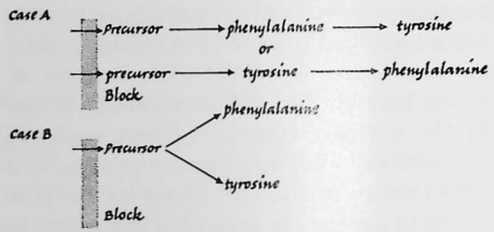


FIG. 10-7. *Possibilities for double-requiring mutant with only one genetic deficiency. Block means that the enzyme system forming the substance is deficient.*

possibilities listed in A or B. In A, if we are allowed only one synthesis block and it comes before the precursor, then the cells should require for growth either tyrosine or phenylalanine, not both. The situation must, therefore, be that of B, i.e., there must exist a precursor common to both phenylalanine and tyrosine which arises by independent pathways beyond this point. If, however, in the block as indicated in Fig. 10-7, we supplied the precursor, then the cell should be able to grow without added phenylalanine or tyrosine since it can readily make either from the precursor substance. Notice that it is possible, if we could identify the

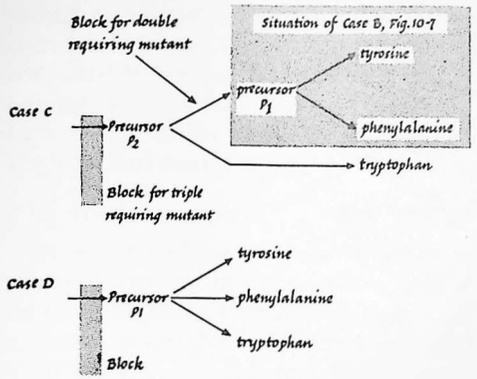


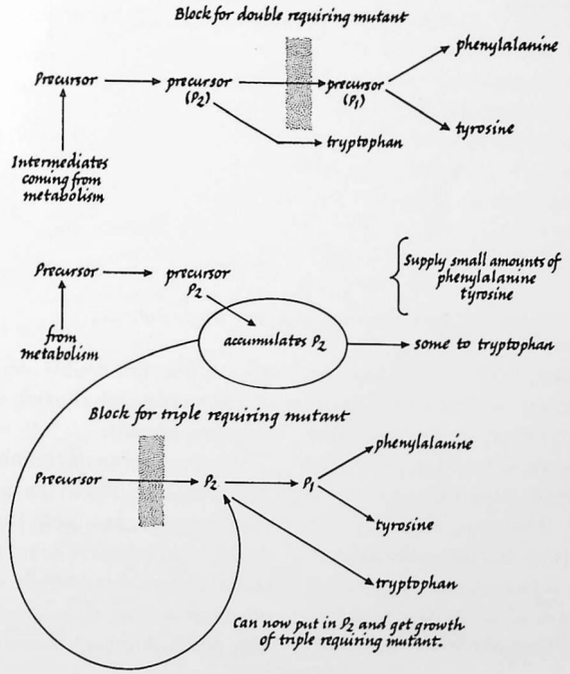
FIG. 10-8. *Possibilities for triple-requiring mutants with only a single genetic block.*

precursor, to trace the pathway of synthesis of phenylalanine and tyrosine.

Of course we could look empirically for related substances and eventually might find the "precursor" substance. But Davis was more ingenious than this. He used triple-requiring mutants. Here, allowing only one block, there could be a common precursor (P_2) for all three substances (situation C, Fig. 10-8). If the situation were as at D in Fig. 10-8, a block in formation of P_1 should not result in a double-requiring mutant. There should be no such mutants, yet we can obtain them. Therefore, the situation must be as at C, Fig. 10-8. It would now be easier to look for P_2 than P_1.

But, after all, the intermediates coming up to make P_2 are all intact, and if we have a double-requiring mutant having a block between P_2 and P_1, then this mutant should accumulate P_2. This situation is illustrated in Fig. 10-9. As such, the P_2 might even be excreted into the medium. This would help to identify

FIG. 10-9. *The use of an accumulation of an intermediate in one mutant to "feed" another mutant blocked earlier in the reaction chain.*



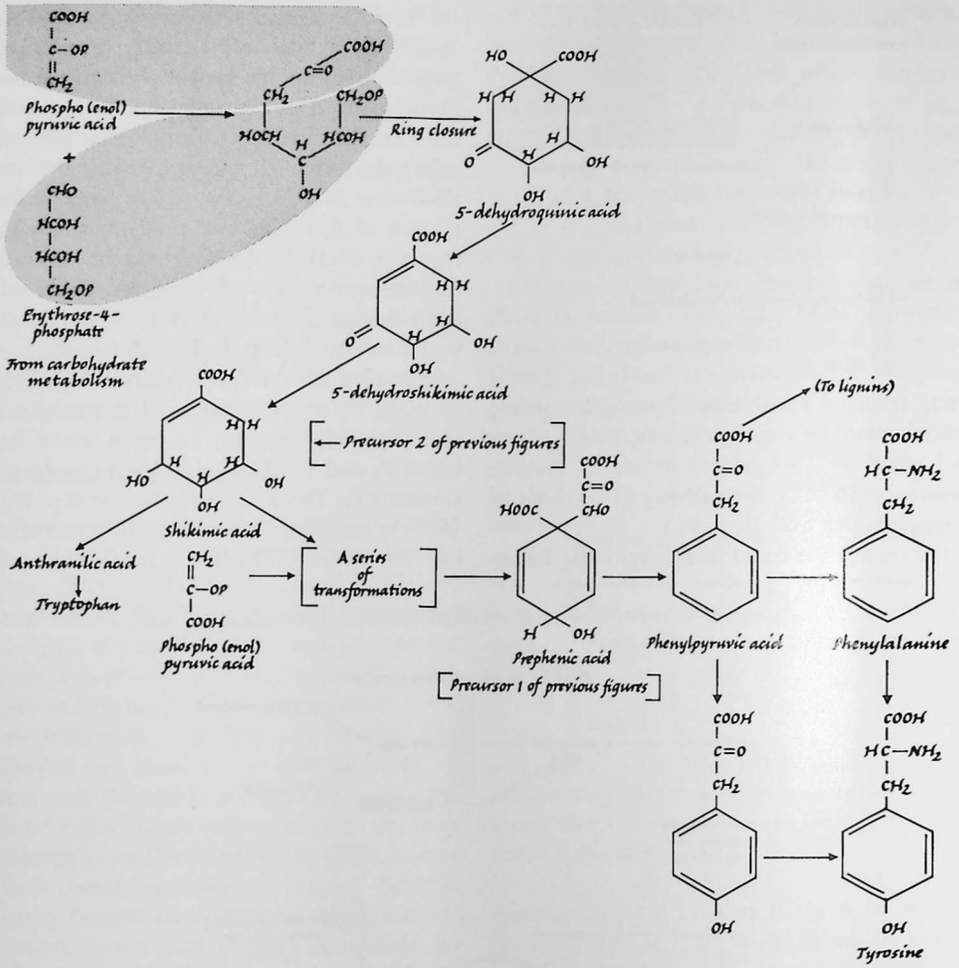


FIG. 10-10. *The pathway of biosynthesis of aromatic ring substances.*

and isolate P_2. Further, if the double-requiring mutant were grown on limited quantities of phenylalanine and tyrosine, it might accumulate P_2, and could serve to *feed* (i.e., to permit growth of) the triple-requiring mutant, as shown in Fig. 10-9. Therefore, not only does this provide a chance of tracing pathways, provided we have some assurance of only one block in each mutant, but it also provides us with a method of preparing the unknown intermediate (P_2), using the double-requiring mutant, and of estimating and assaying it, using the triple-requiring mutant.

When we get to quadruple-requiring mutants with only one block the possibilities become more complex, but using these, combined with double- and triple-requiring mutants, Davis and his collaborators evenutally worked out the path of aromatic ring structure synthesis shown in Fig. 10-10. Although knowledge of its fundamental origin from sedoheptulose requires the use of isotopes, and the pathway outlined was confirmed by isotopic tracers, in the main the outlines of the synthetic pathway were derived from studies of the growth requirements of singly blocked mutants.

The Synthesis of RNA

Most of the **ribose nucleic acid (RNA)** of any cell is present in the cytoplasm. Here it exists in two forms. One is of relatively small molecular weight, generally referred to as soluble RNA. The other is of much higher molecular weight and is associated with a particle containing proteins and other substances which can be centrifuged down in high-speed centrifuges. In animal and plant cells these particles are called **microsomes.** In bacteria and yeasts they are called **ribosomes.** Other microorganisms have not been extensively studied.

RNA, as it occurs in nature, is composed of four bases—adenine, guanine, uracil, and cytosine—four molecules of ribose, and four phosphates. Their linkage is:

base-ribose-P
|
base-ribose-P
|
base-ribose-P
|
base-ribose-P

In 1955 it was discovered that RNA could be made from the enzymatic condensation of the diphosphonucleotides, i.e.,

adenine-ribose-P-P = (ADP)
guanine-ribose-P-P = (GDP)
uracil-ribose-P-P = (UDP)
cytosine-ribose-P-P = (CDP)

An enzyme was obtained from *Azotobacter vinlandii* which, when given an equimolecular mixture of ADP, GDP, UDP, and CDP, would make an RNA very much like that of the *Azotobacter*. The highly purified enzyme still has RNA tightly bound to it and it may be presumed that this "bound" RNA serves as a template in controlling the condensation of the four dinucleotides so the RNA ends up with the adenine, guanine, uracil, and cytosine in the proper order. If this enzyme is given only ADP it makes an RNA having only adenine in it, i.e., an adenine polynucleotide. The purified enzyme system synthesizes RNA or polynucleotides much more rapidly if a small amount of RNA is added; the polynucleotides serve as agents for the growth of RNA chains by the successive addition of mononucleotide units. It appears, therefore, that once the diphosphonucleotides are synthesized in the cell, they can be condensed enzymatically to RNA, the specific nature of the RNA formed being under the control of the cell.

Synthesis of Fatty Acids

As a product of the metabolism of glucose and the decomposition of pyruvate, a substance acetyl CoA was formed (see Chapters 8 and 9, especially Fig. 9-2). This substance is involved in both the synthesis and breakdown of fatty acids but the pathways of synthesis and those of degradation are not exactly the same; moreover, there are some exceedingly complex transformations involved. To understand the principle, however, we may look more closely at

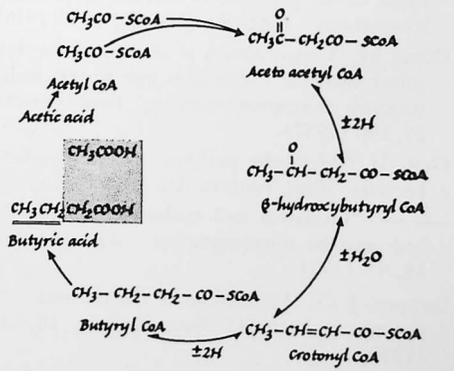


FIG. 10-11. *Pathway of synthesis of butyric acid from acetic acid.*

the less complex stages, those involving only a few carbons, recognizing that as we go up the scale and deal with fatty acids of longer and longer chain lengths, there will be other reactions and reaction mechanisms added.

The simplest case is that of the synthesis of butyric acid from acetic acid, as illustrated in Fig. 10-11. Acetic acid is converted to acetyl CoA or the acetyl CoA is taken directly from other phases of metabolism. Two of these are condensed to form acetoacetyl CoA. This is reduced to β-dydroxybutyryl CoA, from which water is removed to form crotonyl CoA. This in turn is reduced to butyryl CoA, which can form butyric acid. By this process the chain length of the fatty acid has been increased by two carbons. The breakdown can proceed in essentially the reverse fashion, i.e., butyric acid to butyryl CoA to crotonyl CoA, etc. The breakdown of fatty acids for higher members of the series, C_6, C_8, C_{10}, etc., is essentially the same process—conversion to their CoA derivatives and oxidation at the CoA end of the molecule to remove two carbons at a time. But synthesis of the higher fatty acids is frequently somewhat more complex.

The important point in these transformations is that it is not the free fatty acid whose synthesis or degradation is involved but its CoA derivative. The CoA seems rather to serve as an agent whereby these substances are rendered capable of reacting.

General Syntheses

There are a variety of mechanisms for synthesis and those illustrated here are only typical examples. But we can state with some degree of assurance that we will expect to find ATP or its derivatives or various coenzyme complexes involved in synthesis and that we should, as a matter of course, look first for these in any synthesis we may be studying. ATP is the general currency of the cell; we will naturally expect it to be employed in such cases where synthesis is to be accomplished.

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For background information, see p. 492.

QUESTIONS

1. Define or identify:

synthesis	sulfhydryl group
decarboxylation	APS
carboxylation	PAPS
CO_2 fixation	aromatic ring
oxalacetate decarboxylase	blocked mutant
transamination	precursor
glutamic gateway	soluble RNA
fumarate gateway	polynucleotide
	acetyl CoA

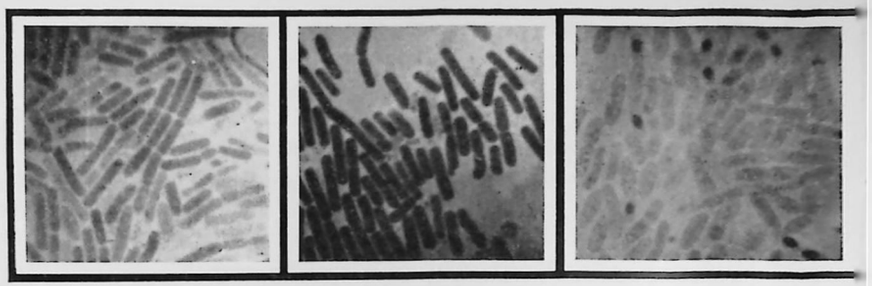
2. The text claims that oxygen taken up in respiration usually appears, not in the oxidized product, but in the water. What kind of a respiratory mechanism will account for this?

3. You have isolated a microorganism which oxidizes fatty acids. It has a "growth factor" requirement whose nature is still unknown. What vitamin would you suspect is required for growth? Why?

4. The text describes an enzyme from *Azotobacter* which is capable of forming polynucleotides. These require a "primer," i.e., a small amount of RNA must be added. Aside from any debate as to whether this particular RNA synthesizing system is the one within the cell which really makes RNA, how would it be possible for DNA (= the gene) to control the synthesis of RNA?

5. You have isolated three mutants, all of which require niacin. In mutant A only niacin will permit growth. In mutant B growth is also possible if tryptophan is supplied. In mutant C tryptophan and indole will also support growth. What kind of a synthetic pathway leading to niacin could you propose on the basis of this data?

Chapter 11



REPRODUCTION AND GENETICS

"Admitting that vital phenomena rest upon physico-chemical activities, which is the truth, the essence of the problem is not thereby cleared up; for it is no chance encounter of physico-chemical phenomena which constructs each being according to a pre-existing plan, and produces the admirable subordination and the harmonious concert of organic activity . . . There is an arrangement in the living being, a kind of regulated activity which must never be neglected, because it is in truth the most striking characteristic of living beings."

BERNARD, *Experimental Medicine*

THE DIRECTIVE FORCE that makes like of like for generation to generation—the force of heredity—is present, too, in the simplest of cells, the bacteria. But this force is essentially a conservative force, and while it is the means of change, the directive force tends to resist change. So, too, we find in the bacteria this constancy, this resistance to change. It is the purpose of this chapter to examine the nature of and the possibilities for change.

Because we shall be discussing the mechanism and means of change, you may have the impression that microorganisms are "unstable"; they have these properties today, and another set tomorrow. This is not the case. We have today strains carried for as long as fifty years

in artificial culture (transferred perhaps once a week, but usually less often) which are still the same as originally described. The vast majority of cells of a species in a given environment will have the same properties. However, change does occur, and two types of change are possible. These we shall call modifications, meaning that they are temporary, reversible, and "adaptive," and mutations, meaning that they are permanent and lasting.

Modifications

It is easiest to understand what we mean by **modification,** or adaptation, by considering some concrete examples. They cover virtually all of the known cases of modification, and seemingly unrelated phenomena can usually be fitted into one or another of the modification classes. Their common character is that the changes are temporary; restore the original condition and the organism is restored to its original properties.

CASE I. MODIFICATIONS IN MORPHOLOGY. When examining the same organisms grown in different media (see Fig. 11-1), you may see differences in cell size and character. Or, examining the same organism in the same medium at different stages of growth, you will see different morphological types, the cells being larger (and more "deformed") during the late lag than during the log phase. But there is adequate evidence to demonstrate that cell division is more sensitive to mild toxicity than is cell growth, and that synthesis of protoplasm and cell membrane is not always exactly synchronous with cell wall synthesis; these differences will cause the same bacteria to appear in different forms. But, transfer the altered form to the media without toxicity and the new growth reverts back immediately to the "normal" cell type. The change is temporary.

CASE II. CHANGES IN STAINING PROPERTIES. It is commonplace, of course, for the Gram reaction to vary—with age of cell or with a variety of other conditions. These variations seem to be due to the presence or absence of an unknown material (magnesium ribonucleate?) which seems to bear some relation to this reaction. But other variations in staining properties are known—see Fig. 11-2. Young cells usually stain more uniformly than older cells.

CASE III. "ADAPTIVE" OR "INDUCED" ENZYMES. What is more impressive, perhaps, and more typical of the variations found has been called **adaptive** enzyme formation. For example, *Leuconostoc mesenteroides,* when grown on glucose, will ferment glucose (i.e., produce acid and gas from it) but not lactose. When grown on lactose, the bacterial cells will ferment both glucose and lactose (Fig. 11-3). The ability to ferment lactose is called "adaptive"; it is only present when the organism has been grown in (or exposed under appropriate conditions to) lactose. The glucose-fermenting system is called **constitutive,** since it is always present. Adaptive enzymes, therefore, are enzymes which are present only when the substrate is present in the growth medium. Constitutive enzymes are always present in the cell.

Four further points need mention. First, it is not an entirely new system of fermentation using lactose which develops in the *Leuconostoc* when grown on lactose. What develops (and what is adaptive about the system) is just the pathway from lactose to glucose-6-phosphate (it happens to be quite complicated, so we shall not specify it precisely). But glucose + ATP forms glucose-6-phosphate, which goes to pyruvic acid by steps earlier discussed. Lactose forms glucose-6-phosphate also, and the cell doesn't care where the glucose-6-phosphate came from; it handles it by the same pathways. Therefore, it is not the whole fermentation

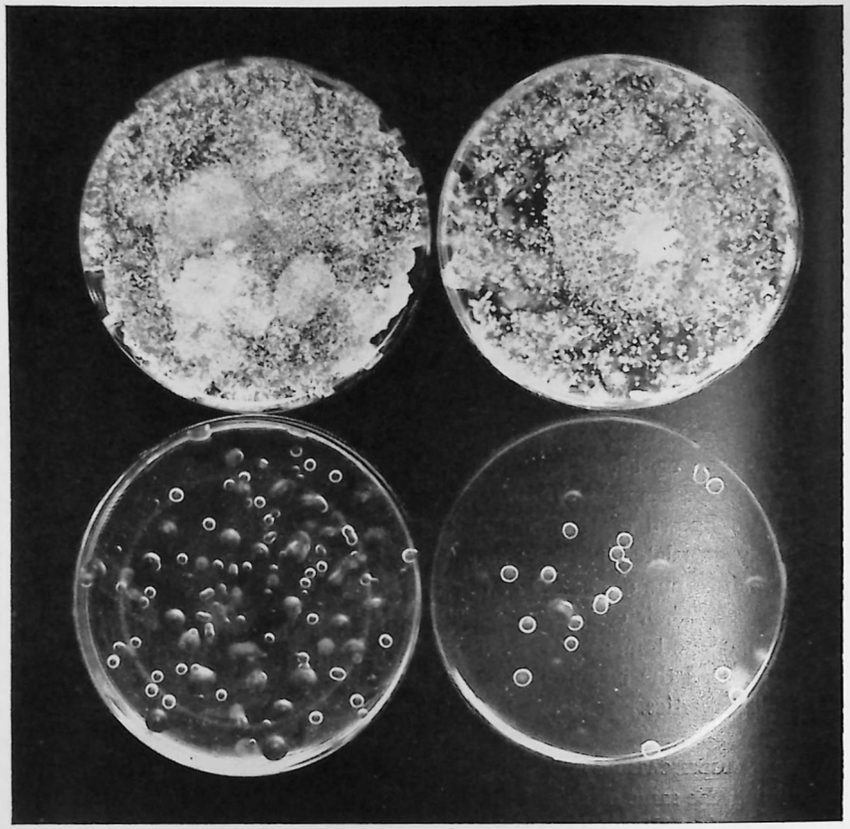
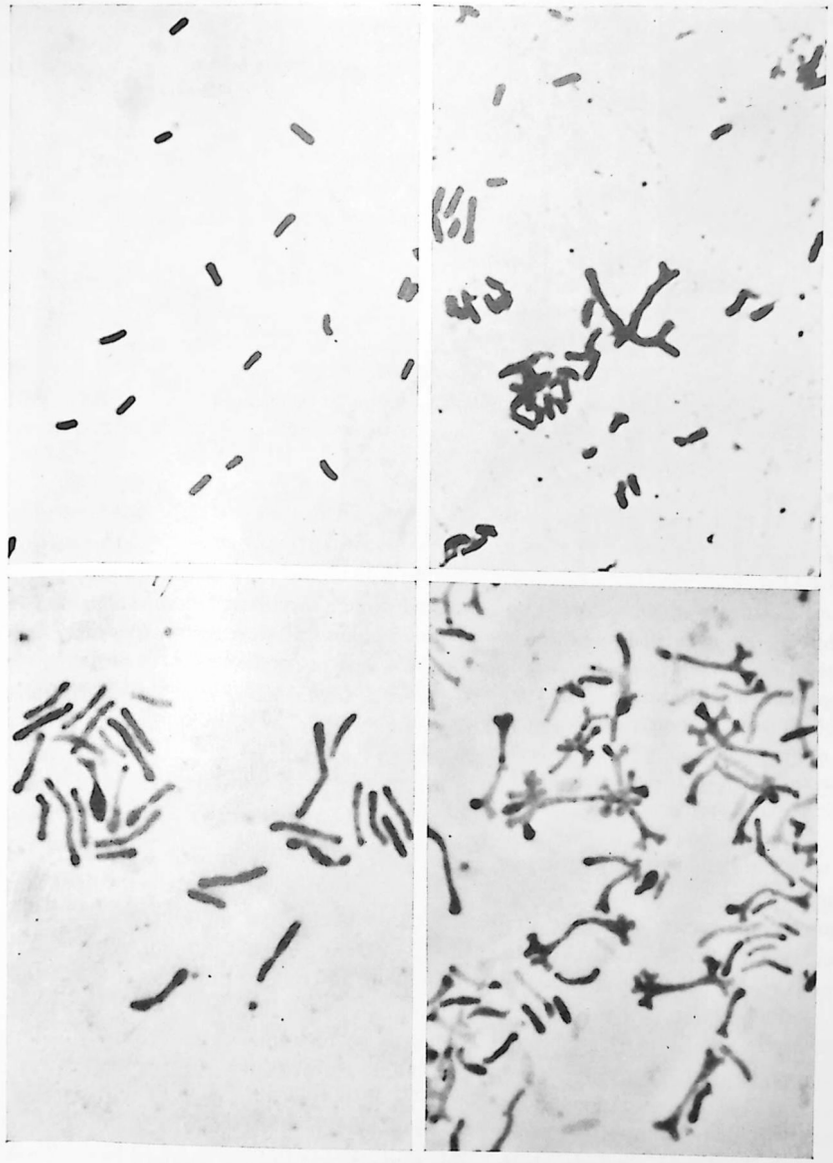


FIG. 11-1. *The influence of the medium upon bacterial cells. Above, the growth on 10% sucrose agar inoculated with a 1/100 (left) and a 1/000 (right) dilution of soil. To the bottom pair of plates 0.005% sodium azide has been added. This has eliminated most of the organisms, but the remainder develop large gummy colonies.* [*From Mayeux and Colmer,* J. Bact., **81,** *1009 (1961). Courtesy of Dr. A. R. Colmer.*] *Opposite page, the two top photos show the same organism* (Butyribacterium rettgeri) *at the same age (3 days), the culture on the right having been grown on a complex medium containing ascorbic acid and lactate while the one on the left was grown on tomato juice agar. Note the distinctly different morphology on the two media. The two bottom photos are of another organism grown on exactly the same medium as were the cells at the top. These are intestinal organisms of uncertain classification and variable morphology, frequently called bifid bacteria. Note that tomato juice medium promoted variable morphology in both the right-hand cultures.* [*Photos on opposite page from Sundman, af Björksten, and Gyllenberg,* J. Gen. Microbiol., **21,** *371 (1959). Courtesy of Dr. Veronica Sundman. Photos taken by Mr. af Björksten.*]



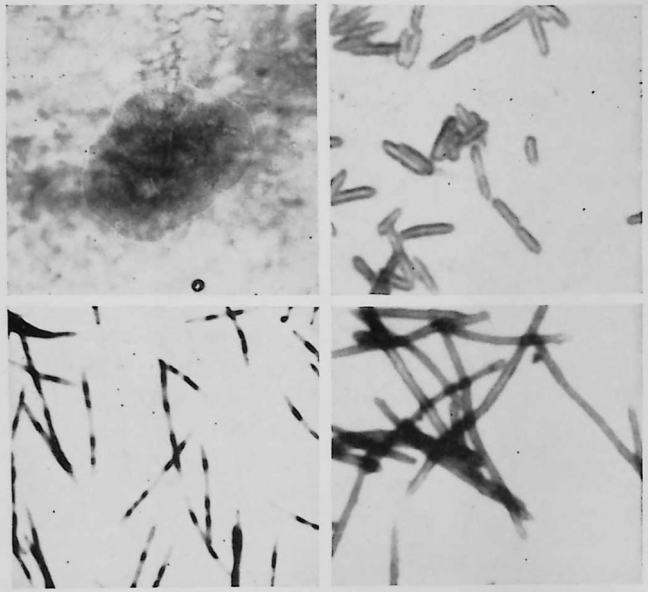


FIG. 11-2. Fusobacterium. *The photographs are picked because they show a curious alteration in morphology depending on the stain used and also to introduce* Fusobacterium, *characterized by cells having "pointed ends"; compare photo lower left. This is a stain with carbofuchsin to demonstrate intracellular granules and pointed ends. At the lower right is the same organism stained to show its cell wall, which gives it a distinctly different appearance. At the upper right is a cell wall stain of a different species of* Fusobacterium, *whose ends look more pointed in other stains. The upper left is a colony of the species shown in the upper right.* [*From Baird-Parker,* J. Gen. Microbiol., 22, 458 (1960). *Courtesy of Dr. A. C. Baird-Parker.*]

mechanisms which are adaptive, only the preliminary maneuvers.

Second, some people prefer to call the kind of adaptive enzyme we have described an **induced enzyme,** i.e., one that appears only in the presence of the substrate (or in the presence of molecules so similar to the substrate as to be mistaken for it). There are, as described in the next section, adaptations to environment (not just to the substrate); hence "adaptive enzymes" should include both changes, while "induced enzymes" should refer only to those induced by a substrate. However, at present there is no clear agreement, and "adaptive enzyme" may mean either kind of adaptation.

Third, the last paragraph implies that one does not need to have growth for the cell to synthesize adaptive enzymes. A cell suspension of *Leuconostoc* grown on glucose and then placed on lactose will not ferment it, even after many hours of contact. But if such a cell suspension is given a little glucose (for energy) and a group of amino acids (from which it can build new protein), the suspension can, after an hour or two of contact with lactose, begin to ferment it. The point is that it is now able to synthesize the new induced enzyme out of the amino acids supplied, even though the entire cell did not grow. Incidentally, studying the rate of adaptive enzyme formation is a convenient way of studying the rate of protein synthesis.

Fourth, we have, in our discussion, taken

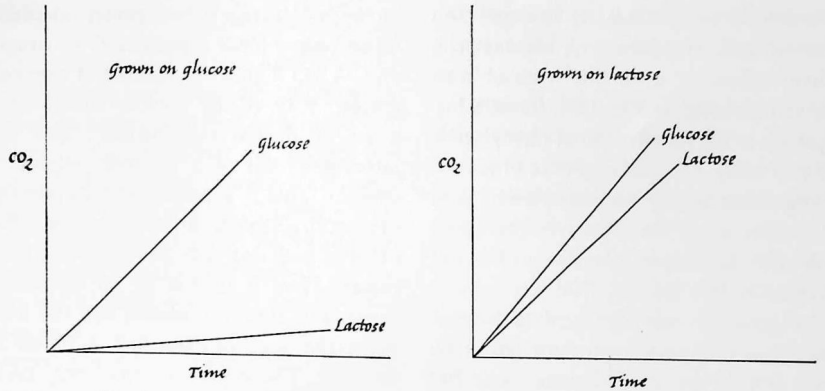
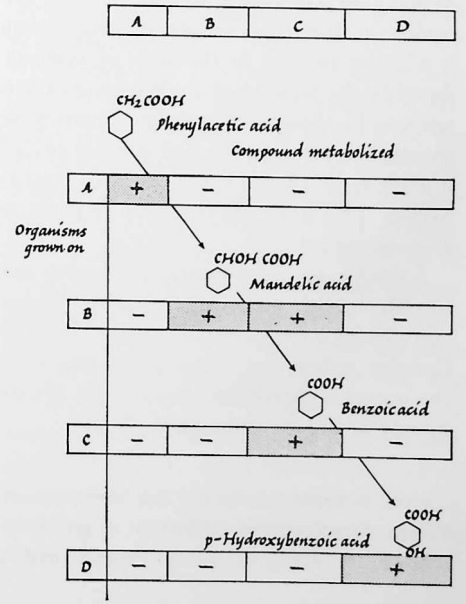


FIG. 11-3. *The production of carbon dioxide from glucose and lactose by cells of* Leuconostoc mesenteroides *grown on glucose and lactose.*

only one enzyme system (lactose fermentation) and only one organism (*Leuconostoc mesenteroides*). This was done for purpose of simplicity and you should not conclude that adaptive enzyme formation is a rare or unusual phenomenon. Not only are many sugar fermentations adaptive, but enzymes concerned with breakdown of amino acids, of aromatic compounds, of penicillin, etc., may be adaptive. Further, an enzyme may be constitutive in one organism and yet be adaptive in another. One rather interesting observation on adaptive enzymes permits us (under certain circumstances) to trace metabolic pathways. If we have a substance A, whose metabolism requires several steps before it reaches a constitutive type of intermediate, and if all of these steps are adaptive, then it is frequently possible to trace that path by cultural methods. If the reaction is A to B to C to D, and D metabolism is constitutive, then organisms grown on A should be able to attack B and C and all organisms should attack D. The reason for this is that when the organism is grown on A, it must make some B, since we postulate that B is the product of the enzyme acting on A. Some B must now be in the medium (or the cell), and since the enzyme attacking it is also adaptive, the enzyme attacking B is formed. We can then, under some circumstances, tell what B is by the following method. (We use an actual experimental case.)

Pseudomonas fluorescens can grow on a vari-

FIG. 11-4. *The use of adaptation to trace metabolic pathways. (See text.)*



ety of aromatic compounds. It can grow on phenylacetic acid, mandelic acid, benzoic acid, and *p*-hydroxybenzoic acid; this series of compounds, as illustrated in Fig. 11-4, forms a logical sequence in the metabolism of phenylacetic acid. If this really were the sequence of metabolism, organisms grown on phenylacetic acid should oxidize all of the others in the series. They do not; hence phenylacetic acid is not metabolized in this fashion. Still, the scheme might be good for mandelic acid to benzoic acid; but it is not, since organisms grown on mandelic acid do not attack benzoic acid. But those grown on benzoic also attack mandelic, hence there appears to be a **simultaneous adaptation** (as this process is called) to mandelic acid when cells are grown on benzoic acid, which is indicative of a pathway between them. Finally, benzoic acid is not the precursor of *p*-hydroxybenzoic acid.

CASE IV. ADAPTATION TO ENVIRONMENT. Many strains of *Escherichia coli*, when grown under anaerobic conditions, will not oxidize acetate. When they are grown aerobically, they will oxidize acetate. The acetate-oxidizing system is adaptive but not in the sense of being induced by the presence of a substrate, since acetate may be added to the medium for anaerobic growth but the organism will still not oxidize it when such cells are later permitted access to oxygen. This is an adaptation in response to an environment.

A similar case is represented by *Bacillus subtilis* which, when grown anaerobically, will convert glucose to pyruvic acid via the Meyerhof-Embden system, but when grown aerobically the organism metabolizes most of the glucose by way of the monophosphate shunt.

CASE V. VARIATION IN COLONY MORPHOLOGY. When *Streptococcus salivarius* is grown on sucrose, it forms a heavy capsule and a "smooth" colony. When grown on glucose, it forms only a small capsule and a "rough" colony. When it grows on sucrose, it uses only the glucose part of the sucrose molecule (composed of glucose and fructose) and piles up large quantities of a fructose polymer in its capsule. This is a particularly important case of smooth $\rightleftharpoons$ rough variation since we shall find other smooth $\rightleftharpoons$ rough variations due to genetic causes. Here it is due to an environmental cause. The organism always has the ability to make the large capsule, but it needs sucrose to do it. The action is completely reversible. Give the cells sucrose, they make the capsule and are smooth; withhold the sucrose, they do not make the capsule and are rough.

Summary of Modifications

Within the five cases cited, one is able to fit most, if not all, of the modifications or "adaptations," changes which are temporary and reversible. These are all a response to a changed environment or condition; restore the original condition and the organism reassumes its original properties. They have been called **continuous variations** in contrast to **discontinuous variations**, which have a genetic origin. But there is another aspect which we should mention. For example, when *Streptococcus salivarius* is placed on sucrose (Case V), all of the cells form capsules, not just one in many as would be the case in a genetic variation. Modifications, then, can be characterized by the following properties.

1. They are a temporary response to a changed condition or environment.
2. They are readily reversible. Restore the original conditions and one restores the original properties.
3. They involve all the cells in a culture. All of the cells respond to the change. This is called continuous rather than discontinuous variation.

Mutations

The permanent and lasting changes which occur in bacterial and other cells are known as **mutations.** In higher cells they have to do with alterations in the genes and this subject may be approached from the viewpoint of animal and plant genetics. If you have had training in genetics, this is helpful and many of the concepts carry over directly. But bacteria are too small to see a dividing nucleus and the chromosomes in it.

However, the subject of mutation can also be approached on simply an observational basis, without concern for genetic theory or the relation to Mendelian inheritance. It is perhaps best to approach it in this fashion even if you are familiar with the genetics of higher organisms. We therefore begin a discussion of mutation without reference to genetics and shall develop the notions of bacterial mutation and bacterial genetics from an experimental viewpoint. Later in the discussion, we shall develop these concepts of genetic theory which seem best fitted to explain the experiments.

EXPERIMENT I. DEVELOPMENT OF RESISTANCE TO STREPTOMYCIN. One organism, *E. coli*, has been cultivated in the laboratory for years. It will not grow in its usual medium when this medium contains 10 μg/ml of streptomycin; streptomycin is toxic to it or, to put it in other words, it is **sensitive** to streptomycin. Suddenly, one day we find that it will grow in a tube containing 100 μg/ml of streptomycin ten times as much as previously killed it, and if we transfer from this tube we obtain a resistant strain, one which will always grow with 100 μg/ml streptomycin. It differs from the sensitive strain only in this property. Otherwise, it behaves as a typical *E. coli*. But it is now "permanently" resistant. Is this an adaptation, or a mutation? Has it occurred in all cells, or in only one? Is it caused by the presence of streptomycin or did the streptomycin only select out, say, one organism in a million which suddenly mutated?

There are two experimental ways in which these questions can be answered. The first of these is illustrated in Fig. 11-5 and is called the **fluctuation test,** but the method is much simpler than the name implies.

From a test tube of medium, we inoculate with a strain sensitive to streptomycin, for example, 10 ml of medium (Fig. 11-5, A), and after the organism is grown for a definite interval, 0.2 ml quantities are plated into each of 50 plates of medium containing streptomycin (at 100 μg/ml) on which, of course, only those organisms resistant to streptomycin will grow. If there has been an adaptive change in the entire culture, growth on the streptomycin will be high. If there has been a mutation, and only one cell became resistant, this mutant could grow in the tube (A) and its progeny would become all mixed up with the rest of the cells; then each 0.2 ml of medium might have 3 or 4 resistant cells.

At the same time, we inoculate a similar 10 ml of medium in the same fashion, but instead of allowing the cells to grow in the 10 ml, 0.2 ml portions are immediately removed and placed in 50 sterile test tubes (Fig. 11-5, B). Each 0.2 ml medium now is by itself. At the end of the incubation period, the entire contents of each tube (0.2 ml) are plated on streptomycin agar. Now if there has been an adaptation, it should occur about equally in all 50 tubes and we would expect to have approximately the same result on the plates, following either procedure A or B. But if there has been a mutation, a drastic change in one cell, this would not occur in all 50 tubes, but only in some of them. While in Part A, these mutants could mix with the rest of the medium, in Part B they could not; only those tubes in which a mutation had occurred would show growth in the medium containing streptomycin. Inciden-

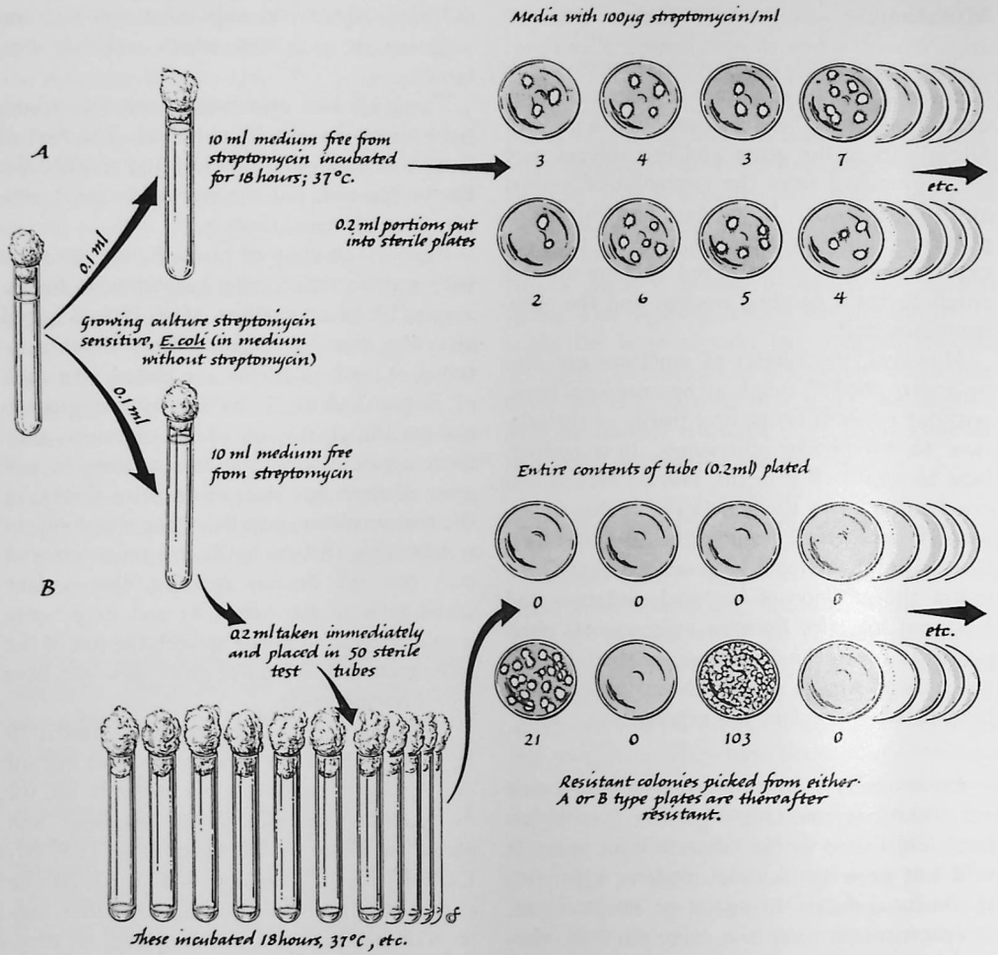


FIG. 11-5. *Diagram of the fluctation test. (See text.)*

tally, the 21 colonies found (Fig. 11-5) would represent a late mutation which had occurred when most of the growth was over, while the 103 colony plate would represent an early mutation, one which occurred when there was still a considerable chance for growth in the small tube. If one picks the colonies from either A or B plates, the cultures are thereafter always resistant to streptomycin.

This experiment, then, tells us that (1) the change occurred in one cell (or a few cells) in the population, and not in all. (2) It was not caused by the presence of streptomycin since none of the media before plating contained streptomycin. The streptomycin was merely a selective indicator to tell us whether or not we had resistant cells.

This experiment provides evidence that the mutation is spontaneous and undirected and that it occurs in only a few members of the population.

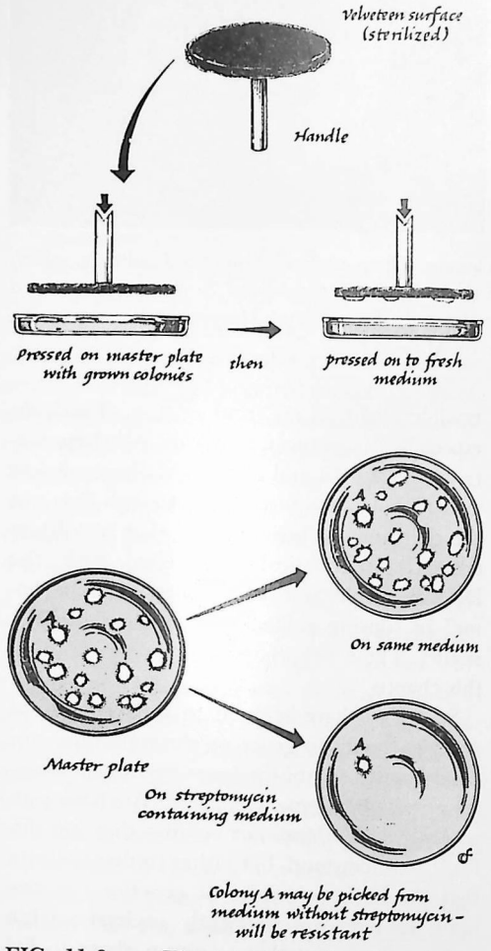


FIG. 11-6. *The principles of replica plating. In the upper half of the figure a sterile disk, covered with velvet or something similar, is pressed down upon a petri dish containing bacterial colonies on the surface. The velvet picks up some cells from the colonies and when pressed to fresh sterile medium inoculates the new plate at exactly the same places where colonies were on the master plate. The lower half shows how this can be used to detect mutants.*

EXPERIMENT II. DEVELOPMENT OF RESISTANCE TO STREPTOMYCIN. There is another method, based on entirely different principles, which can detect mutations. It is called **replica plating**. The procedure is illustrated in Fig. 11-6. If a mutant occurs in any of the colonies on the original "master plate," it will, of course, be confined to this colony; hence this particular colony will be the only one which will contain cells capable of growth when transferred to the drug-containing medium, so only this one colony will grow.

Note how, in this case, it is possible to pick the colony on the master plate (or the no-drug control) corresponding to the resistant colony on the drug plate and thus obtain resistant cells *which have never been in contact with the drug*. Other uses for replica plating will be described later, but at this point we simply point out what this experiment demonstrates. (1) The change to resistance occurred in one cell (or a few cells), represented by the individual colonies. (2) It was not caused by the presence of streptomycin, but occurred in its absence.

EXPERIMENT III. COLONY SECTORING. You may sometimes see colonies like those illustrated in Fig. 11-7, in which a sector of the colony is different from the rest. This results from a mutation at the apex of the sector; early and late mutants can sometimes be distinguished. While these phenomena are not really rigorous enough to be proof of any genetic mutation, they are explainable most easily—possibly *only* —in terms of a mutation. We point them out merely as very simple evidence of mutation which does not require elaborate experiments for observation.

Rates of Mutation

By both replica plating and by the fluctuation test, it is possible to calculate how many cells of a population mutate. From both methods, it comes out that 1 to 3 cells out of every

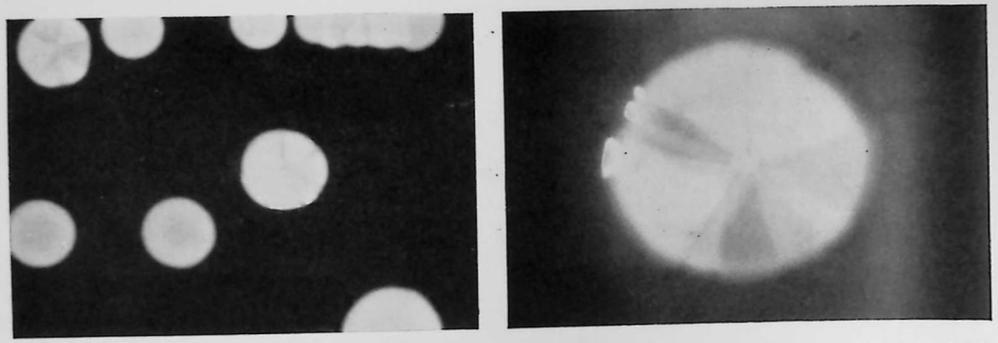


FIG. 11-7. *Sectored colonies of* Staphylococcus aureus. *These are from lyophilized cultures, which seem to promote an otherwise rare occurence. The darker portions correspond to orange pigmented portions. At the left, note that not all colonies are sectored.* [*From Servin-Massieu,* J. Bacteriol., **82,** 316 (1961) *and unpublished. Courtesy Dr. M. Servin-Massieu.*]

10^9 cells, i.e., 1 to 3 out of 1000 million; in the United States we call this a billion. While this varies with the gene, it is reasonably constant, so much so that we speak of *the* mutation rate being 10^9—one cell out of 10^9 cells will be a mutant.

Is this too small to be of importance? Suppose that we have 10 ml of broth inoculated with *E. coli* and grown to the stationary phase. There will be close to a billion cells per milliliter (10^9) or the 10 ml will have 10 billion cells (10^{10}); thus this culture will contain from 10 to 30 mutants. No dense culture is genetically pure. But only when conditions are such that the mutant can outgrow the parent or when a drug such as streptomycin prevents the parent from growing at all, is it possible for the mutant to become an appreciable part of the population. However, sometimes even a slight change in the medium may prove selective and bring about a complete change in the population in a few transfers.

The rate of mutation, 10^9, is, as mentioned, reasonably constant. We know of nothing that will decrease or slow down the rate of mutation. But there are agents which increase the mutation rate—called **mutagens**—which include ultraviolet light, X-ray, and certain chemicals, especially the nitrogen mustards. All these materials react with and alter deoxyribose nucleic acid (DNA). For this reason, though it is not the only one, we believe today that hereditary character is chemically associated with the DNA molecule, and that a mutation is an "error" in copying a DNA, so a different DNA results. These details we will discuss later in this chapter.

So far we have referred to no concepts of genetics, rarely to genes or chromosomes, nor have we used any of the language of genetics—genotype, phenotype, clone, etc. We have purposely avoided them, not because they are difficult to understand, but rather to demonstrate that the basic principles of genetics (or any other science) are frequently understandable without the jargon that grows up about it. Of course this jargon is of some importance; really it is sort of a shorthand. You are now so used to using the terms flagella, spore, growth phase, media, substrate, etc., that you no longer realize that they are part of the terminology of bacteriology, i.e., the jargon of the subject. Admittedly, the introduction of new terms is confusing at the start and this is why we have avoided those terms peculiar to genetics in this initial discussion of bacterial genetics.

Those of you familiar with genetics may wonder why we have taken so much trouble to demonstrate that there are mutations in bacteria. Is this not obvious? Certainly there are permanent changes in bacterial cells, but since we were unable to see dividing nuclei with the detail possible in higher cells it was not at all certain that one was dealing with the same thing. Indeed, some of the aspects of bacterial genetics which we shall go into a little further on are not found in higher cells. It was therefore necessary to provide independent proof that the changes were not only long-lasting, but that they (1) occurred at a constant and determinable rate, (2) occurred independently of the detecting agent, (3) occurred in relatively few cells of a population rather than many cells. These properties are characteristic of mutations in higher organisms, and so the long-lasting changes in bacteria were undoubtedly due to the same cause.

There are, of course, many different types of mutations, and not just those to drug resistance. A mutant usually loses a property possessed by the parent; the mutant may no longer make a pigment or it may lose the ability to synthesize an amino acid. Occasionally a property is gained, as in drug resistance, but usually there is a loss. It is perhaps likely that there should be a loss in function. To use a simple analogy: If you were to throw a monkey wrench into a printing press going at high speed, what are the chances that you would *improve* its operation, rather than harm it?

It is understood, further, that when a mutation occurs, the progeny (the **clone** = all those descendants derived from this cell) have the same properties. The culture "breeds true."

Practical Methods of Detecting Mutants

The main problem in most genetic studies is separating out the mutant. When this is a matter of resistance to chemicals or drugs, this separation is fairly easy, as for example the use of streptomycin resistance to select mutants. But when the mutant is a deficient one—a cell that cannot synthesize an amino acid, for example—then the problem becomes more difficult. One of the most widely used methods for detecting deficiency mutants is based upon a curious property of penicillin; penicillin kills growing organisms by interfering with cell wall synthesis, but not organisms that are not growing. The method works as illustrated in Fig. 11-8. The UV light is used to enhance mutation. The survivors are allowed to grow in an enriched medium containing amino acids, etc. which the deficient mutant might require for growth. The grown culture is then transferred to minimal medium, one on which the parent can grow but on which any deficient mutant might not. As growth of the parent begins, penicillin is added; this kills the parent cells which are growing, but does not kill the deficient cells which cannot grow. The survivors are then transferred to the minimal medium plus an enrichment (without penicillin), where the deficient mutant can now grow.

If the number of mutants is especially high, we may sometimes obtain deficient mutants by replica plating, as illustrated in Fig. 11-9.

Changes in Genetic Construction by Means Other than Mutation

Bacteria differ from higher forms in possessing three processes for changing their genetic constitution which differ from mutation; so far as is known these processes do not exist in animal or in plant cells. These phenomena are called transformation, recombination, and transduction.

Transformation

To discuss this phenomenon, we must take up some still undescribed properties of the

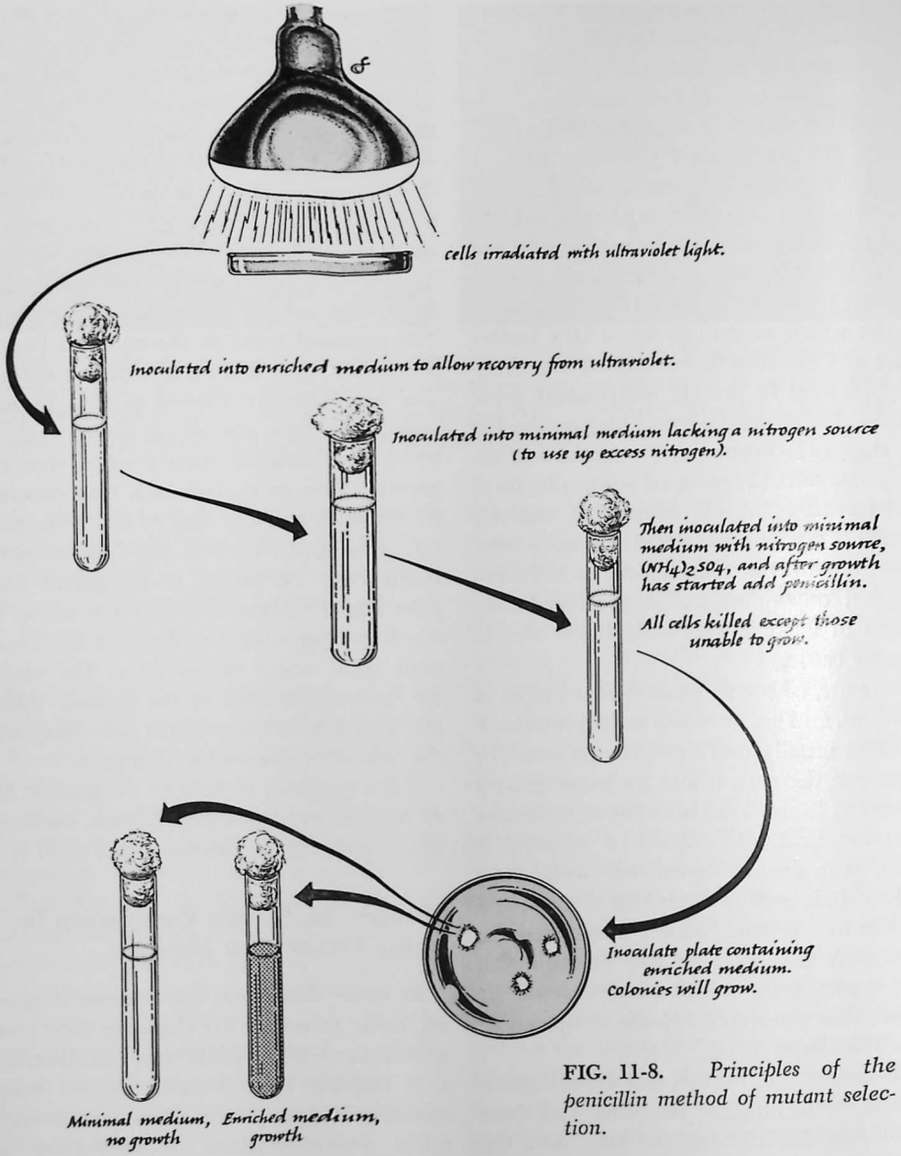


FIG. 11-8. *Principles of the penicillin method of mutant selection.*

Diplococcus pneumonia (**pneumococcus** for short). These organisms possess a capsule, which causes the colonies to look smooth on agar. After cultivation on artificial media they sometimes lose the ability to form capsules; the colonies become rough. Sometimes the rough colonies become smooth again, but most often the change is from smooth (S type or S colonies) to rough (R type or R colonies). This change S → R is called **dissociation.** This property of S or R is a real genetic property and is not dependent upon sucrose in the environment, etc. The capsules of various strains of the pneumococci have a somewhat different composition, and the differences can be detected immunologically. There are Types I, II, III, etc.—today we know at least thirty. When any of the S types become R, the R strains are closely alike. Of the S strains, Types I, II, and III are the most important since they are responsible for most lobar pneumonia. "Typing" of pneumococci was once an important part of hospital laboratory work, since antisera against Type I were not effective against Type II, III, etc. It was therefore important to know which type of pneumococcus a patient was infected with so the proper antiserum could be used for treatment. Today, antibiotics and sulfa drugs have eliminated much of the necessity for "typing."

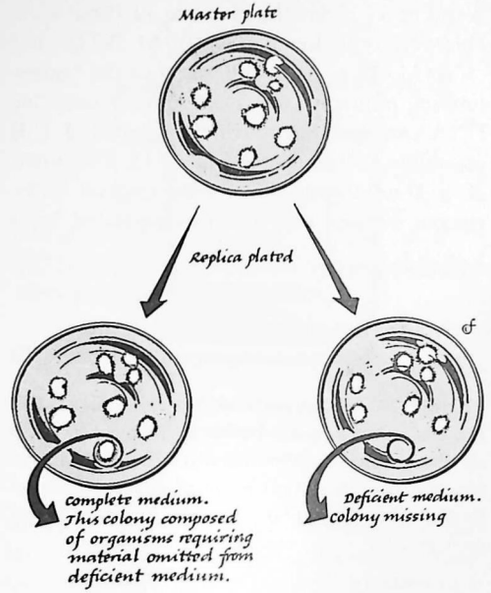


FIG. 11-9. *The replica plating method used to select deficiency mutants.*

If killed capsulated cells of Type I pneumococcus were injected into an animal, the blood would contain an antibody which would react only with Type I pneumococcus and not Type II or with the rough strains. In 1928 it was found that if one inoculated a mouse with live R cells (which were generally not virulent and usually would not kill the mouse) and the mouse died, one usually isolated not R cells from it, but S^{I}. (The S^{I} means a smooth colony with capsules of Type I.) This reversion of R to S^{I} took place in the animal. Of course, this could be explained by assuming that every R colony had a few S^{I} in it, and the conditions in the animal body permitted the S^{I} to grow, i.e., the S^{I} were selected out. This could even be an "I" mutation followed by selection, but in 1928 there was no other kind of evidence for a mutation so this notion was not popular.

Even more interesting was the fact that if one injected live R cells with *killed* S^{I} cells, one could isolate live S^{I} cells. If one injected the same R cells with killed S^{II} cells, one could isolate live S^{II} cells! You may imagine what a buzz this caused. Later (15 years later) it was found possible to carry out this same transformation in the test tube, where there was no question of the animal's selecting anything.

Living R cells + heated killed Type I S cells ⟶ Type I S cells (S^{I})
Living R cells + heated killed Type II S cells ⟶ Type II S cells (S^{II})
Living R cells + heated killed Type III S cells ⟶ Type III S cells (S^{III}), etc.

Either something was coming from the living cells (R) which revitalized the dead cells (S)

or something was coming from the dead cells (S) which converted the living cells (R) to S forms. This something was found to be coming from the S cells and converting the R to S. It was called the "transforming principle." By 1945 it was found to be DNA. This was the first direct evidence that DNA was the ultimate genetic determinant.

Streptomycin-sensitive living cells + DNA from streptomycin-resistant strains ⟶ Streptomycin-resistant living cells

Bacteriophage-sensitive living cells + DNA from bacteriophage-resistant strains ⟶ Bacteriophage-resistant living cells

We may achieve similar transformations, as in the examples shown above.

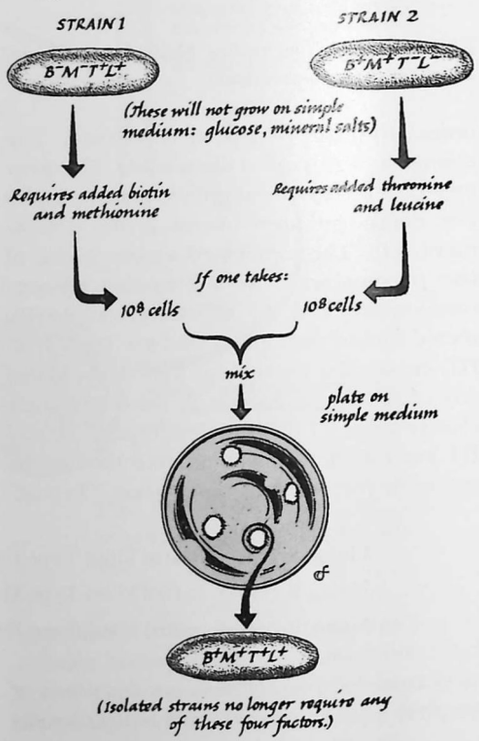
It has now been adequately demonstrated that the phenomenon of **transformation** consists of the absorption and integration of the DNA from a mutant, which processes confer the mutated property upon the nonmutant.

Recombination

E. coli is a gram-negative fermentative rod which grows on a simple medium of mineral salts and glucose; it can synthesize its amino acid and vitamin requirements. A variety of mutants can be obtained from it. For the moment we are experimentally concerned with two such mutants. One of these requires biotin (B^-) and methionine (M^-) for growth. The other requires threonine (T^-) and leucine (L^-) for growth. Neither, of course, can grow on the simple mineral salts-glucose medium. If sufficient numbers (about 10^8) of both kinds of cells are mixed in such a synthetic medium, in which neither can grow alone, between 1 to 200 cells (out of the 2×10^8) supplied will grow, and these cells no longer require any of these factors. This situation is summarized in Fig. 11-10.

When first found, this was supposed to be another example of transformation, but no extracts or heat-killed cells of $B^+M^+T^-L^-$ would convert $B^-M^-T^+L^+$ to $B^+M^+T^+L^+$ (see Fig. 11-10 for the meaning of these symbols). Nor would extracts of $B^-M^-T^+L^+$ act on $B^+M^+T^-L^-$. But still, perhaps the "transforming principle" was a particularly sensitive DNA. An experiment which eliminated this possibility is illustrated in Fig. 11-11. Two arms of a U-tube were filled with mineral salts-glucose medium and the arms separated by a

FIG. 11-10. *Recombination experiment. In this case two mutants, neither of which can grow on the simple medium, are mixed and the combination permits the exchange of genetic material so that the new mutants formed are capable of such growth.*



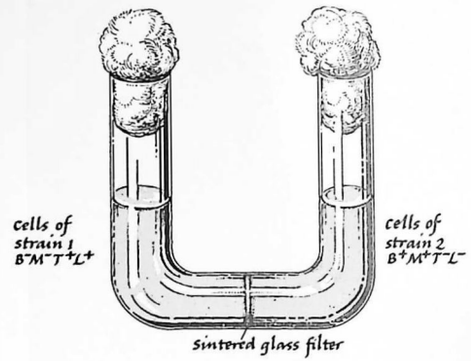


FIG. 11-11. *U-tube experiment which demonstrates that transforming principle is not involved in recombination. (See text.)*

sintered glass filter which prevented the bacteria from passing from one arm to the other. One side was inoculated with $B^-M^-T^+L^+$ and the other with $B^+M^+T^-L^-$ and the media flushed back and forth. If the cells were giving off anything in the medium which would act like a transforming principle, it should be evident under these conditions. But no organisms capable of growth (i.e., $B^+M^+T^+L^+$) were found. If the filter was removed, the recombined organisms were easily detected.

The new strains (i.e., $B^+M^+T^+L^+$) had characteristics of both parents. Perhaps there was a combination of cells. Perhaps this was "sex" in bacteria, or at least gene recombination. If so, there would have to be direct contact between the strains. And this contact has been seen, as shown in Fig. 11-12.

Recombination, then, is the transfer of genetic materials from one cell to another by direct contact, presumably with a movement of DNA from one to the other.

Transduction

While the experiment with *E. coli* described in Fig. 11-11 never succeeded in yielding "recombinants" (i.e., $B^+M^+T^+L^+$) so long as the filter was intact, experiments in 1952 with a *Salmonella* culture did yield positive results. Again it was first supposed that this might be caused by a transforming principle. But it turned out that one of the parent strains possessed a bacteriophage and that the mutant strains had received bits of genetic material from the phage. At this point it is difficult to explain the mechanism of this carry-over since we have not described the curious properties of the bacterial virus (the bacteriophage) or of viruses (see Chapters 28, 29). It will suffice simply to state that in **transduction** the DNA, as material of the gene, is carried by the bacteriophage.

Genetic characteristics are normally transmitted to the offspring through the DNA in the nucleus, being divided equally between the daughter cells. The bacteria have, in addition, three other ways of transmitting genetic material from one cell to another. These are:

Transformation—where DNA in solution can enter the cell and "transform" it.

Recombination—where the cells must be in contact so that the nuclear material may flow from one to another in the protoplasm.

Transduction—where the DNA is carried by a bacteriophage infection.

Note: With regard to recombination we must point out a source of confusion. Some geneticists use the term "sexual recombination" for the phenomena described above, and the term "'recombination" itself to mean any transfer of genetic material and its integration into the genetic machinery of the recipient cell. To these geneticists, transformation, transduction, and sexual recombination are all called recombination and are merely subgroups of a larger process. For our purpose we prefer the three names transformation, transduction, and recombination, and they will so be used in this book. We point out the alternative meaning of "recombinations" so that in your future studies

FIG. 11-12. *Genetic contact in* Escherichia coli. *To demonstrate a real contact between two organisms such that a change of genetic material is possible, one must show more than just juxtaposition of the cells. Here are two cells of* Escherichia coli *of two different strains. One absorbs phage (see Chapter 28) and is here labeled with inactivated phage (note the attachment of the polyhedral particles to the bacterium by their tails). The other strain is resistant to the phage and does not absorb it. Genetic Studies show that characters of the second strain are transferred to the first strain, even in the absence of phage. [From Plate I of Anderson, Wollman, and Jacob,* Ann. Inst. Pasteur, **93**, 450 (1957). *Courtesy of Dr. Anderson.]*

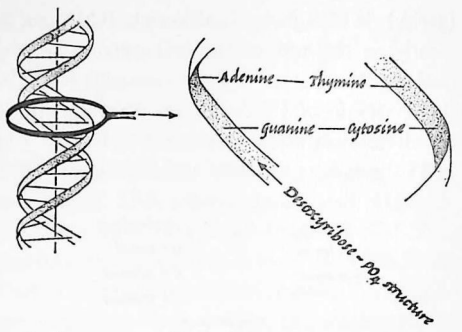


FIG. 11-13. *The unit structure of DNA.*

you may understand that it may be used in a wider sense.

Theory of the Genetic Process

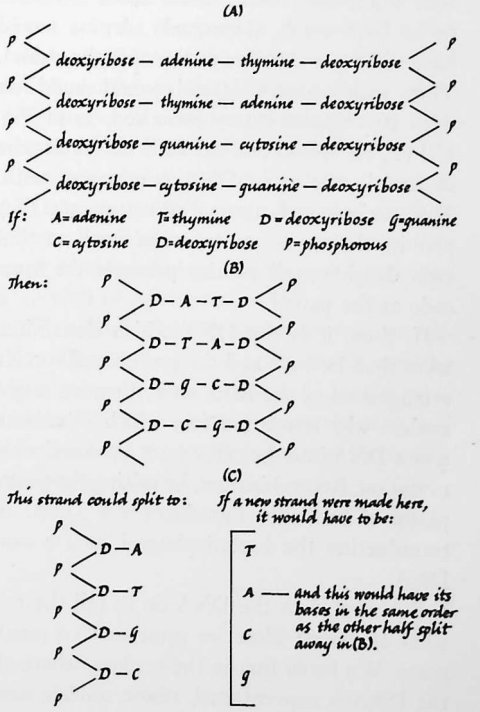
A wide variety of carefully considered evidence can today be brought together to provide a picture of the genetic process. This picture may be wrong in a few details, but it seems, for the most part, to be close to the truth. We present this picture as theory, not because there is not "proof" of it, but because this is not the place where the evidence can be cited.

What modern study has shown is that the hereditary units (called **genes**) which in higher cells are strung in a chain (called the **chromosome**), exist in bacterial cells, also strung on a similar chain, and that in all known cells the genes are composed of deoxyribose nucleic acid (DNA). It is this DNA which had the staining properties by which cytologists could see the chromosome. The DNA does two things. The DNA provides a **code** which "tells" the cell what structures and compounds to manufacture. The DNA also replicates itself so that two daughter cells each have the same code; these cells, therefore build the same substances and structures and are thereby alike.

The chemical structure of DNA and its physical existence in space is such that it provides a chemical means for replicating itself, or for providing the basis on which the cell can make the same kind of DNA. The DNA molecule has generally only four bases: adenine (A), guanine (G), cytosine (C), and thymine (T), and these are paired (Fig. 11-13) across two chains; for each adenine there is a thymine and for each guanine there is a cytosine.

These bases are attached to a sugar—a curious one, deoxyribose—phosphate, giving the structures represented by Fig. 11-14. A certain chain may be like Fig. 11-14, B, C, and presumably the code depends on the arrangement of the bases. That is, if one has a single strand of DNA with adenine-adenine-adenine-cytosine (AAAC), this tells the cell, by a process we shall describe farther on, to make, say, the amino acid histidine. But a DNA with adenine-

FIG. 11-14. *Diagram of the structure and replication of DNA.*



guanine-adenine-cytosine (AGAC) tells the cell to make arginine. We believe today that it is in the arrangement and order of the bases of the DNA that the code lies.

But how does the cell make more of this code? How does it provide DNA, of exactly the right kind with the bases all in the right order, for each of the daughter cells? After all, if mutation, which we regard as faulty copying of the code, occurs only once in a billion divisions, the rest must have divided and transmitted the code correctly.

It happens that the two chains of DNA, which are held together by "hydrogen bonds," are complements of each other, although they are not mirror images. If they were to come apart, as illustrated for one small unit in Fig. 11-14, C, we would have two strands—one ATGC, the other TACG. But if each strand served as the template upon which a new DNA were built, the ATGC would cause a TACG to be built on it, since each adenine would have to have a thymine in the opposite strand. Thus each separate strand would build on itself its complementary form and, as in Fig. 11-14C, we would end up with two molecules of exactly the same DNA one started with. This curious and unusual structure of DNA permits the exact replication of itself so that each daughter cell obtains precisely the same code as the parent.

If, then, it is the DNA which determines what shall be built and the precise and specific arrangement of the bases on it, then we might explain why transformation, which introduces a new DNA into the cell, causes a transmissible mutation. Recombination, by mixing the protoplasm of two cells, introduces new DNA. In transduction the bacteriophage injects a new DNA.

But what does the DNA do to tell the cell what to build? Here we must make a small guess. We know that in the nucleus, where all the DNA is concentrated, **ribose nucleic acid (RNA)** is also formed, although RNA exists mostly in the rest of the cell outside of the nucleus. The guess we have to make is that the single strands of DNA can, on occasion, build on themselves a complementary RNA; Fig. 11-15 shows how the bases and the sugars differ.

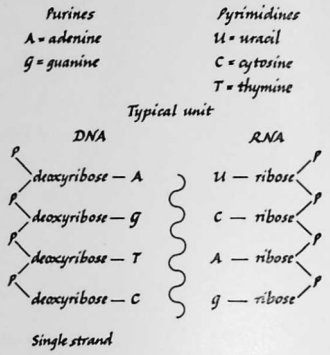


FIG. 11-15. *A possible way to form RNA from DNA.*

It is this RNA made in the nucleus which is coded. At least it has a specific arrangement and order of its bases.

But how does this ordered or coded RNA act to build a cell? Of course, what a cell makes depends on what enzymes it possesses. As all enzymes are proteins, we may ask: Could the coded RNA tell the cell which proteins (enzymes) to make? It could indeed—and it very likely does, most probably in the following fashion.

Proteins are long chains of amino acids, and most proteins have the same amino acids. The proportions of each amino acid may vary, but we believe that it is the order in which they occur which determines the protein. A protein chain starting with alanine-glycine-histidine-alanine-alanine is different (and probably a different enzyme) from one which starts with glycine-alanine-histidine-alanine-alanine.

It has been known for years that RNA had something to do with protein synthesis, but

only within the past few years are we beginning to find out what. There seem to be three steps involved. The first of these is the activation of the amino acid (Fig. 11-16) in which it reacts with ATP to form the adenylo-amino acid. There is a different enzyme activating each amino acid. This adenylo-amino acid, which is somewhat akin to a small fragment of RNA, transfers this amino acid to a small chain of RNA, a soluble nucleic acid complex. We guess that these are each specific, i.e., soluble RNA (type I) will combine with methionine (the

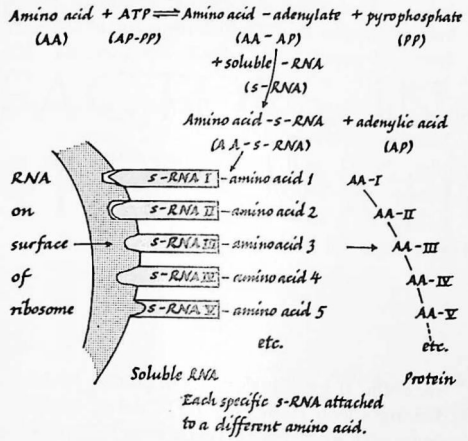


FIG. 11-16. *Conception of protein synthesis.*

one illustrated in Fig. 11-16); soluble RNA (type II) with histidine, etc. We do not know this for sure because we do not know the detailed structures, but the concept has considerable indirect experimental evidence to support it. These processes have converted amino acids to amino acid RNA complexes, and if each one is different, each amino acid now has a different and distinct handle on it. But these are not yet protein.

Protein itself is synthesized from these soluble RNA–amino acid complexes apparently on the surface of very small particles in the cell, called microsomes or ribosomes. These particles are rich in RNA and we suppose that on their surface they have long chains of RNA. Indeed we suppose that their surface is made up of the ordered or coded RNA which was made on the DNA of the nucleus.

We think that the amino acids, with their specific handles, form a temporary chain along the coiled RNA chain, each handle fitting into its proper slot as represented in Figure 11-16. When the necessary and regular slots are filled, the amino acids at the outer end unite—forming a protein. The soluble RNA leaves the surface of the coded RNA to pick up more activated amino acids. But the order of the amino acid in the protein depended on the slots in the RNA of the ribosome, which order also depended on the DNA of the nucleus. Whether we obtained a protein starting with alanine-glycine-histidine or glycine-alanine-histidine depended then upon DNA for its coding. And, therefore, DNA controlled what kinds of proteins—and therefore what enzymes—were made by the cell. And this in turn determined what kind of cell it was.

While these theories are somewhat speculative today, they give us a reasonable working hypothesis as to how it is possible for the gene, and the DNA it contains, to carry the code, and how this code may work to control the formation of a specific cell.

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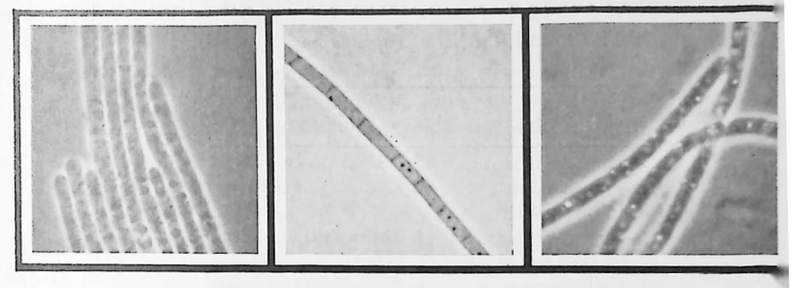
For background information, see p. 493.

QUESTIONS

1. Define or identify:

heredity	transformation
mutation	dissociation
gene	pneumococcus
adaptive enzyme	type I
constitutive enzyme	recombination
induced enzyme	transduction
simultaneous adaptation	adenine
smooth colony (S)	guanine
rough colony (R)	cytosine
fluctuation test	thymine
replica plating	deoxyribose
colony sectoring	RNA
DNA	amino acid activation
clone	adenyloamino acid
ribosome	soluble RNA

2. If a mutation occurs in 1 out of every 10^9 divisions, and one has an organism which grows to 10^8 organisms per milliliter, then there should be 1 mutated organism in 10 ml of medium. If we transfer this organism by transferring a loop (0.01 ml) to 10 ml of medium, once each week for 10 years, how is it possible that the culture is the same over this ten-year period?
3. If the mutation rate was that of question 2, how could we detect the mutant? Is any culture ever genetically pure?
4. Professor von Klagowitz, of the East-Ural Institute of Microbiology, claims that he can alter the genetic constitution of a microorganism by shaking it at a high velocity. What evidence would you insist upon before you would accept this conclusion?
5. *Serratia marcescens* possesses a red pigment. This pigment will not develop in certain media, but will appear in most media and is especially prevalent in a medium containing 5% salt. In studying this organism you find a sectored colony. How would you determine whether or not this was a mutation?



Chapter 12

ENVIRONMENTAL FACTORS IN DEATH-STERILIZATION

". . . we are too much accustomed to attribute to a single cause that which is the product of several, and the majority of our controversies come from that."

VON LIEBIG

HERBERT SPENCER once defined life as "a continuing series of chemical reactions among molecules of great complexity." While this definition has some limitations, it can serve as a starting point to emphasize that anything which can interfere with the continuing reactions or with the molecular complexity can kill. Or, put in another way, life is dependent upon the proper balance between energy-yielding and energy-utilizing reactions. Anything which interferes with these reactions or with the agents which carry them out—enzymes—will kill.

But this leaves still a further factor. Not all of the reactions of a cell occur in all parts of it. There are structures within it—and interference with these structures can kill. For example, a bacterial cell has a rigid cell wall, and if the integrity of the cell wall is destroyed, the cell may be killed.

Yet, to be more precise, one must recognize that one cannot say simply that heat will kill, for example; obviously one must specify how much heat. There are, in fact, two groups of factors affecting killing—one group having to do with the killing agent, the other group with the organism involved.

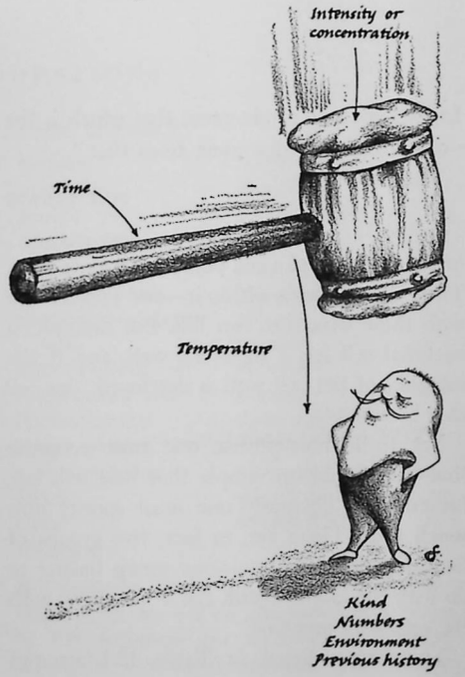
The factors listed in Table 12-1 are put

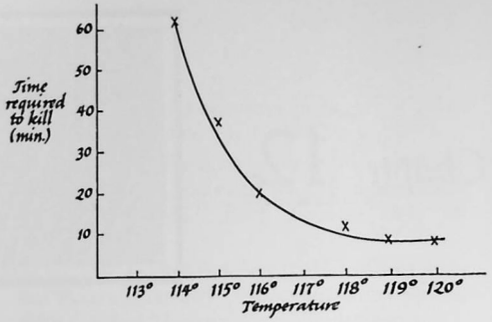
TABLE 12-1. *Factors which Influence the Ability to to Kill Microorganisms*

Related to Killing Agent	Related to Organism to be Killed
1. Intensity (if physical) Concentration (if chemical)	1. Kind of organism
2. The time during which the agent can act	2. The number of organisms to be killed
3. The temperature at which the agent acts	3. The conditions (especially the environment) in which the killing takes place
	4. The previous history of the organism

together in Fig. 12-1 in a sort of cartoon, to help you remember them. In the following paragraphs we shall discuss each factor separately, taking convenient illustrations where-

FIG. 12-1. *Factors which influence the ability to kill microorganisms.*





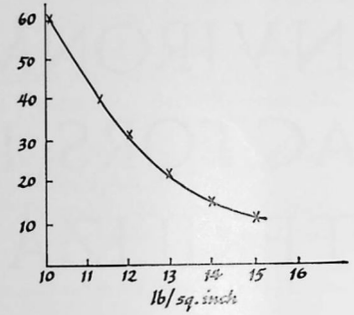


FIG. 12-2. *Time and temperature required to kill* Clostridium botulinum.

ever necessary, without regard as to whether the killing factor is physical or chemical, or whether, indeed, anything at all is known about the mechanism by which it acts.

1. *The intensity or concentration.* The term intensity is applied to physical factors. As an illustration of intensity we might use temperature. The most heat-resistant living thing is the spore of *Clostridium botulinum*. At 100°C it may be boiled for hours, with little effect. At 113°C, it can be killed in 60 minutes; at 114°, in 39; at 116°, in 29 (Fig. 12-2). Since intensity and time are interrelated, we can express the effectiveness of killing in terms of the time required to kill off different populations of the same size. Obviously the higher the temperature, the more effective the agent.

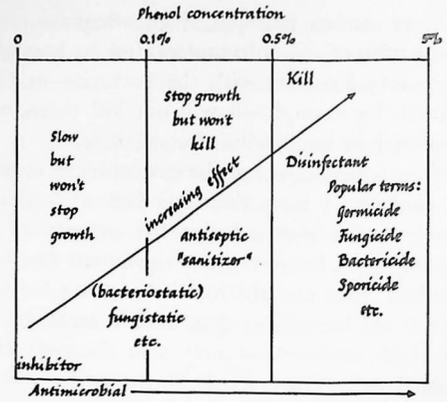


FIG. 12-3. *The killing action of phenol.*

With chemical agents we express this "intensity effect" as concentration. Fig. 12-3 illustrates the killing action of phenol. At a concentration of 0.1%, phenol will not kill *E. coli* but will only prevent its growth. At 10 times this amount, at 1%, it will kill in 20–30 minutes. At 5% phenol, it will kill in 2–3 minutes.

2. *The time an agent can act.* The effect of time of action is clearly evident in the two examples given under the previous discussion. Sixty minutes were required for spores of *Clostridium botulinum* at 113°C, while 29 minutes sufficed at only 3° higher (116°C). Phenol would kill in 30 minutes at 1% concentration, and in 3 minutes at 5%. In short, turning to the cartoon of Fig. 12-1, a heavy head on the hammer requires a shorter handle to do the same job, or a long-handled hammer can do with a lighter head.

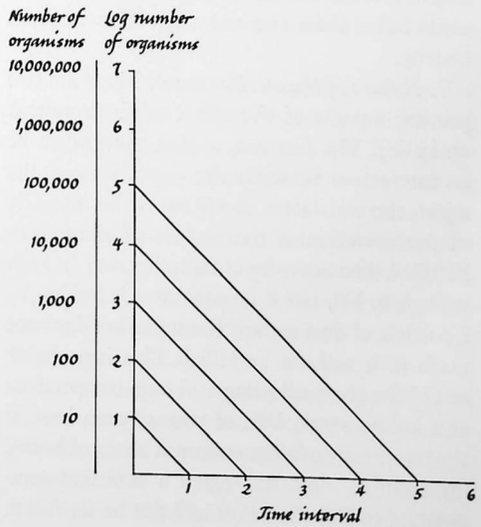
3. *Temperature.* The higher the temperature, the greater the killing effect of any agent. Usually the rate of killing is about doubled with each 10°C rise in temperature.

4. *The number of organisms.* Bacteria die in a logarithmic order of death. As illustrated in Fig. 12-4, this has some important consequences in the killing time. If, as illustrated, 10 cells are killed in 1 minute, it will take only 2 minutes to kill 100—not *10* minutes as one might expect with 10 times as many organisms. Of course, the more organisms, the longer it will take to kill them all off. But the relationship is not a simple one. As a matter of fact, the log death rate works to our advantage; 10 times as many organisms take only twice the time to kill, not 10 times the time.

There is, however, an additional factor which must be considered under practical conditions where mixed populations occur (i.e., different numbers of many different kinds of bacteria are present), as in soil or potatoes, plant tissue, or beef. The greater the numbers of organisms, the greater the chances that one will have some kinds with higher heat resistance. The practical rule is, therefore, to always keep the number of organisms (sometimes called the "load") as low as possible.

5. *The kind of organism.* While the vegetative cells of most bacteria are killed by exposure to 60°C for 10 minutes, the spores are more resistant. Here, however, resistance varies

FIG. 12-4. *The consequences of logarithmic order of death.*



greatly; the spores of some species are killed (in 10 minutes) by boiling water (100°C), while others require 10 minutes in steam under pressure (15 lb/sq in = 121°C).

6. *The previous history (and condition)* of the organism. There is a variety of data to show that cultural conditions, such as growth of the bacteria on one medium compared to another, influence the sensitivity to a given toxic agent; but there are some effects which are large ones and which overshadow most of the information on "previous history." One is that the age of the culture markedly influences its sensitivity; another, that the toxic factors may combine to enhance sensitivity.

Taking the first situation, it is well known that dividing microbial cells are more sensitive to any deleterious agent than are nondividing cells; thus, over a growth curve, from the period of late log to that of the stationary, the organisms can be more readily killed than in the stationary phase. Growing (i.e., dividing) cells are easier to kill.

Second, suppose we have a medium in which the organisms stopped growing because of the accumulation of a toxic factor—acid, for example. A culture under such conditions is more easily killed than one not subject to medium toxicity.

7. *Nature of the environment.* There are two primary aspects of the effect of environment on killing. The first one is that there must be an interaction between the organism and the agent; the real factor in killing is the intensity or concentration at the surface of the cell to be killed. The intensity of radiation may be high enough to kill, but if an organism is hidden by a particle of dust so that the radiation does not reach it, it will not be killed. This is probably one of the great advantages of high temperature as a killing agent, but, of course, even here, if the temperature in the center of a can of beans, for example, does not reach a sufficient temperature to kill, the beans will not be sterilized. Or, as another example, if an adequate concentration of a disinfectant cannot be brought into actual contact with the bacteria—in the throat, for example—it will not kill them, no matter how good a disinfectant it is.

The second aspect of the environment is also a complex of more than one factor. A given environment may enhance the activity of a killing agent because the environment itself is slightly toxic, and the toxicity of the environment and the killing agent add to each other. A given environment may also decrease the activity of a toxic agent. One way in which this is done is that the environment may actually inactivate the toxic agent—adsorb it on colloids, or actually chemically combine with it. Another way is for the environment to be such as to prevent the agent from acting. An opaque fluid is not suitable for sterilization by radiation; all the radiation is absorbed in the first thin layer. Again, some chemicals act only under acid conditions; some only under alkaline.

Terminology of Sterilization Processes

In Fig. 12-3, in which the effect of phenol at different concentrations was illustrated, it may be noticed that at 0.5 g/l, phenol would delay but would not stop growth. At 5 g/l the phenol would prevent growth, but would not kill organisms already grown. At twice this level (10 g/l) phenol would kill; the higher the level above this point, the more rapid the killing. Here, then, is the same material showing three different responses (depending upon its concentration):

1. Slowing growth.
2. Stopping growth but not killing cells already grown.
3. Killing grown cells.

There are names in common use describing these conditions; while they are sometimes used

loosely and incorrectly—and therefore their meaning has become a little fuzzy—there are a few terms whose definitions are of importance.

A **disinfectant** (= remove infection) is an agent capable of killing microorganisms already grown. It does not necessarily mean capable of killing spores and "to disinfect" does not necessarily mean "to sterlize," since the latter means to render free from all forms of life. The disinfectant, then, corresponds to condition 3 above.

An **antiseptic** means an agent which stops the growth of organisms, but does not necessarily kill off those already grown (corresponds to condition 2 above). Antiseptic applies to the inhibition of disease-producing organisms in contact with body surfaces.

A **bacteriostat** means an agent which prevents the growth of a bacterium (a fungistat for fungi, etc.), and in this sense is like an antiseptic; however, a bacteriostat is not necessarily usable on body surfaces.

An **inhibitor** merely slows, but does not necessarily prevent growth (corresponds to condition 1).

Further, there is an array of other words in common use, but they are used so loosely and so inconsistently that it is futile to try to make fine distinctions between them. Without defining them, we list in Table 12-2 a few of the common terms, clustering them about the main distinctions.

TABLE 12-2. *Common Terminology of Sterilization Processes*

Inhibitor	Antiseptic	Disinfectant
Slows growth	Stops growth	Kills
		Germicide
Antimicrobial		Microbicide
Antibacterial	Bacteriostat	Bactericide
Antifungal	Fungistat	Fungicide
Antiviral		Virocide
Antispores		Sporocide
Antipathogen		Sanitizer

In Chapter 6 we considered the factors of environment—water (including osmotic pressure and surface tension), temperature, oxygen, and acidity. All of these are always present in any environment, and it is only at the extremes that they become agents of death. In addition, there are other physical factors which may be introduced into the environment—radiation, pressure, etc., which are capable of killing. These too will be considered here.

Temperature is a factor always present in the environment of any creature. But for some, the internal temperature may be controlled (as in the human body, for example) by elaborate mechanisms, and it may differ considerably from the external temperature. But the microbial cell has no way of maintaining itself at temperatures lower or higher than those of its environment. Heat generated by metabolic processes is usually lost immediately to the surrounding media. Within a few thousandths of a degree except under conditions of very restricted heat flow, the temperature of a microorganism is the same as that of the medium that surrounds it, and this is virtually the same as the environment in which the medium is placed.

All organisms, including bacteria, survive and grow over a temperature range. Below and above this range, growth ceases and killing may start. Within the range, conditions are not all the same; at the edges (the minimum temperature or the maximum temperature) of the range, conditions may be poorer than in the center (or some other) portion of the range, generally called the "optimum," i.e., the optimum temperature, for example. We have earlier (Chapter 6) considered the effect of temperature on growth. We now wish to discuss the use of extreme temperatures to inhibit or to kill.

It seems probable that man knew that high

temperatures were toxic before he learned to control fire. It is a curious fact that in all societies the use of physical factors in the destruction of microorganisms—in the preservation of food, for example—is more widespread than the use of chemical factors. Cooking, salting, and drying of foods found widespread use in most civilizations. And heat is still the most widely used method of sterilization. The reason for this is illustrated in Fig. 12-5. Here the coagulation temperature of a protein (egg albumin) is plotted against the per cent moisture in the preparation. At 10% moisture, a temperature of 147°C is required before the protein coagulates; at 25% water it coagulates at 76°C. At 50% moisture—more comparable to living tissue, which is 80% water—the protein coagulates at 56°C (i.e., boiled eggs!). In Fig. 12-6, the times required to kill the spores of *Clostridium botulinum* by moist heat and by dry heat are plotted against the temperature. Moist heat kills them all in 10 minutes at 120°C, while dry heat requires 2 hours at this temperature. Baking in ovens, then, is not nearly as effective as heating under steam pressure. Most sterilization is done in the laboratory by steam pressure, and we shall therefore devote a special section to it. We should point out that the word **sterile** (and the process **sterilization**) means the complete absence of living things.

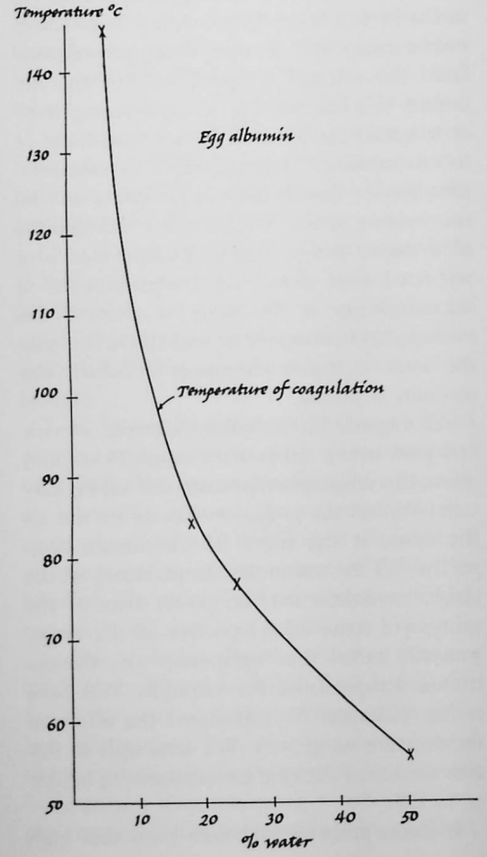


FIG. 12-5. *The temperature required to coagulate egg albumin at different water contents.*

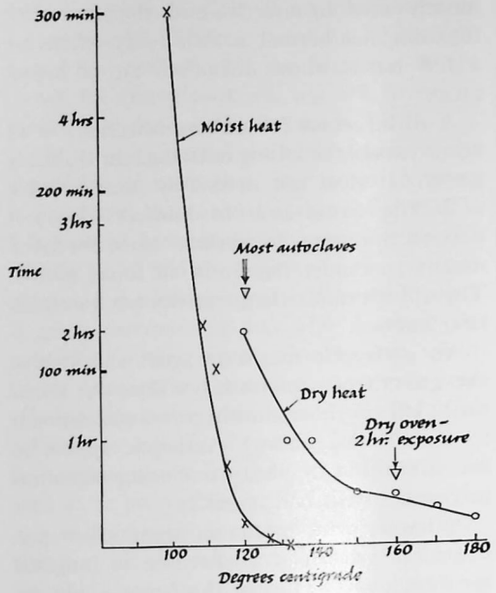


FIG. 12-6. *Time and temperature required to kill spores of* Clostridium botulinum *with moist heat and with dry heat.*

Sterilization by Moist Heat

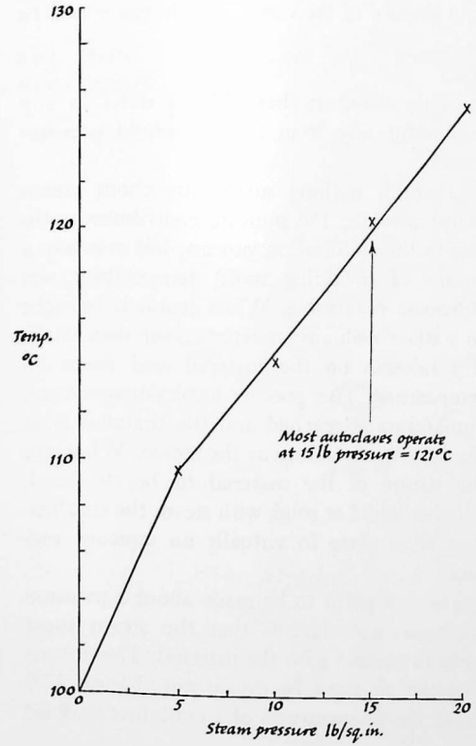
The living thing most resistant to heat, which is thus suitable as a standard, is the spore of the anaerobe *Clostridium botulinum*. Further, this organism, if not killed, can grow in a wide variety of situations; under many conditions it produces a very poisonous toxin and is responsible for the generally fatal disease, botulism (see Chapter 20). If we are preserving food by heat, naturally, any system we use must be good enough to kill all the spores of this organism.

There are other organisms whose heat resistance is as great as that of *Cl. botulinum*. Among them are *Bacillus stearothermophilus*, some other thermophiles, and a putrefactive anaerobe—#3679—but none exceed it. Therefore, any sterilization system must first be sure that it can kill spores of *Cl. botulinum*.

From Fig. 12-6 it is evident that, at the temperature of boiling water at sea level, we must boil for 5 hours before the spores are killed. But if we can raise the temperature to, say, 120°C, only 10 minutes are required. Fig. 12-7 shows the relation between the pressure of steam and the temperature. This shows that at 15 lb/sq in of steam pressure, the temperature reaches 121°C. Hence, sterilization employs steam at 15 lb pressure; the autoclave you will see in every bacteriological laboratory operates at 15 lb/sq in.

The effect of pressure is most important since the speed of killing is so sharply dependent on temperature. For example, see the following data, which also appear in Fig. 12-2.

FIG. 12-7. *Relation between steam pressure and temperature.*



lb/sq in	Temperature	Time to kill 10 million spores
15	121	10
14	119	14
13	118	19
12	116	28
11	114.5	39
10	113.5	60

Thus, while the relation between temperature and pressure is a straight line, the relation between temperature (or pressure) and killing is a steep curve (see Fig. 12-6).

The common laboratory autoclave is shown in a schematic diagram in Fig. 12-8, so you can see what the various valves on your laboratory autoclave really do. The principle is essentially that of a pressure cooker except that most modern autoclaves do not generate the steam in the autoclave, as does a pressure cooker, but take it from a steam line. There are, however, gas-fired and electrically fired autoclaves in which the steam is generated within the

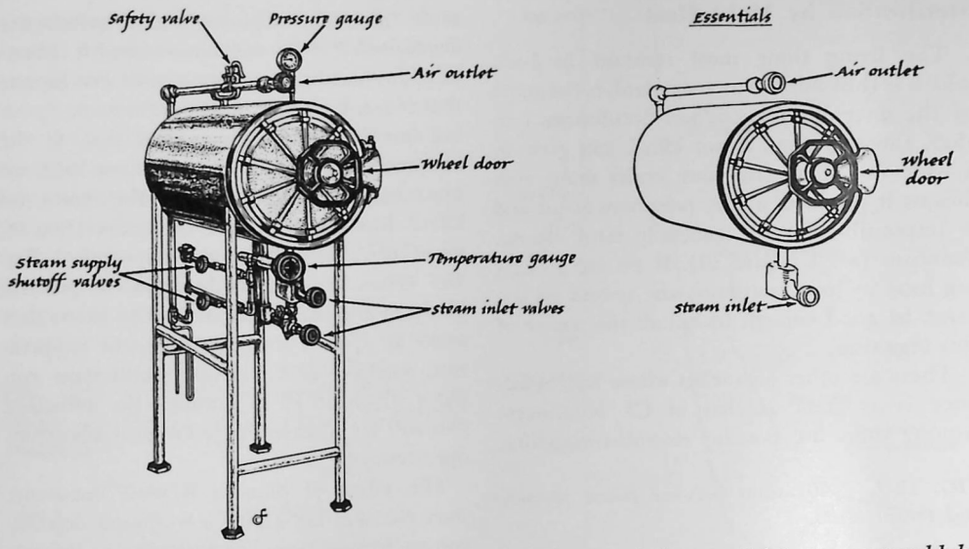
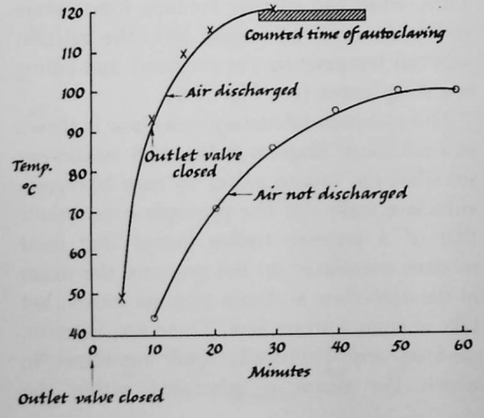


FIG. 12-8. *A common laboratory autoclave with a diagram of the essential parts you would be required to manipulate.*

FIG. 12-9. *The time required to reach various temperatures when the air has been discharged from the autoclave and when it has not.* [*Redrawn from Perkins, p. 778 of G. F. Reddish (Ed.) Antiseptics, Disinfectants, Fungicides, and Chemical and Physical Sterilization, Lea and Febiger, Philadelphia, 1957.*]



pressure chamber; these do not differ in any significant way from the household pressure cooker.

There is nothing mysterious about steam under pressure; the pressure contributes nothing to the sterilization process, but is solely a means of obtaining moist temperature not otherwise obtainable. When steam is brought in contact with any material cooler than itself, it condenses on the material and raises its temperature. This goes on until a temperature equilibrium is reached and the material is at the same temperature as the steam. Whatever the nature of the material to be sterilized, whether liquid or solid, with steam the sterilization takes place in virtually an aqueous medium.

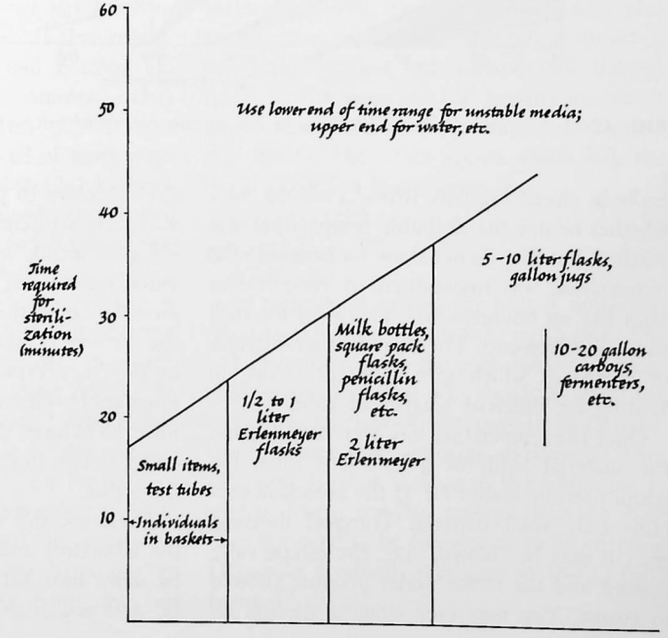
The first point to be made about a pressure cooker or autoclave is that the steam must come in contact with the material. This means that the air must be driven out. Figure 12-9 shows the temperatures of a container of fluid

inside an autoclave with a jacket—in one case where the air has been let out for 10 minutes, at which time live steam is flowing out of the escape valve, and in another case where the escape valve has never been opened. Obviously, unless the air is pushed out, one cannot even reach sterilization temperatures. The *time* of autoclaving is counted from the time the temperature reaches 120°C.

Figure 12-10 shows the approximate time required to sterilize different materials at 15 lb pressure. The reason these times vary is that the heat must penetrate throughout any container one uses. At an external temperature of 120°C, the center of a test tube may take five minutes to reach 120°C, but a 5 liter flask may require 40 minutes before the center reaches 120°C; since both must remain at this temperature for 10 minutes, the test tube will be sterile at the end of 15 minutes, but the flask will have to be autoclaved for 50 minutes.

There are now available a variety of sterilization indicators which will tell you—assuming the indicators are judiciously placed in each autoclave load—whether the heat penetration has been adequate. One such indicator is a tube of bacterial spores, but of course you must culture them to see if they have been killed and this takes time—sometimes time enough so that the remainder of the material autoclaved may show growth if autoclaving was inadequate. Or you may insert thermocouples from which the temperature may be measured electrically, while the autoclave is on. Chemicals of known melting point (sulfur, m.p. 115°C; acetanilide, m.p. 116° C; succinic anhydride, m.p. 120°C), usually mixed with a dye, are inserted at various places in the autoclave, gen-

FIG. 12-10. *Time required for the sterilization of different materials at 15 pounds per square inch steam pressure.*



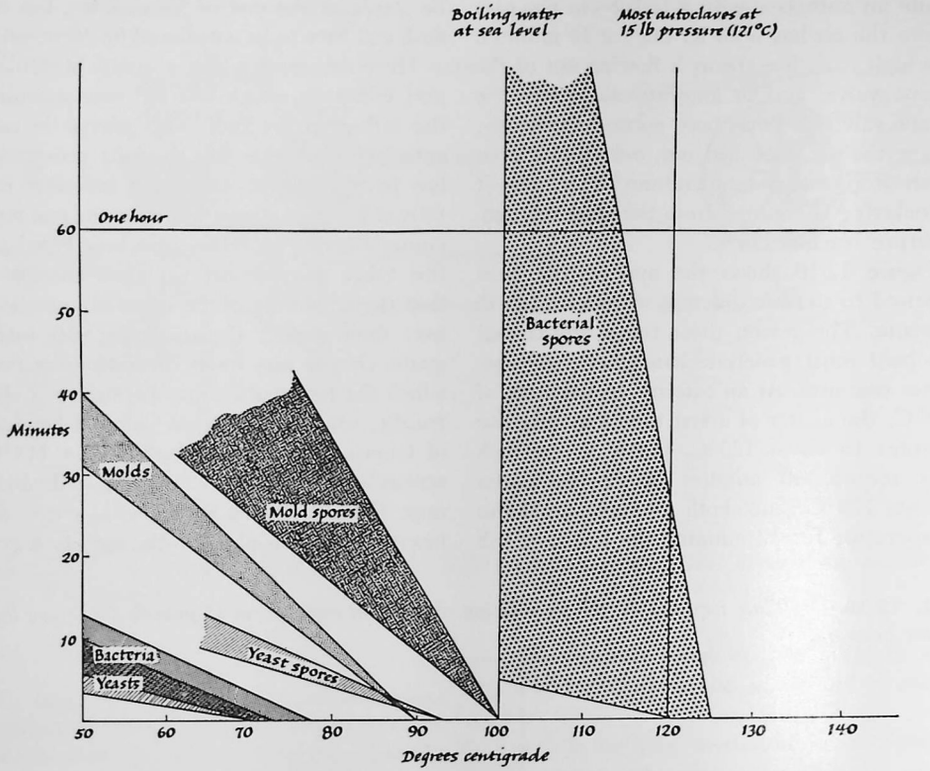


FIG. 12-11. *Temperatures required to kill various classes of microorganisms.*

erally in closed capillary tubes. They do show whether or not the desirable temperature was reached, but they do not show for how long the temperature was maintained. A modification built like an hourglass has been used for such time measurements. There are further mixtures of chemicals which give different colors on heating for different lengths of time.

Once the temperature has been reached, and the material held for a sufficient time, the steam pressure is shut off. If the autoclave contains only solid material (surgical dressing, etc.) it may be "blown," i.e., the escape valve opened and the steam under pressure allowed to escape. You may even draw a vacuum on the autoclave to pull out the moisture which may have saturated the materials. But if there is liquid media, or melted "solid" media, the autoclave cannot be "blown" because the medium (now at 120°C) will boil violently if the pressure is rapidly reduced. In fact, capped bottles may explode, but generally, at least plugs are blown out. Rather, the steam pressure must be reduced slowly, either by allowing the entire system to cool, or by slowly bleeding off the steam.

These are the methods commonly used in the laboratory and in industry for sterilization by moist heat. Of course, some things cannot be sterilized in this fashion: powders and oils

impervious to steam; cotton in a closed container into which steam does not penetrate—but moist cotton is readily sterilized; rubber gloves, which can not go above 115°C; leather materials, etc. For these things other methods have to be devised, but for many, many things, steam under pressure is the best system of sterilization. Most media, test tubes, flasks, instruments, etc. in the laboratory, canned peas, beans, corn, etc. in industry—all use steam.

All of the above processes are designed for getting rid of *all* the organisms, including the most resistant. The most resistant, of course, are spores, and these are much more resistant in the bacteria than are vegetative cells; this is true in yeasts, molds, and bacteria, as shown in Fig. 12-11. But you should not have the impression that *all* bacterial spores require 120°C for killing in 10 minutes. The spores of most spore-formers are killed in 10 minutes at 100°C, i.e., by boiling water, and only the most resistant species survive longer. This, of course, is the basis of the use of boiling water for sterilizing instruments and fruit jars for canning. Further, note from Fig. 12-11 that vegetative cells of yeasts, molds, and bacteria are killed by 55°–60°C for 10 minutes. Many materials which cannot stand greater heat (milk, beer, etc.) may be freed of most vegetative cells (but not rendered sterile) by heating to lower temperatures. This process is called **pasteurization.** It is designed to kill pathogenic microorganisms.

There is a further process, today not widely used, for sterilization, in which intermittent heating is employed, which was used especially on materials that could not stand boiling. In this method (sometimes called "fractional sterilization" or "Tyndallization") the material is subjected to free flowing steam for perhaps 30 minutes or its temperature is raised to 60°C for 20 minutes. This kills all the vegetative cells. The material is then incubated for an interval (usually 24 hours, sometimes less but long enough for spores to germinate). In this interval, the living spores still present germinate and become vegetative cells. The process is now repeated, i.e., heating to 60°C. to kill the newly formed vegetative cells, and then reincubating to allow any further spores to germinate. About three repetitions usually provide a sterile material.

Throughout the discussion of the effects of heat, we have always had to say 120°C for 10 minutes or 60°C for 30 minutes—that is, both time and temperature were involved in the killing action. There are, in this respect, two kinds of indices used to express heat tolerance or sensitivity. These are the **thermal death point,** defined as the lowest temperature required to kill a "standard" suspension of organism in 10 minutes; and the **thermal death time,** defined as the shortest time required to kill a "standard" suspension of organism at a prescribed temperature. Neither term is very helpful; we mention them here only because you will see them referred to in books on bacteriology and sterilization. You may also see reference to the term "thermal death rate," which usually (but unfortunately not always) refers to the slope of the logarithmic death curve, the effect of heat being to coagulate the protein; like other agents which kill, the death curve is a logarithmic one.

Dry Heat

As mentioned earlier, dry heat is much less effective as a killing agent than moist heat. The lack of water reduces the probability of protein coagulation. Dry heat acts mostly by oxidizing or burning up the cell materials. In its extreme, this is incineration—burning up the cells. This, of course, is what is done in the laboratory to sterlize loops, needles, forceps. But dry heat does not need to be this drastic to be effective. Glassware is usually sterilized at 160°C for 2 hours.

It is common custom to sterilize petri dishes, etc., in this fashion, rather than autoclaving. If we examine the reasons for this custom, we find it based on some degree of irrationality, and to some degree upon conditions no longer obtaining. The prejudice against autoclaving for petri dishes seems to be based on two things. First, the petri dishes coming from autoclaves are sometimes moist, but they can, of course, be readily dried. Second, it is thought that steam tends to etch them, so that they become cloudy and no longer useful. Heating in an oven avoids this. Actually, modern pyrex dishes etch so slowly with steam heat that this argument no longer applies.

Low Temperature

While the zero of life is essentially 0°C, and no living process appears to go on below this point, microorganisms are singularly resistant to freezing and many survive such treatment. Apparently the chief danger is that ice crystals, forming in the cell, may cut the membrane or coagulate the proteins, but the real situation is undoubtedly more complex. Quick freezing or instantaneous freezing tends to prevent this; hence, cultures frozen for lyophilizing are "shell frozen," as shown in Fig. 12-12.

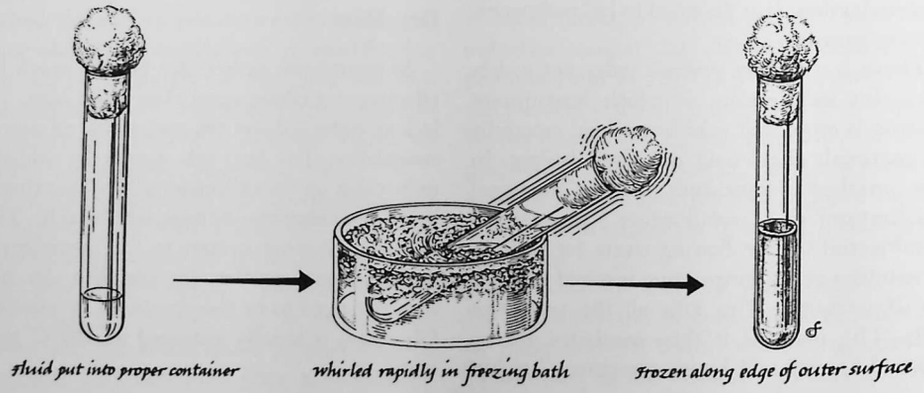
When the situation is looked at closely, it is found that, of a given population being frozen, many—sometimes half, sometimes 90%—are indeed killed, but the remainder are unharmed. Frozen foods, therefore, are not sterile, nor is ice cream or frozen desserts. While organisms do not grow at these lower temperatures, they are present, and when conditions change and the temperature rises, they are ready to grow. Curiously, gram-positive organisms seem to survive freezing better than gram-negative organisms. But chilling, rather than freezing, is harder on the gram-positive type.

Moisture

Just as water and its availability is a condition of life, so its lack is a factor in death. Excess water does not kill—bacteria do not drown—but of course too much water means too little food and they may starve. But too little water is a killing agent. All organisms subjected to drying suffer some loss in viability except bacterial spores. These may be dried and kept for years under laboratory conditions and undoubtedly have kept for years, perhaps centuries, in nature, without significant loss in viability.

With vegetative cells the loss of viability

FIG. 12-12. *Shell freezing.*



is rapid and substantial and may continue in subsequent dry storage. As an agent of sterilization, drying is not adequate; for generally some organisms survive. Especially since pathogenic organisms may remain viable when dried. A handkerchief infected with sputum containing the organism causing tuberculosis may still be infectious, even if dry. Skin, fortunately, contains long-chain fatty acids which, combined with drying on skin, act as a disinfectant, especially to gram-negative organisms.

Desiccation, then, is antiseptic but not disinfectant. The same is true of removal of available water—by bound water or by osmotic pressure; it is antiseptic, not disinfectant.

Osmotic pressure above the allowable range may cause the cell to shrink, but on the other hand rarely causes the cell to burst, if its cell wall is intact. Osmotic pressure by itself rarely kills microbial cells but mostly prevents their growth. It frequently may prevent food from penetrating and thus eventually the organism may die, apparently by starvation, but by itself osmotic pressure is bacteriostatic, not disinfectant.

Similarly, surface tension is not by itself toxic, but chemical agents, which are themselves toxic, may have their toxicity enhanced by lowered surface tension. Apparently the lowered surface tension increases the accumulation of the toxic agent at the cell surface, and this, in effect, increases its concentration.

Acidity

This factor, always in the environment, has (as discussed in Chapter 6) the following ranges of *p*H:

	Lower limits	Optimum	Upper limits
Bacteria	4.5	7.0	9
Streptomyces	4.5	7.0	9
Yeasts	3.5	5.5	8
Molds	3.5	5.5	8

Curiously, by itself, exposure to hydrogen ion concentrations greater than these (i.e. lower *p*H from 1–3) is not especially toxic to most microorganisms. They do not grow, of course, but they also do not die off rapidly, unless the acid has some other toxicity associated with it. For example, the organic acids such as acetic, propionic, butyric, etc. are mildly toxic, and, at a given *p*H, are more active than inorganic acids such as HCl, H_2SO_4, etc., due to the toxicity of the organic part of the molecule. Organisms may die off in culture media not entirely because of the acidity, but because of the organic part of the acid producing this acidity.

Actual data on killing by acid are a little hard to find since most of the studies are on inhibition of growth; those actually studying killing leave much that is still obscure. Acid by itself (i.e., low *p*H) is generally considered exceedingly toxic and it is surprising how little experimental evidence there is for this belief. However, there is plenty of evidence that acid inhibits growth, that it may enhance the effect of other toxicities, and that among the organic acids the remainder of the molecule may have a profound effect.

Introducible Environmental Factors

Certain lethal agents, not always a part of the environment in which a bacteria may grow, may be introduced from the outside. Some are useful for killing microorganisms.

RADIATION. Certain portions of the radiation spectrum are lethal to microorganisms, especially the ultraviolet. The helpful effects of bright sunlight as a killing agent have been known for generations. Just how radiations act to kill is not exactly known; there are some very strange phenomena observed, some of which are not explainable by anything we yet know of natural laws. Figure 12-13 shows the broad band of electromagnetic radiations known. Of

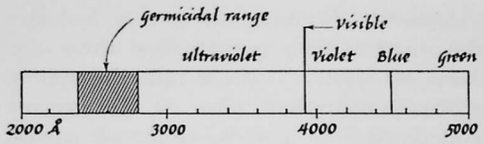


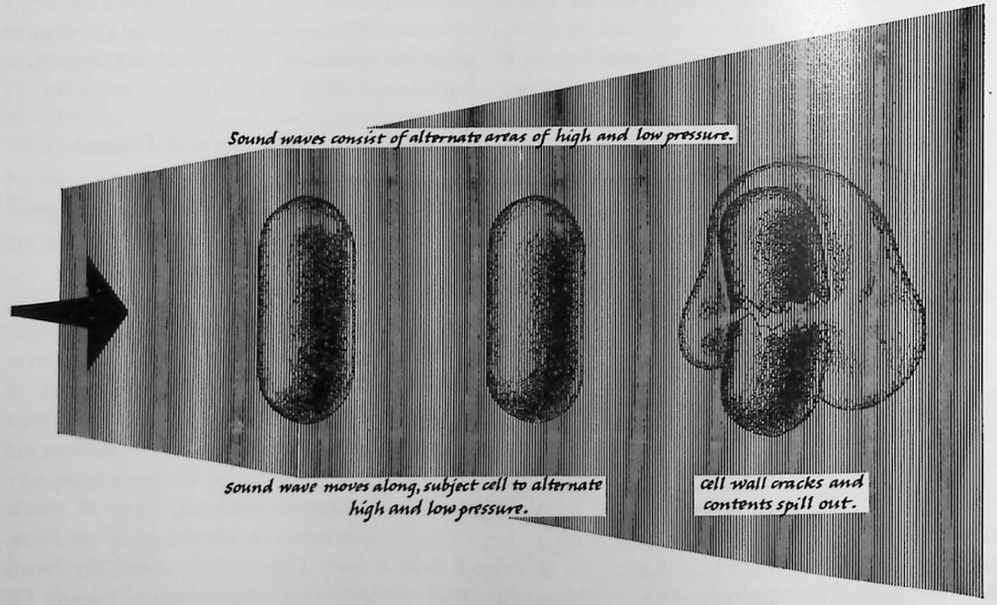
FIG. 12-13. *The lethal spectrum.*

these, a relatively narrow band is lethal—between .2400 Å and 2800 Å (= angstrom unit). It happens that mercury arc quartz lamps have a maximum emission at 2537 A and these are frequently called "sterilamps" or sterilizing lamps. The ultraviolet radiation is as toxic to spores as to vegetative cells. While spores are resistant to heat, they are usually as susceptible to ultraviolet radiation as are vegetative cells. There is, however, some controversy about this point, the results obtained being largely dependent upon the exact method of irradiation.

Ultraviolet light, then, over the wavelengths 2400–2800 A, is a powerful disinfectant, and is especially useful in "sterilizing air." The chief difficulty is that since ultraviolet light penetrates poorly (it is cut off by most glass, for example) it can only act on direct contact. An organism in a liquid is generally not affected at distances an inch or so below the surface, although ultraviolet light may penetrate clear well water from 6 inches to 2 feet. In air, ultraviolet light is effective against air-borne organisms, but much less effective against dust-borne organisms. It has, nevertheless, found a place in the irradiation of hospital or schoolroom air.

How ultraviolet radiation kills is not known. It is true that the wavelength of highest activity are just those of maximum absorption by nucleic acids; and ultraviolet is a potent stimulant of mutation. But there is one phenomenon, called **photoreactivation,** which so far defies "rational" explanation. The effect of photoreactivation is the following: a cell suspension is ir-

FIG. 12-14. *Possible way in which supersonic sound acts upon bacteria.*



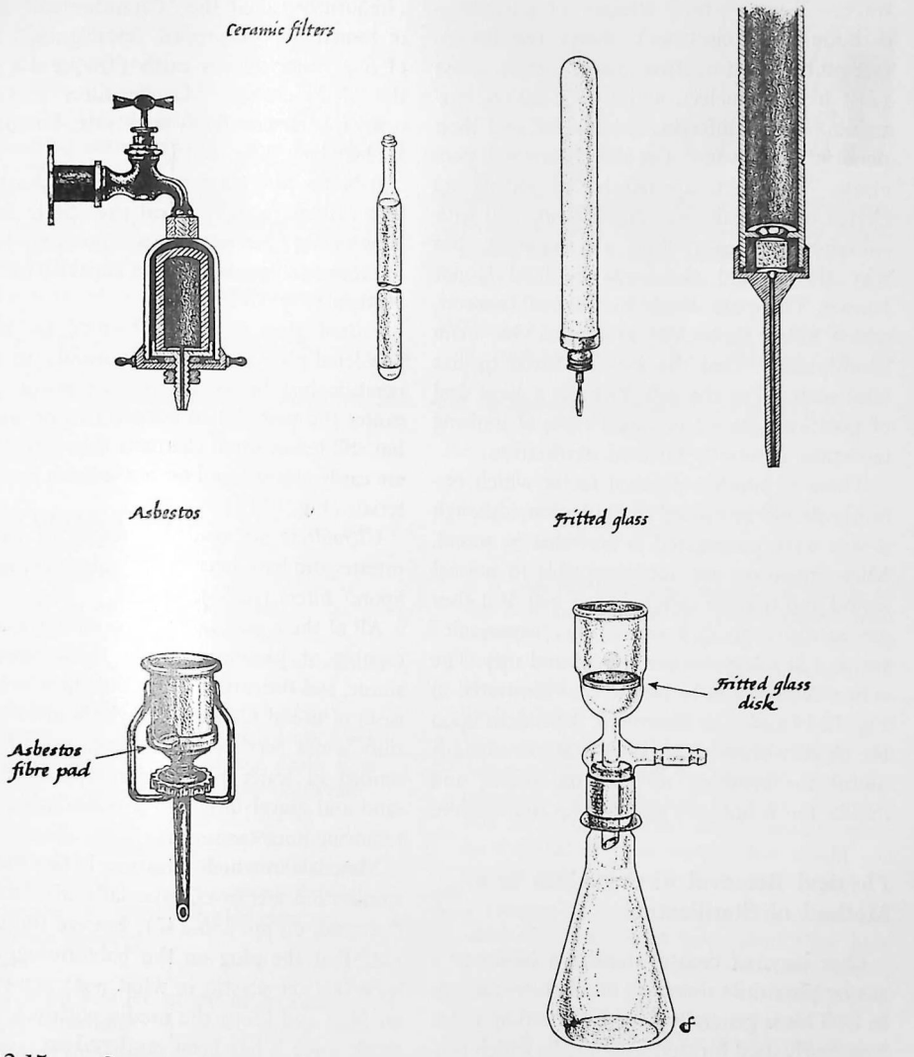


FIG. 12-15. *Some types of bacteriological filters.*

radiated in ultraviolet light until 99% of the organisms are killed, as evidenced by plate count; i.e., they will no longer form colonies on plates. A portion of this same suspension is exposed to visible light for perhaps 30 minutes, and plated. Many more organisms are found on the plate. That is, there were organisms "killed" by ultraviolet radiation but restored to viability by visible light! We are still searching for a reason why this should happen.

Other types of radiation having lethal effects are the ionizing radiations: electromagnetic

waves—X-rays, γ-rays; streams of particles—cathode rays (electrons), β-rays (electrons), fast protons, fast neutrons, fast electrons, α-rays (fast helium nuclei), and atomic fission fragments. These differ in their nature and their penetrating power; notice how X-rays will penetrate. They act, apparently, by setting up chains of ion pairs within the living cell with subsequent "free radical" development, but why this should necessarily be fatal is not known. There are several indications, however, which link their toxicity to oxygen. One main assumption is that the ions produced oxidize vital material in the cell. There is a great deal of practical interest in varied types of ionizing radiation, especially for food sterilization.

There is another physical factor which certainly should be classed as a radiation, although it is a wave propagated in air—that is, sound. Microorganisms are not susceptible to normal sound and may be shouted at at will. But they are sensitive to "ultrasonic" or "supersonic" waves (20 kilocycles per second and up). The action is supposed to be like that illustrated in Fig. 12-14 and is, as illustrated, dependent upon the *rigidity* of the cell wall. Ultrasonic sound is useful for breaking up bacteria, yeasts, and molds, but is not very practical for sterilization.

Physical Removal of Organisms as a Method of Sterilization

One may, of course, sterilize a liquid or a gas by physically removing all of the organisms in it. This is generally done by filtration and is frequently used for sterilizing media which contain materials destroyable by heat. Naturally if such filters remove bacteria they will also remove larger organisms. There are four major types of filters in everyday use for this purpose.

Ceramic filters are composed of porcelain (frequently called the "Chamberland filter") or sometimes compressed "kieselguhr," a type of fine diatomaceous earth (frequently called the "Berkefeld" or "Mandler filter"). The porosity is such that fluids penetrate, but bacteria are held back (Fig. 12-15).

Asbestos filters are composed of matted asbestos fibers (usually called the "Seitz filter"). How such a filter works is not precisely known, but seems to be related to its capacity to adsorb bacteria (Fig. 12-15).

Fritted glass filters are formed by heating powdered glass (in a suitable mold) to a temperature just below its melting point, which causes the particles to adhere to one another, but still leaves small channels through it. They are easily cleaned and do not adsorb many materials (Fig. 12-15).

Ultrafilters are made primarily of cellulose nitrates and have been described earlier as "millipore" filters (p. 53).

All of these may be used to render materials capable of passing through them absolutely sterile, and they are so used. But there are many cases of useful filtration in which absolute sterility is not necessary, and here, as will be described in water purification (pp. 316–317), sand and gravel filters do a remarkable job in removing microorganisms.

Materials in which filtration is *the* means of sterilization are gases, especially air (methods discussed, on pp. 236–237), but we might here note that the plug on the bacteriological test tube (cotton, plastic, or what not) serves as an air filter and keeps the media within the tube sterile once it has been rendered so.

We may remove many (but usually not all) organisms by centrifugation, and this is sometimes used industrially (as **clarification**) to reduce the load of a material to be subsequently sterilized by some other means.

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For background information, see p. 494.

QUESTIONS

1. Define or identify:

   intensity vs. concentration
   killing time
   load of organisms
   vegetative cells vs. spores
   disinfectant
   sterilize
   antiseptic
   bactericide
   bacteriostat
   inhibitor
   germicide
   optimum temperature
   botulism
   autoclave
   sterilization indicator
   pasteurization
   Tyndallization
   thermal death point
   thermal death time
   thermal death rate
   shell freezing
   radiation
   photoreactivation
   supersonic sound
   fritted glass

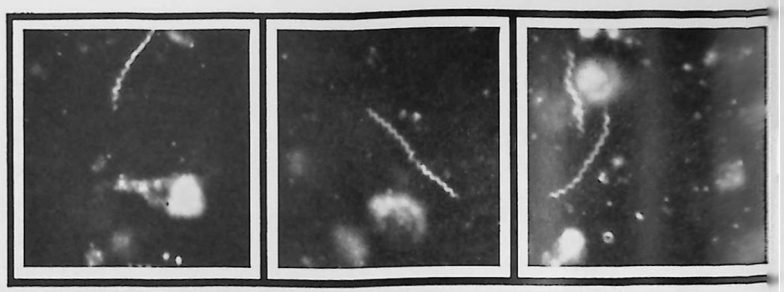
2. Why must you let air out of an autoclave before sterilization?

3. A process for sterilizing canned liquid low-calorie diet has been in operation since September. It is now the following August and several of the cans are blowing up. This happens with materials canned last September as well as those canned now. What would you suspect? How would you improve the sterilization process to prevent this?

4. You are traveling with an expedition to the Antarctic Wastes and you plan to study the intestinal microbiological flora of penguins. You are being air lifted into temporary camp for a period of three weeks. How would you sterilize your media, glassware, and utensils?

5. A cannery has come to you with a problem which they ask you to help solve. They have decided to put out a large "family size" can of dog food and find that while their usual process works well with the smaller can, over half of the large cans spoil. What would you suggest as the cause and what measures would you take to solve the problem?

Chapter 13



THE CHEMICAL FACTORS OF DEATH

"The scientist is a practical man and his are practical aims. He does not seek the *ultimate* but the *proximate*. He does not speak of the last analysis but rather of the next approximation. His are not those beautiful structures so delicately designed that a single flaw may cause the collapse of the whole. The scientist builds slowly and with a gross but solid kind of masonry. If dissatisfied with any of his work, even if it be near the very foundations, he can replace that part without damage to the remainder. On the whole, he is satisfied with his work, for while science may never be wholly right it certainly is never wholly wrong; and it seems to be improving from decade to decade.

"The theory that there is an ultimate truth, although very generally held by mankind, does not seem useful to science except in the sense of a horizon toward which we may proceed, rather than a point which may be reached."

LEWIS, *The Anatomy of Science* (1926)

OLIVER WENDELL HOLMES, in *Medical Essays* (1883), says that medicine learned from a monk how to use antimony, from a Jesuit how to cure agues, and so on down a long list. The monk was Basil Valentine (partly mythical) who noticed that pigs eating food containing antimony grew very fat; he promptly tried it on some monks who had become emaciated

through fasting and they died. This apparently did not discourage anyone and "tartar emetic" (antimony potassium tartrate) was used for centuries at the "start of fever" and does indeed have an effect on several protozoan infections although it has the disadvantage that it has a variable toxicity. Undoubtedly, many people so treated suffered the same fate as the monks—they died. But Holmes' Jesuit is even more to our point; the Jesuits imported to Europe from the Peruvian Indians, Jesuit's Bark or quinine. Brought to Europe in 1632, it "did for medicine what gunpowder did for war"—it changed the approach. If there was a substance which would cure or relieve the ague, then were there not other substances, could one but find them, to cure all the ills the flesh was heir to?

Poisons were known to the ancients, but antimony and, even better, quinine, had a more important property. They had specific activity, a selective toxicity. They could inhibit the malarial protozoan (it was later discovered that malaria was caused by such an organism) without harm to the host.

From ancient times, then, man has been interested in and searching for substances of medicinal value. But against pathogenic microorganisms the most interesting and valuable substances were those which possessed a specificity—that is, were able to attack and inhibit or kill one kind of organism but not another. This selective toxicity was of most importance. Yet even poisons, without specificity, could be useful, especially in killing microorganisms. These chemical substances, which are capable of killing or inhibiting microorganisms, we may regard as chemical factors which influence their death. By this term we mean really to consider those substances introduced into the environment of living cells with the view of killing or inhibiting them. Certainly the chemical substances used for this purpose have been varied but for our purpose we may divide them into three large groups.

The first are those substances showing little specificity—soaps, alcohols, halogens, phenols, heavy metals, etc., whose attack on one organism may be somewhat more effective than on another, but which cannot be used within the body tissues. The second are intermediates in the metabolism of the cell but are yet so closely related that the cell mistakes them for the real thing, and thus builds into itself a false molecule which will eventually fail when called upon to do a critical task. Some of these, for example, the sulfonamides, have a great deal of specificity and are capable of inhibiting bacteria within the animal or human body; many others are of much less interest because they have much less specificity. The third group are the antibiotics, substances formed by one organism which are toxic to another; among these we find some of the highest specificity. We shall discuss those with low specificity and the antimetabolites in this chapter, together with the kinds of methods one may, and sometimes must employ to discover, develop, and evaluate these materials. The antibiotics have a chapter to themselves—Chapter 14.

Substances Showing Little Specificity

These are substances toxic to microorganisms which are also toxic to higher forms. This does not mean that such substances are not useful; it means only that they cannot be used on or in higher cells. It is rather difficult to classify them and to organize the information concerning them because there are so many types and such variation in "mode of action" that schemes of "related substances" or "related activities" are generally less useful. For our purpose, we shall first begin with factors in the environment such as acids and alkalis which may become overbalanced, go from here to the soaps, detergents, and surface tension reductants, thence to

the alcohols, both aliphatic and aromatic, the aldehydes and related substances, whence we shall have to speak of gaseous agents, and finally to the halogens and heavy metals, ending with a few minor agents that have some interest to us. This for the most part must be regarded as more a detailed compendium of fact about each of the major groups of nonspecific compounds than as a tightly organized discussion of principles. We have already defined antiseptic, disinfectant, etc. (p. 219).

Acids and Alkalis

Acids represent an imbalance in the environment. Living cells are always in water, and there is always hydrogen ion. Acids, then, represent only more of something which is already present—more hydrogen ion, less hydroxyl ion. *Alkalis* represent a similar, but opposite imbalance, i.e., less hydrogen ion but more hydroxyl ion. In acids there is, of course, the hydrogen ion and the negatively charged ion. Most mineral acids (HCl, H_2SO_4, HNO_3, H_3PO_4, etc.) act primarily as generators of hydrogen ion, and it is only at exceedingly concentrated solutions of these acids that the negatively charged ion plays any role. Of course, organisms are killed by concentrated sulfuric acid. It chars (abstracts water from) them just as it chars sugar. Of course, concentrated nitric acid kills bacteria because it actually nitrates proteins and ring structures in the cell. But at more dilute levels, say at concentrations below molar, the effects of most mineral acids depend solely upon their *p*H; therefore the microorganisms' ability to survive at low *p*H and ability to grow at low *p*H, which are two different things, are discussed under *p*H, which is called a "physical factor" (p. 227).

But many acids are used as antiseptics—for example boric acid, benzoic acid, propionic acid—which are only weakly ionized and do not have much effect in altering the *p*H. These acids act, indeed, because of the undissociated or the negative ion part of the molecule and it is only by chance that they are acids and classed as such. Most of them are mild antispetics; although the rest of the molecule is the important factor (rather than the hydrogen ion) there is no pertinent notion as to why they inhibit. This does not prevent their widespread use. Benzoic acid was widely added to foods before it was legally excluded. At present sodium (at times calcium) propionate (not even the acid itself) is sprayed over the surface of bread to prevent mold development. Table 13-1 shows the activity in killing a particular bacterium as related to the structure of the acid; the longer the chain, the less is required, but the *p*H actually rises.

TABLE 13-1. *Activity in Killing a Particular Bacterium*

No. of C	Organic Acid	Conc. to Kill	*p*H
2	Acetic	0.3 *M*	2.7
3	Propionic	0.25 *M*	2.8
4	Butyric	0.12 *M*	2.9
5	Valeric	0.05 *M*	3.1

Alkalis (NaOH, lye, lime, white-wash) are not especially useful because they are also corrosive. They appear to act mostly by dissolving the cell wall or membrane, of both bacteria and skin. A milder alkali, such as NH_4OH, seems to act by entering the cell where NH_4^+ seems to be "toxic." Both the animal and the plant, and to some extent the bacteria, have mechanisms for reducing the ammonium ion content of the body fluids as rapidly as possible, but why the ammonium ion should be so "toxic" is not really known.

Soaps act partly by producing mild alkaline reaction but they also lower surface tension, which we have earlier classed as a physical phenomenon. *Detergents*, as such, other than soaps, sometimes have antiseptic properties and while this may be desirable in washing dishes

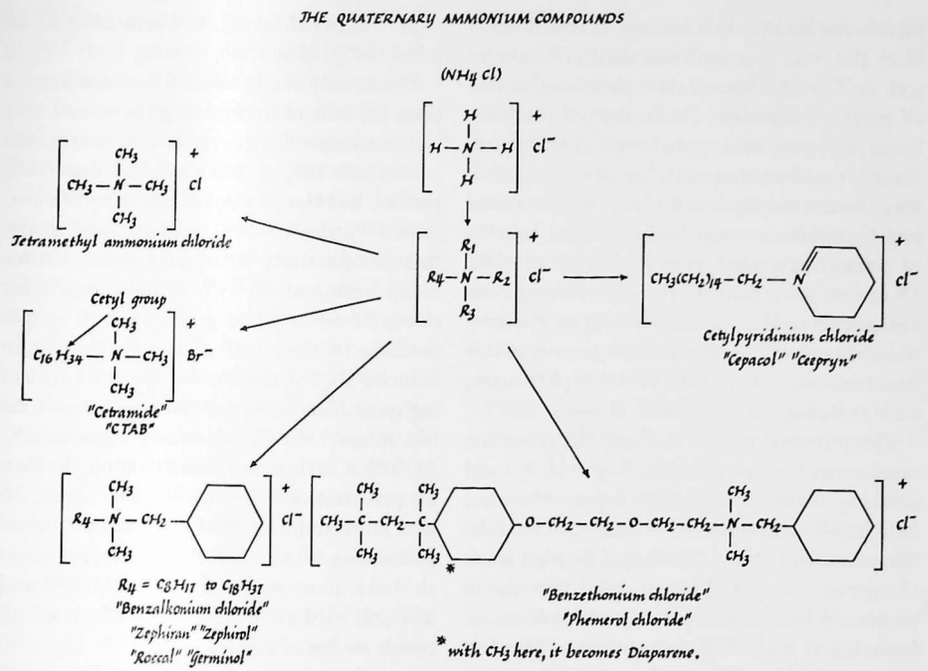


FIG. 13-1. *The quaternary ammonium compounds.*

and clothes, it can cause difficulties when these are introduced into a sewage disposal system where one wants bacteria to grow. Most of the anionic and nonionic detergents have little antiseptic value, but cationic detergents or cationic wetting agents—cationic meaning that they carry a + charge and thus move to the negative pole or cathode—frequently have antiseptic properties. These include the *quaternary ammonium compounds* (Fig. 13-1), in which $R_{1,2,3,4}$ are carbon-containing groups rather than hydrogen, as they are in ammonium salts. The simplest would be tetra methyl ammonium chloride, which has some value. If one of the methyls is replaced by a cetyl group (to yield cetyl trimethyl ammonium salt) and the X^- is a bromide ion, the compound is called "Cetrimide," "CTAB," or "Cetavlon" (trade names). If one of the methyls is replaced by something a little longer (an alkyl group) and another by a benzene group, the compound, dimethyl alkyl benzyl ammonium chloride, is known by the trade names "Roccal," "Zephirol," "Benzalkonium chloride," and others. In general, at least one of the four R's should be a carbon chain from C_8 to C_{18}. These compounds were originally examined closely because they had activity in the presence of serum.

The substances act more readily against the gram-positive bacteria. For these it requires 0.1 to 0.5 mg/ml compared to 1 to 20 mg/ml required to kill gram-negative organisms. These substances seem to exert their action in two ways. One is that they all lower surface tension, which means that they tend to accumulate at the cell surface. The other is that they somehow react (we are not quite sure how) with the cell

membrane leading to a leakage of constituents from the cell. This has been used in biochemical studies to "chemically" dissolve the cell at suitable intervals to obtain the cell contents. Some biologists believe that even at concentrations lower than those capable of causing leakage, there is an effect on the oxidative enzymes and that these enzyme inhibitions are capable of preventing growth even if they do not kill. Of course, since many of the oxidative enzymes are located in the cell membrane, their inhibition may prevent the normal functioning of this structure even before gross evidence of damage, such as leakage, is observed.

The principal uses of the quaternary ammonium compounds are cleaning eating and drinking utensils, especially dishwashers and food-handling machinery. While they are relatively nontoxic, they are affected by high levels of organic matter and hence are of little use in tissues. While effective for gram-positives at from 1/500 to 1/1000 concentration, they are not particularly effective against gram-negative organisms and thus do not offer adequate protection against enteric diseases.

Aliphatic Alcohols

Ethyl alcohol, and to a lesser extent isopropyl alcohol, seems to act mostly by dehydrating and thus denaturing cell protein, and this action leads to some curious consequences. First, in spores, where the water balance has already been stabilized, alcohol has little effect. Spores have survived for 20 years in 70–100% alcohol, so although alcohol has some activity on vegetative cells, it has essentially none on spores. Second, proteins are most unstable to external influence when they are partially hydrated, i.e., fresh egg white will readily coagulate on heating, but dry egg white can be heated longer before coagulation. A little water makes the proteins more unstable than either a lot of water or no water at all. As such, 70–95% alcohol is more effective than, say, 40% alcohol or absolute (100%) alcohol.

The activity of ethyl alcohol has been known since the time of Koch, during the second half of the nineteenth century. At this time it was known that 70% alcohol was better than 95% alcohol, and thus, for not exactly scientific reasons, 70% alcohol came to be accepted as the best concentration. Actually, it appears that for many organisms 50–75% is quite good; for others 60–95% is also good. There is ample evidence to show that alcohol is effective in reducing the bacterial flora of skin (not removing spores, however), and since it is quite volatile, it leaves the skin dry almost immediately. At 70% it is thus used with safety on the skin for preoperative treatment, but it is not much used on instruments (except clinical thermometers) since it lacks action on spores. Isopropyl alcohol is about as effective as ethyl alcohol and is widely used as "rubbing alcohol." Other alcohols are less effective.

Aldehydes and Gaseous Sterilization

It was discovered that formaldehyde in solution was toxic; "formalin," a 30–40% (usually 37%) solution stabilized with methanol, or paraformaldehyde (or "paraldehyde"), a polymer of formaldehyde, became useful disinfectants in the early 1900's. The action seemed to be to combine with amino groups in proteins and seemed to resemble the tanning process. However, it also hardened the skin and, since formaldehyde was a good reducing agent, it frequently caused bleaching.

When it became evident that some infectious diseases were air-borne, there was naturally a great desire to sterilize the air. In the late 1800's the practice of fumigating the sickroom was widespread (and still lingers in some communities). Sulfur dioxide and chlorine were first tried but even when used as dilute gases they damaged many materials in the rooms. Such

fumigation, even with formaldehyde, which replaced these gases in about 1895, often failed to sterilize, and from about 1910 on, the practice decreased. By this time more effective ways were found to treat infectious diseases and sheer cleanliness was demonstrably quite effective.

However, there are many materials which could be conveniently sterilized by gas—instruments, radio sets, plaster casts, phonograph records, shoes, plastics (as plastic petri dishes), arteries and "catgut" for surgical use, oil paintings, books, to name a few—and some, indeed, in no other way; so formaldehyde is still used for this purpose on materials that it does not react with. The usual practice is to pack the materials into a chamber such as an autoclave, which is filled with formaldehyde vapor, and leave them for 6–12 hours. The procedure only works with a few materials since the penetrating power of formaldehyde is poor.

Other gases have proved to be better. The principal one in use today is **ethylene oxide**, which acts presumably in a manner similar to formaldehyde by combining with amino and sulfhydryl groups. Ethylene oxide has high penetrating ability, penetrating even into eggs through the shell, and while it reacts with a few things (streptomycin and some vitamins, but not with penicillin), it is becoming widely used as a sterilizing agent. It has several disadvantages. First, its action is slow and exposure must be 6–8 hours. Second, it is flammable, i.e., it will burn; this is overcome by diluting it with carbon dioxide or fluoridized hydrocarbons. The third disadvantage is its toxicity to man. Its inhalation toxicity is not too high—about of the same order as ammonia; but one must, for example, provide for the rapid removal of the gas from the atmosphere when it is released from the autoclave. The substance is also a vesicant, i.e., it will blister the skin on contact. It has the tendency to remain in "solid solution" in some things—especially rubber, and these materials can become irritants when subsequently used next to the skin. It cannot be used on most liquids. However, gaseous sterilization opens ingenious possibilities. The sterilization of many kinds of materials may be accomplished with very simple apparatus. Ethylene oxide, with the proper amount of dilutant, can be packaged in pressure cans, such as used for insecticide sprays, and the proper amounts may be released into steel barrels or even plastic sacks, in which the material to be sterilized is packed. Much of the complicated machinery required for present-day sterilization may be largely replaced by more simple devices.

Other gases such as methyl bromide, an insecticide, chlorpicrin, a soil fumigant, or ethylene sulfide have been proposed and worked on, but so far only one, beta-propiolactone, seems to be better than ethylene oxide, largely because it has less persistency. Its safety has still to be worked out.

Ozone (O_3) is bactericidal and has some limited application, but it is difficult to generate and handle and is damaging especially to rubber. In common with all gaseous agents so far devised, it is irritating to the eyes in very low concentration.

Halogens

Of all the halogens, only chlorine and iodine are of practical use. Bromine is strongly irritant and difficult to handle; fluorine is even more so. The salts of the halogen acids (HCl, HI, HBr, and HF) are not bacteriostatic except for the fluorides. The use of fluorides in drinking water and in teeth treatment is not based upon any bacteriostatic property but upon the construction of a more resistant tooth enamel when fluorides are available in the proper concentration.

Chlorine has a long history as an antiseptic and disinfectant, and around the 1790's was introduced as a fumigant in hospitals—note that this was before there was any bacteriology. It was used too as hypochlorites as a liquid disin-

fectant, called "eau de javelle." Today it is useful for disinfecting water and sewage, for the disinfection of dairy equipment, dishes, glasses, etc. in restaurants, and as a household "bleach" and "disinfectant." Chlorine gas as such is used only industrially (as in water treatment plants); elsewhere hypochlorites are used. Actually, they amount to the same thing, since chlorine reacts with water to produce hypochlorites:

$$\underset{\text{chlorine gas}}{Cl_2} + H_2O \longrightarrow \underset{\text{hypochlorous acid*}}{HClO} + \underset{\text{hydrochloric acid}}{HCl}$$

$$\downarrow \text{ slowly}$$

$$HCl + [\tfrac{1}{2}O_2] = \text{"nascent oxygen"}$$

* Its salts are hypochlorites.

If gaseous chlorine is run into slaked lime, $Ca(OH)_2$, calcium chlorides and hypochlorites are formed:

$$Cl_2 + Ca(OH)_2 \longrightarrow Ca\begin{matrix}OCl\\Cl\end{matrix}$$

or a mixture of the above plus

$$CaCl_2 \quad \text{and} \quad Ca(OCl)_2$$

These also decompose to yield "nascent oxygen." The calcium hypochlorites are called "bleaching powder" or "chlorinated lime." Similarly, sodium and potassium and other hypochlorites which are more water-soluble are prepared and used, for example, in the disinfection of swimming pools, etc. "Dakin's solution," a mild antiseptic used in wound treatment, is a solution of calcium hypochlorite, sodium carbonate, and boric acid.

In addition, there are some organic chlorine compounds, collectively called chloramines (Fig. 13-2), which are not as reactive chemically and thus are bactericidal for long intervals and can be carried, for example, on explorations and used to disinfect drinking water. The action of chlorine in killing or inhibiting microorganisms is due to the nascent oxygen, i.e., probably oxygen before it combines with itself to form

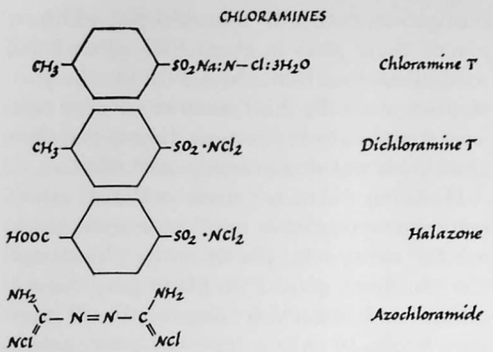


FIG. 13-2. *The chloramines.*

O_2, possibly in the form of the free radical, "—O." The same "—O" is responsible for its bleaching properties. The concentrations and amounts of chlorine employed vary from 0.5 to 20 parts per million (mg/l) of free chlorine, depending on the conditions. In addition to the *p*H, the main condition influencing the amount required is the organic matter content; both the chlorine and the oxygen resulting from its action with water react readily with organic matter, and are thus used up. In fact, one of the great values of chlorine treatment as the final step of sewage treatment is that it provides an extra "shot" of oxygen to decompose any last traces of organic matter present.

Iodine. As tincture of iodine (2.5% I_2, 2.5% KI in 90% ethyl alchohol, or 2% I_2 in 70% alcohol) this halogen has been a household disinfectant for wounds, cuts, and scratches, for fifty years. It acts by direct iodination of proteins or other organic matter rather than principally by oxygenation, as does chlorine. It is essentially as active against spores as against vegetative cells and is also active against viruses, fungi, and yeasts. It is irritating and toxic to tissue, but not forbiddingly so. It can be used on the skin (but not internally) without appreciable damage except for certain individuals who are allergic to iodine and may be killed by shock from very small amounts of it. On

cuts it stings, and, of course, leaves a stain. There is, therefore, an array of iodine compounds and complexes which retain its wide range of microbial toxicity but reduce its toxicity to tissues. One of the oldest of these, iodoform (CHI_3)[1] is widely used. It has a characteristic "hospital" smell but, curiously, by itself it seems not to be antibacterial. In contact with the body it slowly releases iodine.

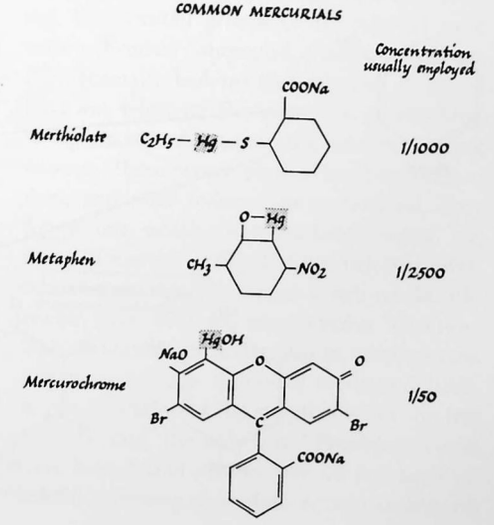
Heavy Metals

A clean penny or a clean dime on the surface of an agar plate seeded with *Staphylococcus aureus* and incubated for 24 hours will show a zone of inhibition about the metals. This is the so-called **oligodynamic action** of metals (*oligos* = Greek for small, *dynamis* = power), a word seemingly perpetuated because of its peculiar nature rather than for any meaning it may have. While most heavy metals (i.e. copper, mercury, etc.) have some slight toxicity, the two important ones are mercury and silver; arsenic, antimony, and copper are poor seconds, but useful in special cases. Their action occurs due to the solubility and ionization of the metal to the dissolved form. Since the metals are hard to dissolve, organic substances with the metal as part of the molecule have been employed, rather than salts themselves. While one may see descriptions of their action as all "combining with —SH groups," they are sufficiently different to warrant separate consideration.

Mercury is toxic in the form of salts and organic mercurials. Mercury chloride ($HgCl_2$, bichloride of mercury) is a standard laboratory disinfectant and while in 1 part to 1000 (1 g/l) it is somewhat irritating to the skin, it is useful for washing down table tops, soaking pipettes, etc. In 1881 Koch popularized the use of mercury bichloride by showing that it readily prevented the development of anthrax spores. The medical world assumed that it now had in its hand an all-powerful germicide that would destroy any disease-producing organism. But by 1900 it was evident that the mercuric bichloride was not directly lethal, for it did not kill the spore; rather, it prevented its development, i.e., it was bacteriostatic. Further, this bacteriostasis could be eliminated and the organism grown again by materials (sulfides, for example) which would combine with mercury. Bichloride of mercury had some additional disadvantages: it was cumulative, it corroded metal instruments, its action was diminished by organic matter and serum, it could not be used internally, and when used externally, as on the hands, over long periods of time it caused dermatitis. Other compounds of mercury, especially organic compounds, were found which were useful. A few of the common ones are shown in Fig. 13-3. The organic mercurials are all inactivated by sulfides, but are less irritating to the skin than mercuric chloride. Most are colored and appear to act by slowly liberating mercury salts in the tissue.

[1] It is of interest that the comparable compound of chlorine, chloroform ($CHCl_3$), has no antibacterial properties but is a potent anesthetic.

FIG. 13-3. *Useful organic mercurials.*



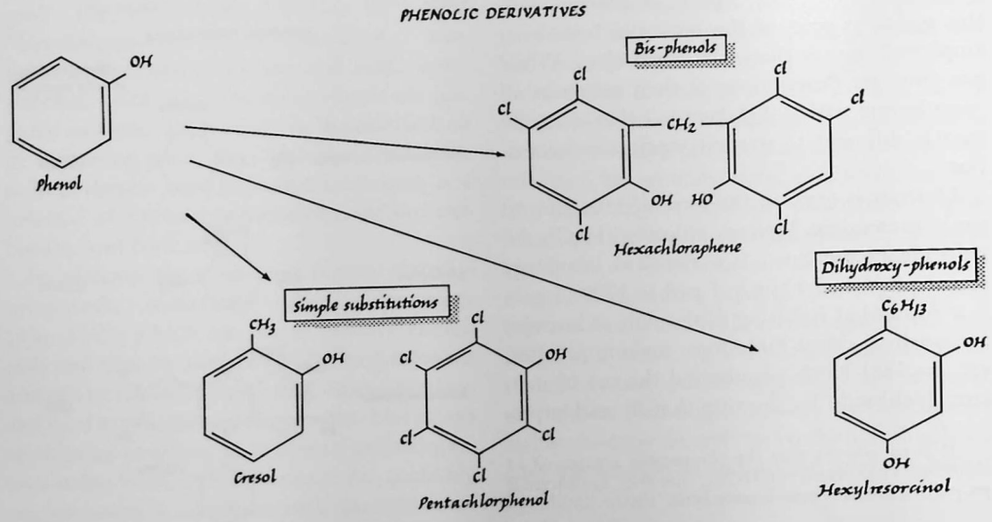
Silver. The mode of action of silver is not known, but there is no doubt that certain kinds of colloidal silver are effective disinfectants in water and on solid surfaces, and are nontoxic to man in even rather high concentration. Silver nitrate on reaction with tissues produces colloid silver in the form of a black deposit and nitric acid, both of which are toxic, but surprisingly, if used dilute enough the nitric acid has little effect. At a dilution of 1/1000 silver nitrate is bactericidal to most organisms. It is a requirement of law that new-born infants receive a few drops of 1% silver nitrate in their eyes to prevent blindness caused by possible gonorrheal infection of the eyes. But colloidal silver preparations are used widely without any real explanation for their effectiveness.

Oxidizing agents having some bactericidal properties are hydrogen peroxide and potassium permanganate. But they are rapidly destroyed by tissues and also are essentially ineffective in the presence of much organic matter.

Phenolics. The parent compound of this group—phenol or "carbolic acid"—is one of the earliest germicides and was used by Lister in 1867 for the development of antiseptic surgery; it is still used as a standard (see phenol coefficient, p. 247). But its name "carbolic acid" has really come to be associated with its property of burning tissues; while it is mildly anesthetic, it is highly corrosive. Further, its activity is markedly influenced by the concentration, temperature, and *p*H. There have been two types of work on the phenol-like compound: one has been an effort to find more active inhibitors by substitution into the phenol structure and attempts to obtain decreasing corrosiveness while retaining germicidal action; the other has been a careful study of the factors which affect its action.

Of these substituted structures, three general families have found wide use. The first are the bis-phenols (Fig. 13-4), relatively mild but nonirritating phenols used widely in soaps ("hexachlorophene" or other fancy names). The second group are the dihydroxy-substituted phenols, of which hexylresorcinol, used in mouth washes, gargles, and cough-drops, is the

FIG. 13-4. *Useful phenols.*



prime example. The third group are the carbon-substituted phenols, of which cresols ("Lysol," etc.) are the major examples.

Other Substances

Among other materials having antibacterial action, one might mention certain dyes; crystal violet and acriflavin, for example, but their action is really too weak to be of practical value. Sulfur (and sulfur dioxide) are sometimes used, but again their action is not impressive. Volatile oils and spices have some antiseptic activity and the widespread use of the latter in cooking was of great importance in the Middle Ages, where the presence of spices not only covered up the smell and taste of advanced decay but also tended to prevent it.

Preservatives, agents preventing the deterioration of materials of economic value, were of interest to the ancients—and not only in embalming; with modern civilization they are of even more importance. The deteriorating agents are all called "mildew," although the organisms may vary considerably. For the preservation of wood and cotton, copper derivatives (as copper naphthenate, copper-8-hydroxyquinoline) and the bis-phenols (dichlorophene and hexachlorophene), cresols (and tars), salicylanilide, pentochlorphenol, and mercaptobenzothiazole are widely used. For wool and other protein fibers, fluorides, salicylanilide, and organic mercurials are used. Paranitrophenol and β-naphthol are useful for leather. Even paints have preservative added: zinc oxide, mercurials, chlorphenols, etc.

Substances Which Are Antimetabolites

By a **metabolite** we mean any substance which is involved in the biochemical processes of the cell. The most important ones for our purpose are those present in the smallest amounts and those which are also concerned with synthetic reactions. Each such metabolite has a chemical structure, and its action, its function, what enzymes it will adsorb to, and what happens to it in the body depends upon the chemical structure. If one makes a slight alteration in the chemical structure of a metabolite, the normal biological activity is usually lost, but in the case of some alterations the new compound prevents the action of the normal metabolite and thus becomes an **antimetabolite.** There are many illustrations of this, but the one best suited for our purpose is a very practical one—the sulfonamide antagonism.

The development of sulfonamides. It would be nice to be able to say that knowledge of biochemical intermediates in the reactions in the body and in the bacteria finally culminated in the discovery of a very useful therapeutic drug. But this would not be true. The firm of Hoechst in Germany, the same firm indeed that supported Ehrlich in the early 1900's, set out, in the early 1930's, on what was then a daring conception: they set out to find drugs effective against disease in an animal rather than against organisms in a test tube. For this purpose they set up an experimental streptococcal infection in mice and then "screened" compounds in essentially an empirical manner. In this screening, they treated groups of 10 infected mice with a chemical compound, chosen at random; they generally had no idea whether it would have any effect on the organisms at all. After trying many substances (the number is not known), these people concentrated on the azo dyes, and after trying over a thousand, they found one which they marketed under the name "Prontosil" (Fig. 13-5) which was effective when injected into mice which would otherwise have died of streptococcus infection. This substance was active also in streptococcus infections in man. Curiously it showed little, if any, inhibition of the streptococci in the test tube. In fact, the only way "Prontosil" could have been found was by way of just such an animal screening procedure as was employed,

since it was not active in the test tube. This work was reported by Domagk in 1935. In that same year bacteriologists at the Pasteur Institute in Paris found that if "Prontosil" was reduced, it was still active in the body, but was now active in killing the bacteria in the test tube. The active agent proved to be sulfonamide (Fig. 13-5).

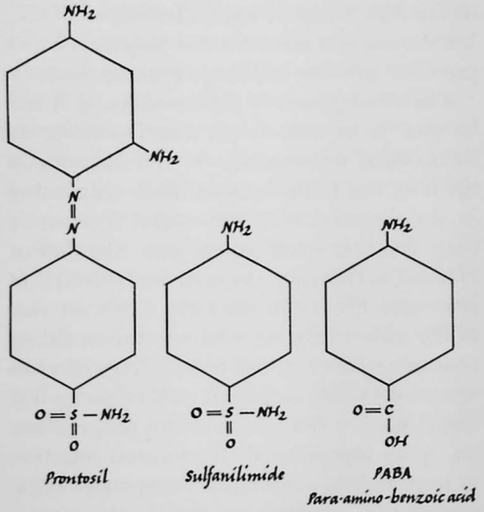


FIG. 13-5. *Prontosil, sulfonamide, and PABA.*

The action of sulfonamide. The discovery of the sulfonamides revolutionized two areas related to medicine. First, in the practice of medicine, an array of previously dangerous diseases came under reasonable control, especially pneumonia. The entire therapy of this disease was based on serum treatment and the whole practice of pneumococcal typing—in fact the entire approach to therapy of the disease—changed. Second, the entire field of **chemotherapy** opened up. This is the treatment of infectious disease by adminstering chemical substances which kill the invading organism without harm to the host. Until this point, there had been much discussion of the theory of chemotherapy, and indeed, some noteworthy progress: Salvarsan in 1910, Acriflavin and Emetine in 1912, Plasmoquine in 1926, Carbarsone in 1930, Atabrine in 1932; but all of these were active against protozoan or helminthic diseases or those caused by higher bacteria and all were slow-acting and not spectacular. But sulfonamide was dramatic and relatively nontoxic; persons with pneumonia, for example, with high fever and indeed at death's door, who formerly would have been hospitalized for months if, indeed, they could be saved at all, were out of the hospital in one week and could even be treated at home. At last chemotherapy was a reality, and the hunt for new drugs was on. It was not stopped by the war; the latter only added urgency to possibility. Further, it appeared that revolutionary drugs could be found by strictly empirical methods; one could bypass the long slow efforts to understand the action of living cells and progress directly to therapy of disease. Simple modifications in the molecule of sulfonamide itself had yielded 5,488 derivatives by 1945, all tested in animals against a variety of diseases. Of these, a few became of widespread practical use; these are illustrated in Fig. 13-6.

Sulfonamide was found in 1935. It was soon known that its activity in the test tube varied greatly with the media employed and that on some "rich" media, it had little, if any, inhibitory action. These media contained a material present especially in peptone, in tissue extracts (as brain, heart, or yeast extracts), and in pus, which abolished the effect of sulfonamide. This material was identified as p-aminobenzoic acid or PABA (Fig. 13-5) by Woods in 1940. The relatively close structure of the two suggested that sulfonamide acted by replacing PABA, that PABA had a function in the bacterial cell which sulfonamide prevented, that, indeed, sulfonamide was an antimetabolite of PABA. This was further probable since the activity of sulfonamide depended on the amount of PABA; at a

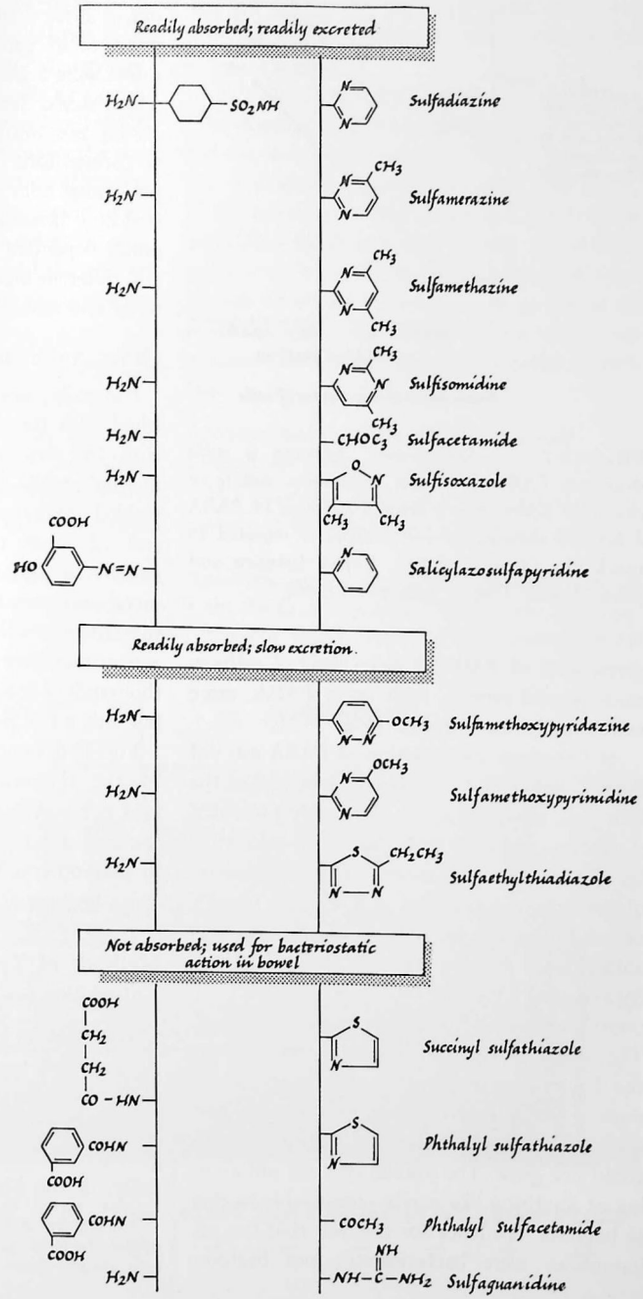


FIG. 13-6. *Clinically useful sulfa drugs.*

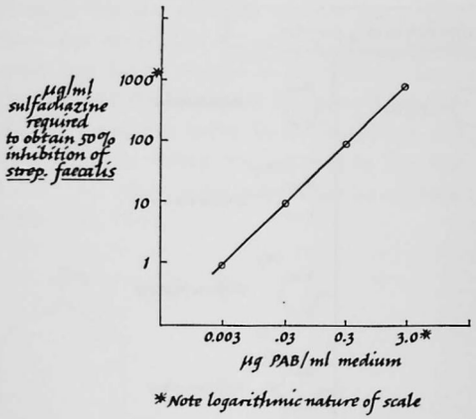


FIG. 13-7. *"Antagonism" between a sulfa drug and PABA. Note the logarithmic nature of the both scales. For a tenfold increase in PABA a tenfold increase in sulfadiazine is required to reach the same inhibition.* [*After Lampen and Jones,* J. Biol. Chem., **166,** *435 (1946).*]

given level of PABA, a given level of sulfonamide would inhibit; with more PABA, more sulfonamide was required (Fig. 13-7).

At this stage, the function of PABA was not known, but later it was shown to be part of the folic acid molecule and sulfonamide prevented the incorporation of PABA into folic acid. Here lay the secret to the most interesting property of the sulfonamides; that is, they were capable of inhibiting the bacteria in the human and animal body without harm to the body. Here, for essentially the first time in a synthetic compound, was specificity high enough to be useful. There were two reasons for this specificity. One, the bacteria were growing; they were making more folic acid, and when sulfonamide prevented their maunfacture of folic acid, they could not grow. The animal cell was not growing at anything like a rate comparable to that of bacteria. Evidence for this was that the sulfonamides were bacteriostatic, not bactericidal.[1] Folic acid would permit growth in the presence of sulfonamides. Two, the sulfonamides were active mostly against those bacteria which made their own folic acid from PABA. It did not inhibit organisms which required preformed folic acid. The animal cell required preformed folic acid and hence was not inhibited by sulfonamide. The action of the sulfonamide depended upon the different growth rate and different metabolic pathways between bacterial and animal cell.

Other Antimetabolites: Chemotherapy

Naturally, research in chemotherapy flourished with the stimulus of sulfonamides and with the demonstration that (1) empirical methods, while they might be wasteful, at least yielded results where other methods did not; and (2) that there was a rational basis—namely, antagonists designed on the basis of metabolite structure—for the design of new substances which one might hope would be active therapeutically. Literally hundreds of thousands of compounds were made and tested, but only a few proved to be of any use.

Fig. 13-6 shows most of the substances out of the thousands tested which proved to have some clinically useful antibacterial or antimicrobial activity. Others, not listed, proved to have other actions and some became useful drugs because of their pharmacological action.

Methods of Testing and Evaluating Antiseptics and Disinfectants

There are, in reality, several kinds of properties we wish to measure. These are: (1) the ability to prevent growth of organisms (antiseptic); (2) the ability to kill organisms already grown (disinfectants); (3) the spectrum of activity, or the kinds of organisms a substance

[1] As defined in Chapter 12, bacteriostatic means to inhibit bacterial growth; bacteriocidal means to kill cells already grown.

acts upon; (4) the toxicity, or the ability to be used in or on living tissues. The testing of antiseptics and disinfectants is somewhat complicated by the influence of a variety of factors: the multiplicity of bacterial species and strains and their varying degrees of resistance, the time and temperature of tests, the nature of the culture media employed, the concentration of the chemical and its reaction with materials other that the organism concerned, and the relation (if any) to the conditions under which the material is to be used in practice.

Measurements of the Ability to Prevent Growth (Antiseptic, Bacteriostatic)

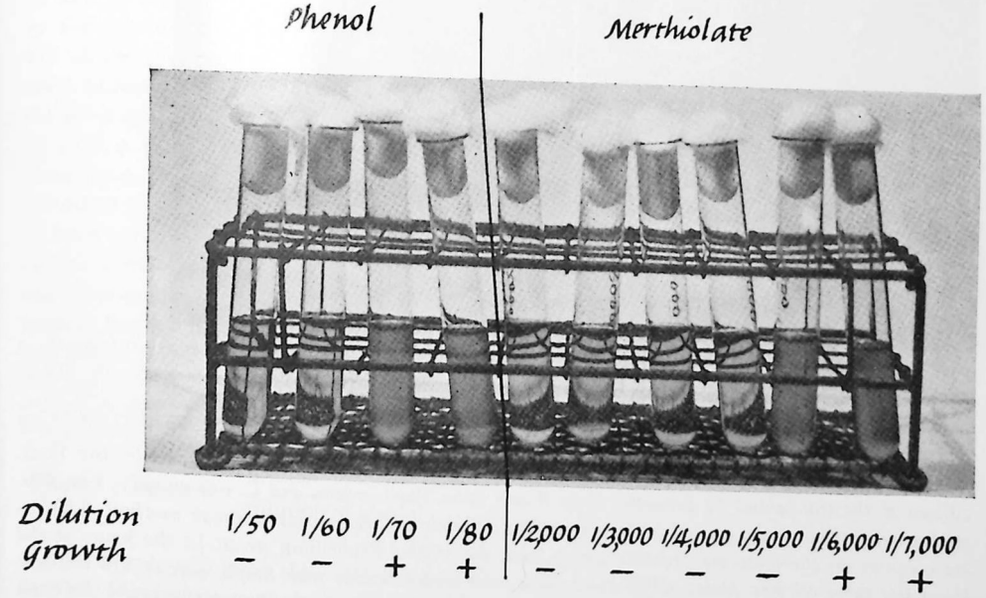
In basic principle, we provide a system whereby the organisms can grow, and determine the concentration of substance required to prevent the growth. In order that results may be comparable from laboratory to laboratory, the strains, conditions, etc. are controlled as much as possible.

Of course, one of the easiest methods is a tube dilution, as illustrated in Fig. 13-8, where the method is illustrated in detail. But there are additional simpler methods: cup (Fig. 13-9), streak, and filter paper disc methods of estimating ability to inhibit growth. All of these are in wide use both for the evaluation of compounds which are known to have an action on microorganisms and to search for new compounds which might possess valuable properties.

Measurements of the Ability to Kill Organisms Already Grown

We grow the organism to be studied in the absence of the substance to be tested, and then

FIG. 13-8. *The tube dilution method. In this experiment* Staph. aureus *was inoculated into all tubes. The first four on the left contained phenol in the dilutions indicated. Those on the right contained merthiolate in the dilutions indicated.* [*From* Research Today, *Vol. 7, 1951. Courtesy of Eli Lilly and Co., Indianapolis, Indiana.*]



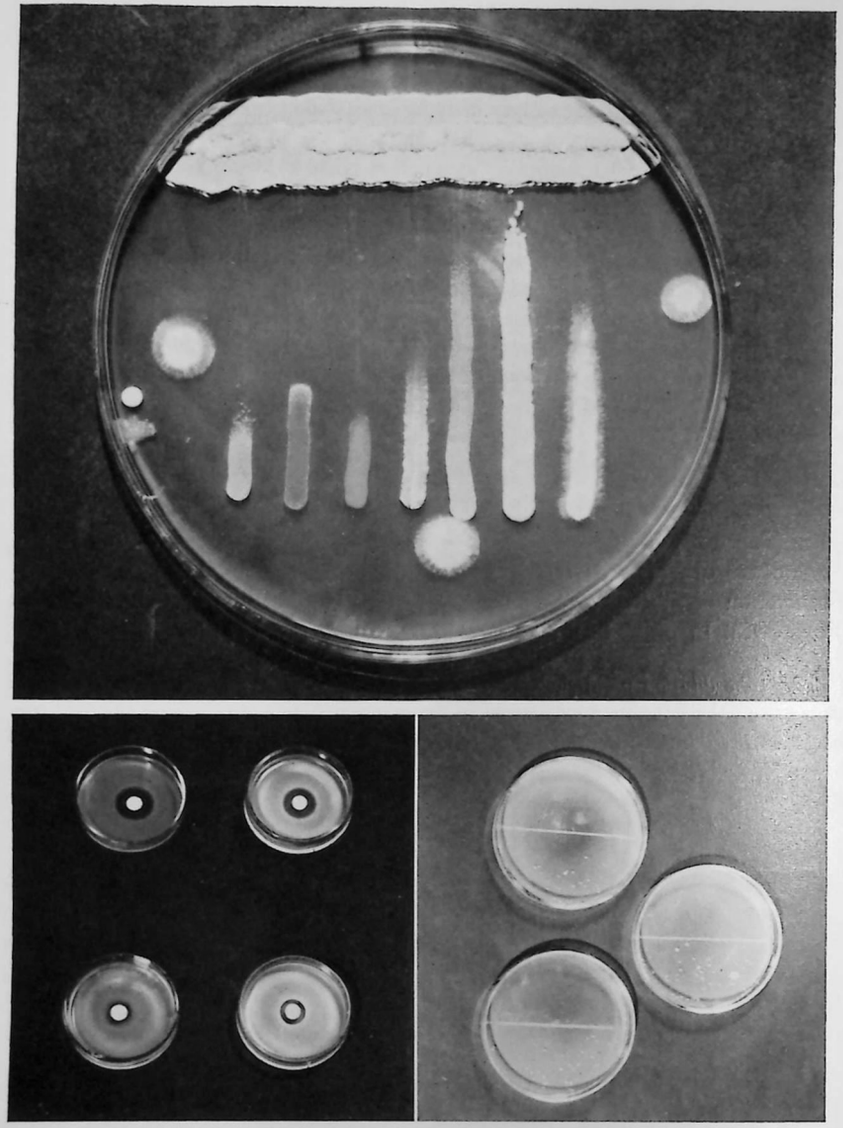


FIG. 13-9. *Three types of methods for estimating bacteriostatic effect. In the top photograph, a culture of a mold, a* Penicillium, *has been streaked across the top and allowed to start growth. Other organisms have been streaked perpendicular to it. They are, from left to right,* Staph. aureus, E. coli, *and* Brucella bronchoseptica (*all markedly inhibited*), M. tuberculosis, Saccharomyces, Candida, *and* Trichophyton; *the last two are pathogenic fungi. The bacteriostatic material produced by the* Penicillium *at the top cannot be penicillin since it acts upon* Staph. aureus *and* E. coli *equally. The substance, on further study, proved to be the antibiotic Patulin. This is a simple streak method. The extra colonies on the plate are probably spores from the original* Penicillium *streak. In the figure at the lower left there are four plates, all of which have been seeded heavily with* Staph. aureus. *On the surface of the plate a filter paper disc has been dipped in the bacteriostat. At the top this is a 1/1000 dilution of Merthiolate; at the bottom a 1/1000 dilution of Zephiran. In the lower right are three plates, half of each of which has been painted with the bacteriostat and the plates then exposed to air before incubation. The upper plate is Metaphen, the middle plate is Zephiran, and the lower Merthiolate. (From* Research Today, *courtesy of Eli Lilly Company.)*

place the grown cells in contact with the substance, measuring the rate of killing. It is the measurement of the rate of killing that is difficult. We can only determine whether a bacterial cell is alive by determining whether it grows. And we must be sure that the toxic chemical has been removed, or its concentration so lowered that the chemical itself does not prevent growth. To determine killing, we may, for example, set up a series of tubes containing organisms and at intervals, after contact with the chemical, dilute with media and determine the time required to completely kill *all* of the organisms in a tube. Or we may make plate counts at intervals.

Phenol coefficient: A somewhat simpler method is the phenol coefficient. In this method a series of dilutions of the chemical to be tested are mixed with the test culture at a constant temperature. At intervals (5, 10, 15 minutes, etc.) we add some of the chemical-culture mixture, for each dilution of the drug, to subculture tubes, and note the time required to kill the organisms so that no growth appears in the subculture. This is actually the time to kill all the organisms in the volume of sample used to inoculate the subculture. A loopful or 0.1 ml is commonly used. Of course, the results of such a method can vary considerably, depending on the amount of sample used, etc. An effort was made to overcome the variability in such conditions by comparing the activity to the activity of phenol. We determine (1) the dilution of the chemical (not its concentration, but its dilution!) required to kill in 10, but not in 5 minutes; (2) the dilution of phenol required to do the same thing under the same conditions.

$$\text{Phenol coefficient} = \frac{\text{dilution of disinfectant}}{\text{dilution of phenol}}.$$

An example will make this clear, using a culture of *Staphylococcus aureus* (Table 13-2).

TABLE 13-2. *Growth Subcultures*

Staphylococcus aureus	5 min.	10 min.
Phenol 1:50	0	0
1:60	+	0
1:70	+	+
1:80	+	+
$HgCl_2$ 1:1000	0	0
1:5000	0	0
1:10,000	+	0
1:20,000	+	+

The dilution of $HgCl_2$ which kills in 10, but not in 5 minutes (growth at 5 minute samples, no growth at 10) is 1/10,000; the dilution of phenol which does the same is 60. The phenol coefficient is therefore 10,000/60 = 166.

Table 13-3 shows the action of an unknown sample on *Salmonella typhosa* (at 20°C). In this case the phenol coefficient is 250/100 = 2.5. This has been for years the standard method of expressing disinfectant action and very elaborate standardized conditions, strains, and media have been specified in order to make the results have more meaning. The test works rather well for those substances much like phenol, but for others the results may be quite misleading. Although qualitatively it will generally demonstrate activity, the *degree* of activity is not generally revealed; more especially, the conditions under which it may be used in practice are not established by such a test.

TABLE 13-3. *Growth Subcultures*

Salmonella typhosa	5 min.	10 min.
Phenol 1:90	−	−
1:100	+	−
1:110	+	+
Test 1:150	−	−
1:200	−	−
1:250	+	−
1:300	+	+

Determination of Spectrum of Activity

This means merely the range of organisms attacked. For example, in Table 13-4, hexachlorophene, a material commonly used in soaps, acts at about 1/1000 to 1/5000 dilution on many organisms but is more effective on others. What is noteworthy is the high sensitivity—i.e., high dilution = less substance required to kill—for *Salmonella paratyphi* A compared with the relatively low sensitivity of *Salmonella typhosa*. There is no specific method for the determination of spectrum, but in general we use whatever method we are employing on as many organisms as is sensible for our purpose.

TABLE 13-4. *Action of Hexachlorophene**

	Concentration required to kill in 10 min (but not kill in 5 min) at 37°C
Staphylococcus aureus	1/5000
Salmonella typhosa	1/3000
Escherichia coli	1/3500
Corynebacterium diphtheriae	1/70,000
Pseudomonas aeruginosa	1/1000
Salmonella paratyphi A	1/30,000
Shigella dysenteriae	1/3000

* Data selected from A. R. Code and W. S. Gump, p. 329, in Reddish, G. F.; see references.

Determination of Effectiveness

We should recognize that the methods employed for the determination of effectiveness of any antiseptic or disinfectant must vary with the use to which the material is to be put. If we are using a substance to prevent mold growth on a painted wall, certainly the methods used to find out whether or not it is effective, and to what degree it is effective, will differ from those employed in evaluating a substance to be used on skin for open wounds. Different properties are desired; different things are looked for. Obviously the main test of any agent is that it does the job claimed for it and that it is not destructive of the material treated nor is otherwise dangerous to use, i.e., that there is little if any chance of it harming plants, animals, or man. We don't sterilize milk pails with sulfuric acid, nor use mercuric chloride on drinking glasses. Different situations demand different approaches, most of which are reasonably obvious once the situation is specified.

But there are certain special methods employed for those agents which are used on or in the body because the situation here is somewhat more complex. An agent useful in this connection must be toxic to the bacteria or other microorganisms but considerably less toxic to the living tissue of the animal or man. It is surprising that there are many simple materials—silver nitrate, iodine, mercurochrome, phenol, etc.—which have a sufficiently selective toxicity to be used *on* the body, although they cannot be used *in* it. There are agents—the sulfa drugs and certain antibiotics—where the selective toxicity is so high that these can even be used in the body.

To evaluate substances to be used on the body, we must determine not only their toxicity to the microorganism, but also their action in or on the animal (or man). This normally involves the determination of toxicity to tissue, which may be done externally by applying it to the skin and seeing what happens, with preliminary tests being made on rabbit ears or on guinea pig skin, as favorite tissues; it also involves the determination of the effectiveness when the tissue is present. That is, we must measure the bacteriostatic or bacteriocidal property under the conditions of use. It does us no real good either to know that substance Q, as part of a gargle, kills "disease-producing bacteria," even after five minutes' exposure in the test tube. What we need is information on how readily it penetrates the tissue, and kills the organisms in the throat when it can only be used for a brief interval. Tissue toxicity and effectiveness in tissue require close cooperation between the bacteriologist and pharmacologist, and in the future this type of information will and should be demanded of every agent to be used on the body surface.

To evaluate those materials to be used inside the body, certain principal things must be determined. Will it actually cure a disease? (And what are the conditions, dosage, etc. required?) What is the immediate toxicity? Are there any long-term effects, any delayed toxicity? In all of these cases, we generally begin by determining the effect of a quantity of the material on the infection of the susceptible organism in an experimental animal.

We may take *Eberthella typhosa* infection in the mouse as an example, and the action of two preparations, corrected to 100% purity, of polymyxin against this infection. As shown in Table 13-5, an inoculum of 5×10^4 organisms per mouse is lethal to half of the mice. At 5×10^7 organisms per mouse, thus dealing with 1000 times the number of cells required to kill half the mice, we see that with this size of inoculum all the mice are dead by the third day; indeed, all except one died the first day. When mice inoculated with 5×10^7 cells of *E. typhosa* are given 0.1 mg of polymyxin A, they survive. At half this amount, almost half are saved. Polymyxin D is also effective in saving most of the mice. Therefore, these antibiotics were effective in this situation.

The *E. typhosa* used was inhibited by 0.5 μg/ml of either of the polymyxins. The acute toxicity of polymyxin A, by the same intraperitoneal route, was LD_{50} 13.9 mg/kg; this is the lethal dose that will kill 50% of the animals. For mice of 20 g an injection of 0.278 mg would kill half the mice. The polymyxin preparations contain a factor which is damaging to the kidney; this has been demonstrated in rat, dog, rabbit, and man by the excretion of protein into the urine and histological section of the tissues. The antibiotic can therefore not be used internally although it is effective in curing the infectious disease. It is this kind of evaluation which is necessary before substances may be selected for use internally.

TABLE 13-5. *Effect of Polymyxins on E. Typhosa Infection in Mice**

Drug	Inoculum	No. of Mice	Day	No. Mice Surviving 1	3	5	7
None	5×10^7	18		1	0	—	—
	5×10^6	6		4	0	—	—
	5×10^4	6		3	3	3	3
Polymyxin A							
0.1 mg	5×10^7	18		17	17	17	17
0.05 mg	5×10^7	18		9	9	9	8
Polymyxin D							
0.2 mg	5×10^7	12		12	12	11	11
0.1 mg	5×10^7	12		12	9	9	9

* Antibiotics given parenterally twice daily for 3 days. After Brownlee, G., S. R. M. Bushby, and E. I. Short, *Ann. N. Y. Acad. Sci.*, **51**, 891, 952 (1949).

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For background information, see p. 494.

QUESTIONS

1. Define or identify:

selective toxicity	organic mercurials
specificity	mildew
quaternary ammonium compound	metabolite
gaseous sterilization	antimetabolite
fumigation	screening
ethylene oxide	prontosil
hypochlorite	sulfonamide
chlorinated lime	PABA
nascent oxygen	phenol coefficient
oligodynamic action	spectrum of activity

2. It has been recently shown that most cases of paint "peeling" are due to microorganisms growing on the dried oil when moisture is present. It takes several years to test out possible agents to prevent this growth if you used paint "test panels," etc. What would you do to devise a simple rapid laboratory procedure for agents which might be useful in this problem?

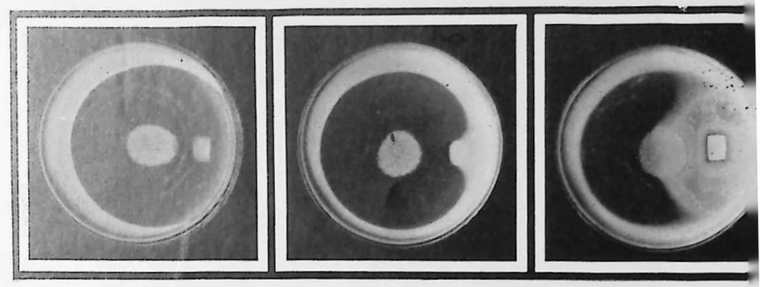
3. What kind of method would you use to *sterilize*:

tennis shoes	orange juice
milk bottles	water
wooden barrel staves	

4. Why would you wish to sterilize the above? Under what conditions would pasteurization be adequate?

5. You are employed as a microbiologist for Space Research, an organization building sensing instruments to be sent into outer space. They have devised a small radio transmitter, built of glass and metal, but containing some plastic insulators. What kind of a process could you think of to prevent this transmitter from contaminating the moon when it lands on it? Why would it be important not to contaminate the moon?

Chapter 14



THE ANTIBIOTICS

"Antitoxins and antibacterial substances are, so to speak, charmed bullets which strike only those objects for whose destruction they have been produced."

EHRLICH

Development of Knowledge

It is rare that a science is revolutionized in a generation—but this has happened more than once in microbiology. Perhaps no more dramatic and far-reaching example of such a change is the subject of antibiotics. The past generation knew them not. Today they are the miracles that are commonplace. The history of their development is not only an important landmark in the history of man, but it is part of the knowledge of microbiology.

It has been known for many years that some organisms exhibited an **antagonism** to others. One of the simplest observations can be made as shown by Fig. 14-1; in this case the organism at the top inhibits the growth of others around it. Of course, there could be many reasons for this; perhaps it grew so fast it used up all the food in the area, or perhaps it produced something which inhibited the growth of other organisms. Instances of both types of inhibition were known. In some cases, the substance produced was known to be acid—acetic acid or lactic acid, especially.

But there were other cases where it was not lack of food or production of some simple material like acids; rather, there were evidently organic substances produced by one organism which would inhibit the growth of another. In the 1930's these things were known, but really attracted little attention. To be sure, such antagonisms undoubtedly occurred in soils or anywhere that mixed populations occurred, but if

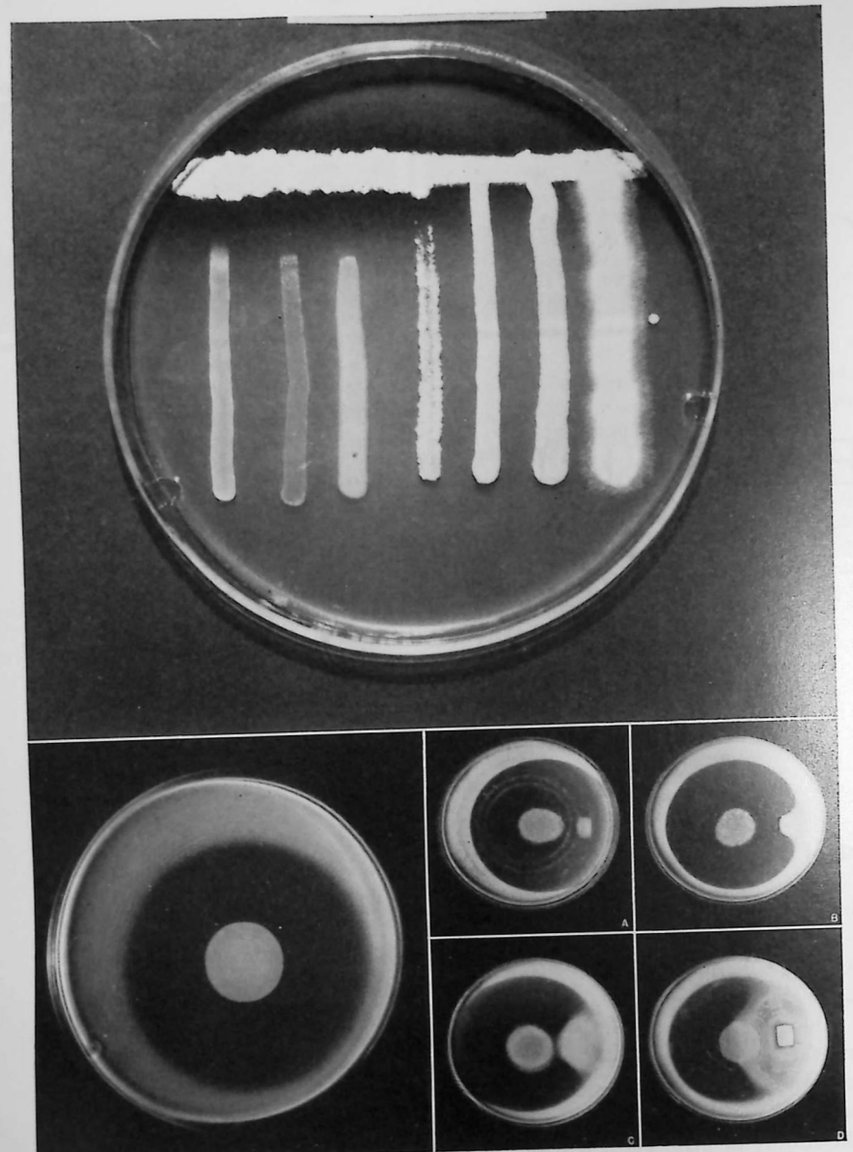


FIG. 14-1. *Antagonism between microorganisms. In the top photograph a* Streptomyces *has been streaked across the top of the plate. Perpendicular to it, seven different organisms have been streaked. The* Streptomyces *has inhibited the development of the first four organisms in the region close to its growth. In the lower left a central colony of a strain of* Agrobacter tumefaciens *(an organism causing tumors in plants) has inhibited the growth of another strain of the same organism seeded evenly over the plate. In the lower right the same method has been used but small blocks of agar containing other materials which might influence the antagonism have been added. The block in A contains glutamic acid, B alanine, C glutathione, and D phenylalanine. Glutathione has a little effect, but phenylalanine (D) completely abolishes the antagonism. [The upper figure is courtesy of Eli Lilly and Co., Indianapolis, Indiana. The lower figures are from Stonier,* J. Bacteriol., **79,** 889 (1960); *courtesy of Dr. Stonier.]*

it ever occurred to anyone that under some circumstances some of these materials might be useful in the treatment of disease, it was kept discreetly silent.

In the early 1930's the prevailing opinion was that chemotherapy was impossible. The prevailing mood was that "life is too much alike"—there could be no substance, aside from antibodies, that could search out and kill the invader without harm to the host. Of course, there were some compounds which possessed this property: atoxyl for trypanosomiasis (found in 1905), "606" or Salvarsan for syphilis (found in 1910), plasmoquine for malaria (1926), and of course quinine, known for centuries, for malaria. But all of these were protozoan diseases; even syphilis is caused by an organism which resembles the protozoa (see p. 290) in that it does not have a rigid cell wall. Besides, most of these things were really somewhat toxic; quinine was the least toxic of the lot, but still it too would cause nausea and ringing in the ears if one took too much. And it was argued too that all these, and especially quinine, acted only on a phase of the life cycle of protozoa; there could be nothing that would do anything to organisms that have no complicated life cycle.

But times were changing, and there was a new spirit in the air. Chemotherapy began in many small ways; to cite who was first and who followed does not tell the true story, since what happened was not a logical progression. And there were many "John the Baptists." But things were happening.

There was, for example, an attempt to treat pneumonia, which in the late 1920's was a real killer, by deliberately searching for and finding organisms containing enzymes which would dissolve the capsule. These did not work therapeutically since the body developed antibodies to the injected enzyme, but still it was a bold attempt. There was, too, the finding that there were substances, produced by some bacteria, which were especially toxic to the pneumococcus. There turned out to be two—gramicidin and tyrocidin. These were unfortunately too toxic to be used in therapy, but their mixture, called tyrothricin, is used externally even today. (Note how preoccupied the work was with pneumonia.)

Actually, antagonism between organisms was demonstrated in 1889 by Doehle in Germany, who published a photograph showing that the anthrax organism was inhibited by a "*micrococcus anthracotoxicus.*" In 1889, as well, it was shown that a culture of *Pseudomonas pyocyaneus* could be used to kill anthrax organisms; an enzyme, pyocyanase, was isolated from it which, while too toxic to be administered via the blood stream, could be used locally, especially by application to wounds. Furthermore, in 1896 an Italian by the name of Gosio obtained a crystalline substance from a species of *Penicillium* growing on moldy corn, which was thought at the time to be the cause of pellagra. Gosio showed that this substance, which we know today as **mycophenolic acid,** inhibited the growth of the anthrax organisms. By 1904 Frost had described at least six different methods for searching for antibiotics. By the start of the World War II a very large number of observations had been made of antibiotic substances, and methods were available for the study. But none of the antagonisms observed were of any real use and the prevailing opinion was that though antagonists and antibiotics existed, they were not usable. Then in 1935 came the sulfa drugs and they worked, so people began to look more seriously at the possibilities of bacterial antagonism.

We have said that the development of chemotherapy did not follow a logical progression. So now let us turn back a few years to Fleming and the discovery of penicillin. Fleming's paper appeared in 1929 in the *British Journal of Experimental Pathology,* a journal not normally read by most microbiologists and

not widely available even in libraries. It is therefore of some interest to examine it more closely. Its title is "On the antibacterial action of cultures of a penicillium with special reference to their use in the isolation of *B. influenzae*." The paper begins in the following fashion:

> While working with staphylococcus variants a number of culture plates were set aside on the laboratory bench and examined from time to time. In the examinations these plates were necessarily exposed to the air and they became contaminated with various micro-organisms. It was noticed that around a large colony of a contaminating mould the staphylococcus colonies became transparent and were obviously undergoing lysis.
>
> Subcultures of this mould were made and experiments conducted with a view to ascertaining something of the properties of the bacteriolytic substance which had evidently been formed in the mould culture and which had diffused into the surrounding mecium. It was found that broth in which the mould had been grown at room temperature for one or two weeks had acquired marked inhibitory, bactericidal and bacteriolytic properties to many of the more common pathogenic bacteria.

Fleming was concerned with the problem of isolating gram-negative organisms in the presence of large numbers of gram-positive organisms and he devoted about 20% of his paper to this problem. But he described methods of production, gave some indication of stability and possible routes of isolation, animal experiments showing activity in vivo, and antibacterial spectrum. The most interesting conclusions were:

> A certain type of penicillium produces in culture a powerful antibacterial substance. The antibacterial power of the culture reaches its maximum in about 7 days at 20° C. and after 10 days diminishes until it has almost disappeared in 4 weeks.
>
> The active agent is readily filterable and the name "Penicillin" has been given to filtrates of broth cultures of the mould.
>
> The action is very marked on the pyrogenic cocci and the diphtheria group of bacilli. Many bacteria are quite insensitive, e.g. the coli-typhoid group, the influenza-bacillus group, and the enterococcus.
>
> Penicillin is non-toxic to animals in enormous doses and is non-irritant. It does not interfere with leucocytic function to a greater degree than does ordinary broth.
>
> It is suggested that it may be an efficient antiseptic for application to, or injection into, areas infected with pencillin-sensitive microbes.
>
> Its value as an aid to the isolation of *B. influenzae* has been demonstrated.

Sir Howard Florey, one of the men involved in the development of penicillin, has stated that the selection of penicillin on which to work, ten years after its discovery by Fleming, was merely one of the several choices. Florey wrote in the *British Medical Bulletin*, 4: 248 (1946).

> In the early part of 1939 a wide investigation of natural antibacterial substances was planned by Dr. Chain and myself, and penicillin was one of the first chosen for investigation. In 1932, Clutterbuck, Lovell & Raistrick had published a paper in which they showed that penicillin could be produced by the mould on a synthetic medium, and that it could be extracted from water into ether if the water was made acid, but that large losses of antibacterial activity occurred on the evaporation of the ether. They had recognized that penicillin was most stable around neutrality, but had stated that it was a very labile substance, and they did not carry the work further. Based on this work, we found, with our colleagues at Oxford, a method for extracting the penicillin and making preparations which, though crude, had high antibacterial activity. After some preliminary biological experiments with these extracts, it became clear that the substance might be of exceptional interest and a team of workers was assembled in the Sir William Dunn School of Pathology at Oxford. In 1940, the first observations on penicillin from Oxford were published (Chain, Florey, Gardner, Heatley, Jennings, Orr-Ewing & Sanders), which clearly distinguished this antibiotic from any of its predecessors. They showed that artificial infections in mice with the streptococcus, staphylococcus and *Cl. septique* could be completely controlled by injections of a penicillin extract, so that there was almost 100% survival from infections which would otherwise certainly have been fatal. In other words, penicillin was a chemotherapeutic drug of great power, that is to say, it was of such low toxicity that it could, without producing any toxic signs, be injected by parenteral routes in amounts which would stop the growth of bacteria in all parts of the body. Many other investigations were made. A full account of these, together with the results of the first clinical trials, was published in 1941 (Abraham, Chain, Fletcher, Florey, Gardner, Heatley & Jennings), and the results of a larger clinical trial in 1943 (Florey & Florey). Two of the facts of especial importance which emerged from the laboratory work at Oxford were, firstly, that extracted penicillin was fully active in the presence of pus and tissue-breakdown products, and of large numbers of bacteria, all of which impaired the antibacterial action of all the sulphonamides known at that time, and, secondly, that even very impure extracts were non-toxic to animals in concentrations hundreds of times greater than those required to inhibit bacteria, and that with increasing purity of the extracts, the toxicity became still less (Florey & Jennings, 1942).

There was no reason, at the start, to expect penicillin to be of any more use than the mycophenolic acid isolated fifty years before, but it proved to be the most important drug ever found. However, there are two aspects of its emergence that should be mentioned. If it had been found in, say, 1889, and shown to be useful in infections, it could not have been produced on a scale large enough to be of much help because the technology of an aseptic fermentation on an industrial scale was not available. Second, there was, at the start of these studies, no reason to choose penicillin from among any of the other antagonists known at that time. But when once it was demonstrated that a chemical substance could actually be put into the body and completely eliminate in a few days diseases which had been highly fatal, and where recovery was previously a matter of months, naturally this stimulated the search for other antibiotics.

Types of Clinically Useful Antibiotics

Several hundred antibiotic substances have been obtained, but only about twenty are sufficiently useful in the clinic to be especially noted. However, it is difficult to classify them. They do not fall into any single chemical class, and there seems to be no relation between chemical constitution and clinical usefulness. For our purpose, it will suffice to divide the antibacterial antibiotics into five groups:

A. *Antibiotics active primarily against gram-positive bacteria.*
   - Penicillins
   - Erythromycins

B. *Antibiotics active against both types of bacteria (broad spectrum)*
   - Tetracyclines
   - Chloramphenicol

C. *Antibiotics with restricted or specialized use*
   - Kanamycin
   - Vancomycin
   - Ristocetin
   - Novobiocin
   - The polymyxins
   - Antinomycetins
   - Azaserine and DON

D. *Antibiotics with a special relation to tuberculosis*
   - Streptomycin
   - Viomycin
   - Cycloserine

E. *Antibiotics active against yeast, mold, or protozoal infection*
   - Nystatin
   - Amphotericin B
   - Griseofulvin
   - Fumagillin

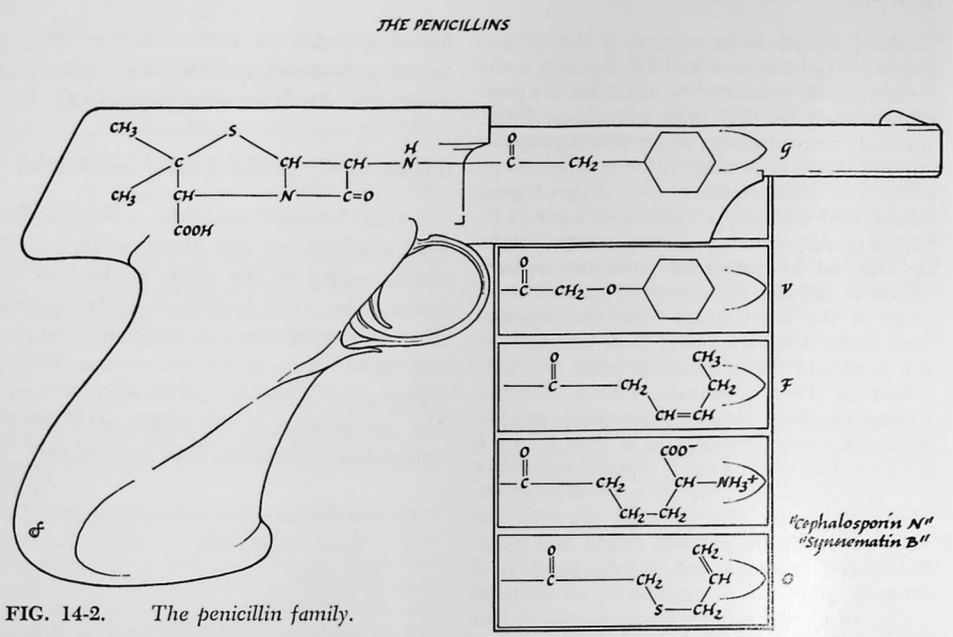


FIG. 14-2. *The penicillin family.*

Each of these will be discussed individually and relations between them noted. So far as one can now discern, this separate, individual discussion reflects precisely the separate and unique nature of each of the antibiotic groups. For this purpose we regard all of the closely related derivatives of an antibiotic as the same antibiotic, even to the extent of considering tetracycline, oxytetracycline, and chlortetracycline as the same agent. We assume further that what differences may exist between these substances in their effectiveness under one or another circumstance have to do with pharmacological matters such as penetration, distribution, and detoxification, rather than with any real difference in their action.

Antibiotics Active Primarily Against Gram-Positive Bacteria

THE PENICILLIN GROUP. It took a great deal of time and work to determine the chemical structure of penicillin, i.e. the family of penicillins; see Fig. 14-2. Much additional work was necessary to find out the best ways of producing it in quantity. All penicillins differ from one another in the group in the side chain, and it has been discovered that if we add certain intermediates to the mold culture making penicillin, we can obtain incorporation of the intermediate into the penicillin. Thus, feeding the mold phenylacetic acid substantially increased the amount of penicillin G (which has the phenyl group in the side chain). As mold strains were selected for higher potency, they tended to make more K than G, but if phenylacetic acid was supplied, then mostly G was formed. The G is more desirable than K. By use of precursors, other penicillins not normally

found in nature could also be formed. Originally only surface cultures of *P. notatum* or *P. chrysogenum* were capable of making appreciable quantities of penicillin, but submerged aerated cultures were developed. While Fleming's original culture produced only traces of penicillin, strains in use today produce several grams per liter. The **lactam ring** originates as shown in Fig. 14-3.

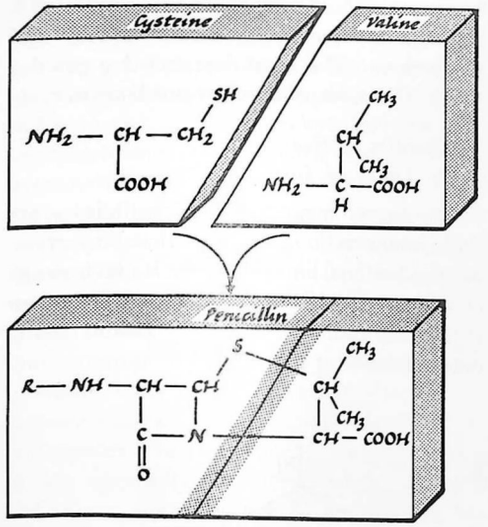


FIG. 14-3. *Origin of the lactam ring in penicillin.*

The action of penicillin is to inhibit the synthesis of a particular constituent (muramic acid) of the cell wall of gram-positive bacteria; thus, if they are not protected osmotically, they will burst. (See Chapter 3.) Some gram-negative cells are also vitally dependent upon muramic acid for their proper function and they too are sensitive to penicillin. Animal cell walls do not have and are not dependent upon this material; hence penicillin can damage sensitive bacteria without harming the animal or man.

Penicillin is highly effective in both acute and chronic gonorrhea, pneumococcal pneumonia, most infections due to streptococci, anthrax, and syphilis. It is effective against staphylococcal infections if the organisms are not resistant, but the incidence of penicillin-resistant staphylococci is extremely high, especially in the hospital environment. This limits its usefulness in these infections, resulting in the need for antibiotics which we have listed in group C.

Inadequate dosage of penicillin may result in the development of resistant strains and it should not be used topically because of this resistance development. Oral administration is somewhat uncertain but widely used, and orally effective forms have been developed—especially phenoxymethyl and phenoxyethyl penicillin. Care must be taken that the spacing of doses is proper. The penicillin should not be taken with the meal; the preferred route of administration is intermuscular doses ranging from 30,000 to 600,000 units[1] every 3 hours or every 8 hours, depending on the circumstances. Penicillin in the crystalline form is stable for several years in the dry unrefrigerated state, but solutions are unstable and should not only be refrigerated, but kept no longer than 3 days.

Because of its low toxicity, penicillin can be used widely. There are, however, individuals who are hypersensitive or allergic to penicillin. Generally the effects, even in these persons, are mild and rarely is there more than a persistent rash. However, on rare occasions a fatal shock may result.

It is doubtful whether mutation to penicillin resistance is of any greater occurence than that to any other antibiotic, but resistance development does occur, especially among the staphy-

[1] One unit of penicillin, originally defined before the pure substance was obtained, turned out to be 0.6 μg of crystalline sodium penicillin G.

lococci. There are some organisms inherently resistant to penicillin, either because the presence of muramic acid in their cell walls is not critical for them or because they possess or can develop an enzyme, **penicillinase**, which rapidly destroys the penicillin. Many aerobic spore-formers are resistant to penicillin because they possess an adaptive penicillinase. But most resistant staphylococci do not have penicillinase and these staphylococci are therefore resistant by some as yet unknown mechanism.

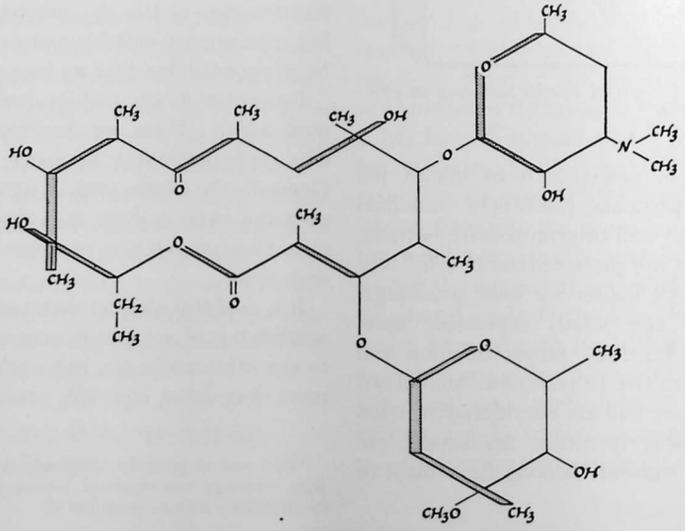
THE ERYTHROMYCIN GROUP. The four principal antibiotics in this group may be grouped together because of common chemical structure. This consists of a large lactone ring called **microlide**—hence the group is sometimes called the microlide group—together with some odd sugars, which are dimethylamino sugars of unusual structure. The chemical formula of erythromycin is given in Fig. 14-4. The antibiotics belonging to this group are erythromycin (including erythromycin B and C), oleandomycin, carbomycin or magnamycin (magnamycin B), and spiramycin (A, B, and C).

In general these antibiotics have the same spectrum as penicillin but since the latter is so effective in pneumococcal and streptococcal infections, erythromycin has its greatest usefulness in treatment of infections caused by resistant organisms. Resistance development is reasonably rapid and, when developed, is generally to the entire group; i.e., a strain which becomes resistant to erythromycin is also now resistant to spiramycin, oleandomycin, and magnamycin. The usual dose is 1–2 g per day orally. The mode of action is not known.

Antibiotics Active Against Both Types of Bacteria

The second major group of antibiotics are those whose action is not restricted to gram-positive bacteria, but which attack a wide range of organisms. These are the broad **spectrum** antibiotics, meaning that they inhibit many different kinds of bacteria.

FIG. 14-4. *Erythromycin.*



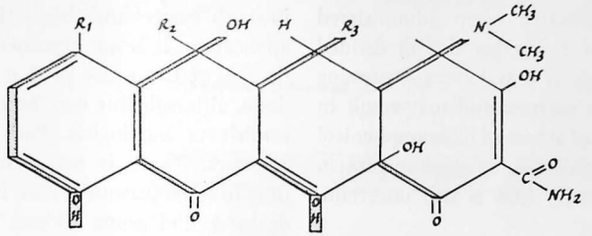


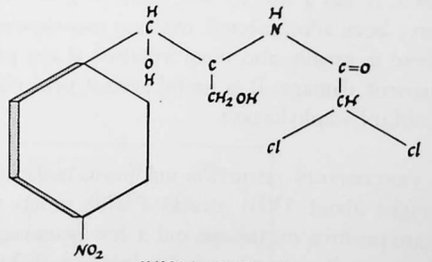
FIG. 14-5. *The tetracycline family.*

THE TETRACYCLINE GROUP. There are four substances in this group: tetracycline (also called achromycin), chlortetracycline (aureomycin), oxytetracycline (terramycin), and dimethyltetracycline; see Fig. 14-5. In tetracycline, R_2 is CH_3, the rest are hydrogens; in chlortetracycline, R_1 is Cl, R_2 is CH_3; in oxytetracycline, R_3 is OH, R_2 is CH_3; and in dimethyltetracycline R_1, R_2, and R_3 are all hydrogens. These are all broad spectrum antibiotics, attacking not only gram-positive but also gram-negative organisms. Chlortetracycline seems also effective against some of the viruses and rickettsiae. The substances are effective orally at a level of 1–2 g a day against typhoid fever, other *Salmonella* infections, gram-negative bacteria causing urinary tract infections—brucellosis or undulant fever, typhus, Rocky Mountain Spotted Fever, psittacosis—streptococcal infections, and staphylococcal infections.

While there is a mild nausea sometimes involved with these substances, the principal difficulty is the rapid development of resistance by staphylococci and the overgrowth by it and in some cases by a yeast-like fungus, *Candida*. The tetracyclines, by attacking such a wide range of organisms, markedly reduce the bacterial flora in the throat or the intestinal tract. The other organism, such as *Candida*, may then grow rapidly and cause diseases which are difficult to treat. The mode of action of the tetracycline is not known. They do, however, inhibit adaptive enzyme formation in resting cells and thus interfere in some as yet unknown manner with protein synthesis. There is, further, some experimental evidence that the tetracyclines interfere with electron transport in respiration. The tetracyclines all combine with magnesium and similar ions and tend to remove them from the reaction mixtures; thus many of the effects reported for tetracyclines are probably artifacts. How it is possible to use the tetracyclines in the animal or in man is not known.

CHLORAMPHENICOL (chloromycetin) is a relatively simple compound (Fig. 14-6), at least compared to other antibiotics, and it can be synthesized. It has a wide range of activity including some rickettsiae and viruses, and thus has a value in typhus and Rocky Mountain Spotted Fever. For typhoid fever it is the drug

FIG. 14-6. *Chloramphenicol.*



of choice. It is effective when administered orally at a level of 1–2 g per day in divided doses. Unfortunately it may have a depressing effect on the bone marrow and may result in anemia. The mode of action of chloramphenicol appears to be to inhibit some steps in protein synthesis, but precisely how is still uncertain.

Antibiotics with Restricted or Specialized Use

The third large group of antibiotics are those that cannot be widely used for one reason or another and are thus restricted in their use. They are, however, useful within the limitation imposed.

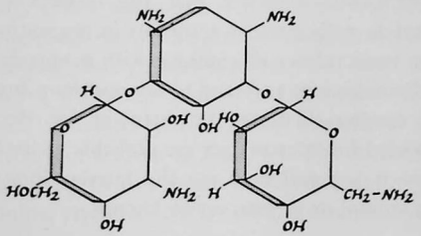


FIG. 14-7. *Kanamycin.*

KANAMYCIN (Fig. 14-7) is an antibiotic related in activity to neomycin and streptomycin, but is distinct from both. It is active against gram-negative organisms, but also against staphylococci. It is poorly absorbed from the intestinal tract and hence is injected. Three grams per day in divided doses are commonly used. It has a toxicity and when 30 g or more have been administered, deafness may develop; there is usually also renal irritation if not permanent damage. It is useful against penicillin-resistant staphylococci.

VANCOMYCIN (structure unknown, molecular weight about 3300) attacks a wide variety of gram-positive organisms and a few gram-negative ones. Resistance to it develops very slightly in staphylococci and this is therefore its major application. It is not absorbed well orally. The dose is of the order of 2 g daily in divided doses, although this may be doubled for endocarditis or meningitis. Pain on injection is common. There is easy sensitization to the drug in some persons. There is also a chance of deafness and some kidney impairment. Its mode of action is not known nor is it known why it can be used in animals or man.

RISTOCETIN (A, B, Spontin, Riston) is an antibiotic of unknown structure and is active against a wide variety of both gram-positive and gram-negative organisms. It is at present restricted in its use to staphylococcic infections resistant to other antibiotics. The dose (intravenous) is about 3 g per day in divided doses. There are harmful effects on the white blood cells. Its mode of action is not known.

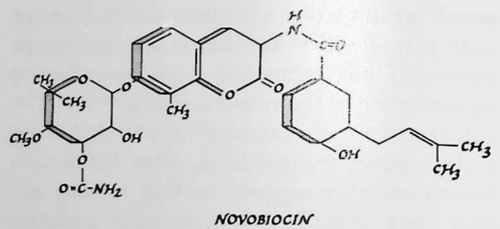


FIG. 14-8. *Novobiocin.*

NOVOBIOCIN (Fig. 14-8), also known as cathomycin, albamycin, spheromycin, vulcamycin, streptonivicin), is active against a wide range of organisms, but it has some drawbacks. Its activity is markedly decreased by serum, to which it is tightly bound, and it frequently causes drug fever. Its use is restricted to antibiotic-resistant infections, especially *Staphylococcus* and *Proteus*.

THE POLYMYXINS. These are a variety of peptide antibiotics, frequently produced by aerobic spore-forming bacteria and known under a variety of names. Tyrocidin, gramicidin, bacitracin, subtilin, polypeptin, licheroformin, aerosporin, circulin, polymyxin, are collectively known as polymyxins. They are all characteristically circular peptides. Two examples are illustrated in Fig. 14-9, the polymyxin group, and Fig. 14-10, the bacitracin group. While each differs in some aspect of chemistry, they have certain properties in common. They are more active against gram-positive organisms than against

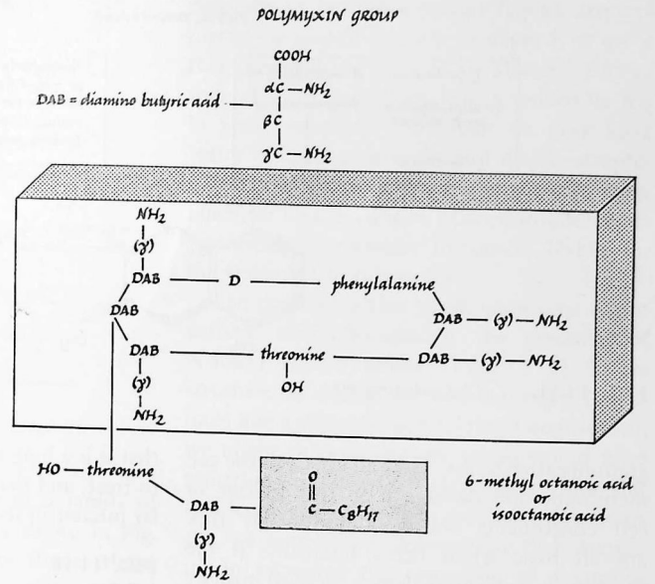
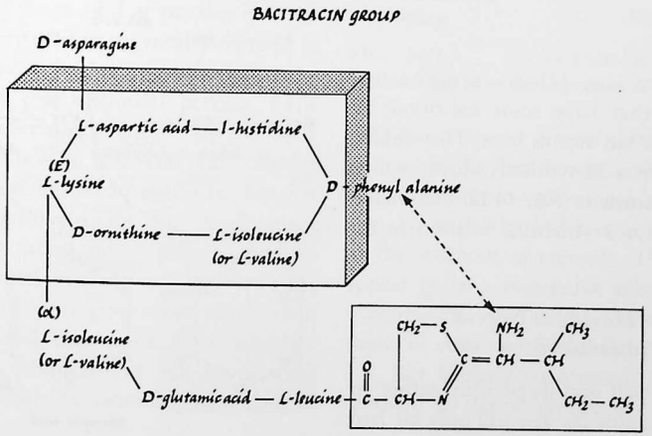


FIG. 14-9. *The polymyxin group.*

FIG. 14-10. *The bacitracin group.*



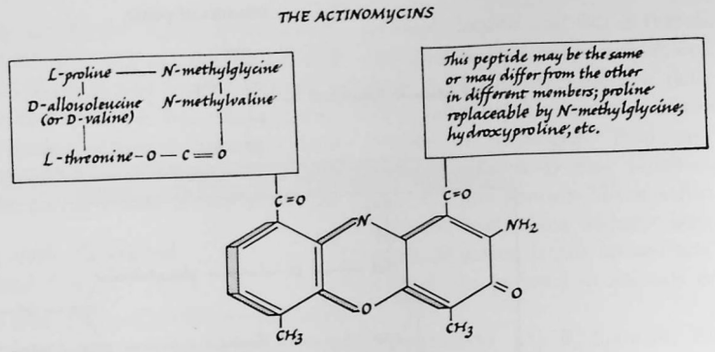


FIG. 14-11. *The actinomycins.*

gram-negative forms. They all attach to the cell membrane and damage it so that leakage of cell constituents occurs. Unfortunately they are all toxic when taken internally; if the reaction is not immediate, a delayed toxicity may develop. They are useful externally in some cases but cannot be used internally.

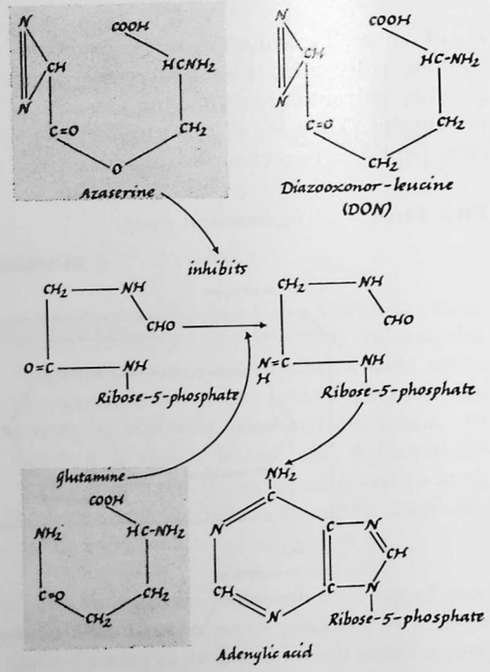
ACTINOMYCINS (Fig. 14-11), of which these are several related members, are colored circular peptides, the color being due to the attached group. They are generally too toxic for internal use but they seem to possess an anticancer activity which, while still not useful clinically, is of some interest.

AZASERINE AND DON (Diazo-oxo-norleucine) are antibiotics that have some antitumor activity in animals but not in man. They inhibit a step in adenylic acid synthesis which requires glutamine, as shown in Fig. 14-12, presumably because they bear a structural relation to the glutamine.

D. Antibiotics Having a Special Relation to Tuberculosis

The disease itself and its peculiar nature are discussed in Chapter 26. We will only say here that it is a long slow "chronic" disease, difficult to treat, and that there was, therefore, particular interest in its treatment.

FIG. 14-12. *Adenylic acid synthesis and its inhibition by azaserine and DON.*



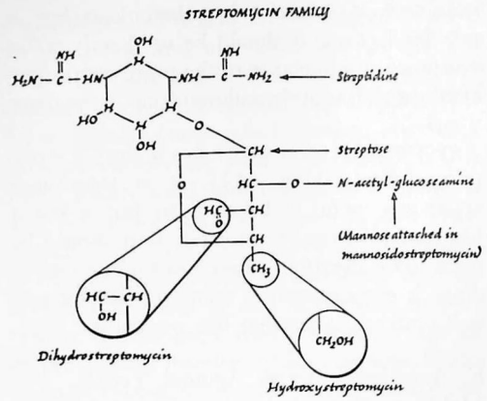


FIG. 14-13. *The streptomycin family.*

STREPTOMYCIN is a member of a family of antibiotics whose structures are shown in Fig. 14-13. Of the four substances shown there, mannisidostreptomycin is less active, and hydroxystreptomycin more toxic, leaving streptomycin and dihydroxystreptomycin as the clinically useful drugs. Many organisms, both gram-negative and gram-positive, are sensitive to streptomycin, but there are several difficulties in its use. It does not actually penetrate the animal cell; not only is it thus poorly absorbed from the intestinal tract, but also it does not attack even sensitive organisms which may be inside of cells. Again, in doses of 1 g per day over extended periods, it causes irrevocable damage to the eighth nerve, resulting in deafness. Streptomycin acts on the vestibular portion, while dihydrostreptomycin acts mostly on the cochlear portion. Thus a half and half mixture of each is still as active an antibiotic, but has only half the neurotoxicity. But development of resistance is rather rapid, perhaps more so than to other antibiotics. In our experience the really resistant strains grow more slowly than the sensitives and thus, even after resistance has developed, streptomycin may be of some use. There is also the curious phenomena of dependence, i.e., some strains require streptomycin for growth and are so dependent on it that they will not grow in its absence. But in spite of these limitations, which reduce its use in many infections for which we now have better drugs, streptomycin and dihydrostreptomycin are still widely used separately, combined, or combined with other antituberculars (principally isonicotinic hydrazide, INH) for the treatment of tuberculosis.

The mode of action of streptomycin is not entirely clear. Because of the presence of N-acetyl glucose amine (Fig. 14-13) in its structure, as well as an unusual sugar (streptose) and a curious structural ring (streptidine), we might suppose that its action would have something to do with bacterial cell walls. But this seems not to be the case. Some few years ago, we were able to demonstrate that streptomycin prevented the formation of a curious phosphorylated compound, now called glycocitramalic acid phosphate (Fig. 14-14). At that

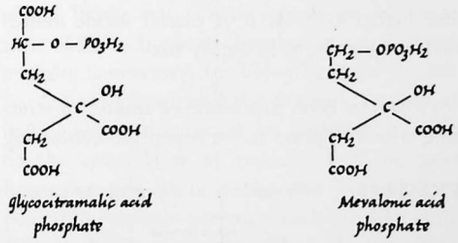


FIG. 14-14. *Glycocitramalic acid phosphate and its relation to mevalonic acid phosphate.*

time there was no notion as to what its function might be, but it was later shown that a related substance—mevalonic acid phosphate, also shown in Fig. 14-14—was an intermediate in the synthesis of steroids. This would then appear to be a reasonable mode of action of streptomycin, particularly since steroid synthesis in yeast can be inhibited by streptomycin. But bacteria have essentially no steroids in them so steroid synthesis ought not to be

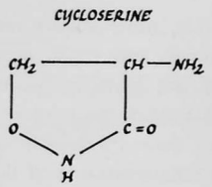


FIG. 14-15. *Cycloserine.*

important to them. However, it is perfectly possible that the glycocitramalic acid phosphate, and mevalonic acid phosphate as well, may be involved in the synthesis of materials other than steroids. One clue as to what these may be is afforded by the observation that frequently there is a leakage of certain material from the cells of streptomycin-treated organisms. We may therefore suppose, at this stage of our information, that streptomycin inhibits the synthesis of some important but as yet unknown constituent of the cell membrane. In spite of these uncertainties, it appears quite clear that streptomycin can be used in the animal or in man because it does not penetrate the higher cells. If it is placed inside animal cells artificially, it is highly toxic.

VIOMYCIN is an antibiotic of unknown structure which appears to be a peptide containing guanidine. It is useful in tuberculosis but is potentially toxic; it should be used only when resistance or intolerance to the more commonly administered antituberculosis drugs is evident.

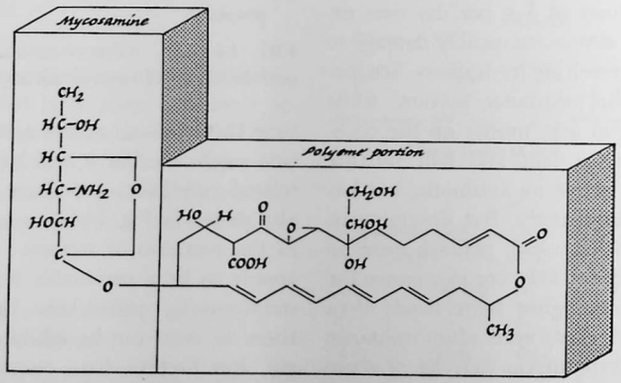
CYCLOSERINE (oxamycin) has a simple structure, shown in Fig. 14-15. It is sometimes apparently useful in tuberculosis but it has a high potential for neurotoxicity and should be reserved for situations where resistance to other drugs is encountered. It seems to inhibit cell wall synthesis, somewhat like penicillin.

E. Antibiotics Active Against Yeasts, Molds, or Protozoal Infection

NYSTATIN (mycostatin, fungicidin) and amphotericin B (fungizone) are active primarily against yeast infections. Both these and pimaricin are polyene antibiotics and consist of a complex polyene structure attached to an unusual amino sugar, mycosamine (Fig. 14-16). Nystatin contains a tetraene and amphoteracin B contains a heptene, but further exact details are not yet known; hence the related structure of pimaricin is shown.

While most yeasts or yeastlike fungi are not pathogenic, the few that do cause disease are difficult to treat. Most of the diseases are on

FIG. 14-16. *Pimaricin as a prototype structure of the polyene antibiotics.*



the skin (called dermatomycoses) but some are in the tissue and may affect any part of the body. They are usually slow-developing and the diseases are usually chronic (see Chapter 16). In moniliasis (now called *Candida* infection) both antibiotics are useful. Amphotericin B is more widely used, and because it is insoluble and not active orally it is given by slow intravenous drip. Nystatin may be given similarly or may be used locally; its usefulness for deep-seated infections is still uncertain. Amphotericin B has been used successfully against cryptococcosis (torulosis), histoplasmosis, blastomycosis, coccidioidomycosis, and systemic moniliasis. The action of nystatin in vitro appears to be on the cell membrane and its effect may be antagonized by steroids.

GRISEOFULVIN (fulvacin, fulcin) is an antifungal antibiotic derived from a fungus. Its structure is illustrated in Fig. 14-17. It is active orally and is useful against infections caused by *Trichophyton*. It is of little use against deep-seated fungal infections.

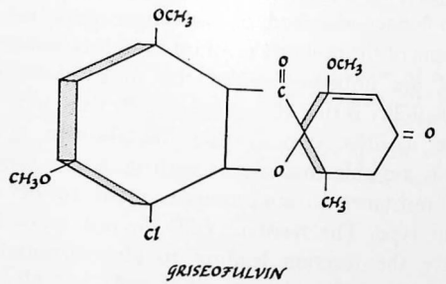


FIG. 14-17. *Griseofulvin.*

FUMAGILLIN (amebacillin, fumidil) is an antibiotic produced by a mold active against amebae. It is poorly absorbed from the intestine, but is active within it. It does not inhibit bacteria or molds.

Organisms Producing Antibiotics

The production of substances antagonistic to other organisms is a widespread property and antibiotics are produced by molds, yeasts, streptomyces, bacteria, algae, protozoan, mushrooms, plants, and even higher animals. However, of the clinically useful antibiotics, by far the majority are produced by streptomyces, discussed in Chapter 15. Of the antibiotics we have considered in this chapter, which are the clinically important ones, only one antibacterial (penicillin) and two others (griseofulvin, fumagillin) were produced by molds. The polymyxin group was derived from bacteria but all of the rest were obtained from streptomyces or very close relatives (as ristocetin from **nocardia**).

Antibiotic Action

It is profitable to consider toxicity in still another way. A chemical agent which is toxic must react chemically with some component of the living cell. There appear to be three areas of the cell where chemical if not physical integrity is necessary for life—the outer boundary, the cytoplasm, and the nucleus. Looked at in this way, we know of no antibiotics acting on the outer layer or capsule. But the next layer, the cell wall, is the site of action of penicillin, cephalosporins, which are like penicillin in structure, and possibly cycloserine. The reason we say "possibly" is that both D-cycloserine and L-cycloserine are very similar antibiotics, but the D-isomer inhibits cell wall synthesis and the L-isomer does not. Hence, we are not quite sure that the inhibition of cell wall synthesis in this case is really the important point.

At a still lower layer, the polymyxin group act by combining with the cell membrane; the polyenes act here and streptomycin apparently prevents the synthesis of some important component in it.

If we regard the cell wall and cell membrane as preventing the cell contents from running out into the environment, we must also think of the cell wall and cell membrane as preventing the environment from running in on the cell. Certainly many toxic agents may not, indeed, be toxic because they are unable to penetrate these barriers. The remainder of the antibiotics must, however, penetrate, since they do have an action, and react with components of nuclear material or cytoplasm, presumably with one or another vital enzyme system. Streptomycin, azaserine, chloramphenicol, and tetracyclines have provided some clues as to the nature of this interaction. In each case the constituent of protoplasm reacting with the antibiotics is a distinctly different one, and there appears to be no general mode of action applicable to all antibiotics.

Further, the reasons why they may be used in animals or man seem also to vary. Penicillin is usable because it inhibits a reaction not possessed by, or at least not vital to, the higher cells. Streptomycin does not penetrate them. Chloramphenicol inhibits a step in protein synthesis which seems to be peculiar to the microorganism. And azaserine inhibits a step in purine nucleotide synthesis apparently in both animal and microorganism, but the microorganism needs to synthesize the nucleotide before it can grow, while the higher cell is not growing under the conditions employed. It is curious that for each antibiotic for which we have any information at all on why they can be used, the mechanism of this use seems to be different.

Searching for New Antibiotics

The notion is current that the search for new antibiotics, the screening, as this process is called, is both an easy and rewarding activity and that it yields immensely valuable and profitable substances. By chance this has been true in the past, but no longer should screening be regarded as an especially useful thing to do. It is true that in many circumstances we do not know how to do anything better, and the situation—as in cancer—is so desperate that we should not leave any stone unturned in the search for antibiotic agents. But with the present array of clinically useful drugs, it is very difficult to find other agents which are better than these.

Resistance to Antibiotics

It is a common finding that microorganisms are capable of developing resistance to antibiotics; strains formerly susceptible are now capable of growing in the presence of the drug. These resistant forms have undergone a change. This change is a genetic one and occurs in all instances studied in the complete absence of the drug; see Chapter 11, for example. But what has the genetic change done to permit resistance?

There are several major possibilities. One is that the resistant strain may possess an altered surface or membrane so that the antibiotic is no longer absorbed or no longer penetrates. Some of the mutants resistant to chloramphenicol are impermeable to the drug. Another possibility is that the resistant cells may bypass the sensitive step in their metabolism, and thus are able to dispense with it. Some types of resistance to streptomycin seem to be of this type. The resistant cells do not seem to have the reaction leading to glycocitramalic acid that is found in sensitive cells, yet glycocitramalic acid is present in the resistant cell. In this case we suppose that the cell has learned how to make this vital substance some other way. Another possibility is that the resistant organism learns how to metabolize or destroy the drug. An example is the development of penicillinase, although there is yet no evidence that this occurs in the resistant mutants from susceptible cells rather than the naturally resistant cells.

Other Applications

To extend the use of antibiotics from the treatment of human and animal diseases to the treatment of plant diseases requires no great stretch of the imagination. It is not especially difficult to isolate antibiotics which are active against plant pathogens, including plant viruses, without being toxic to the plants, but there are not too many in use. This is partly because of cost and partly because of difficulties of penetration, application, and movement in the plant.

Other applications are the use of antibiotics to preserve fish, for example, and other foodstuffs. A somewhat surprising use is the growth-stimulating properties in animals of the tetracyclines; they therefore have a wide use as supplemental foodstuffs in animal husbandry, rather than for the treatment of disease.

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For background information, see p. 494.

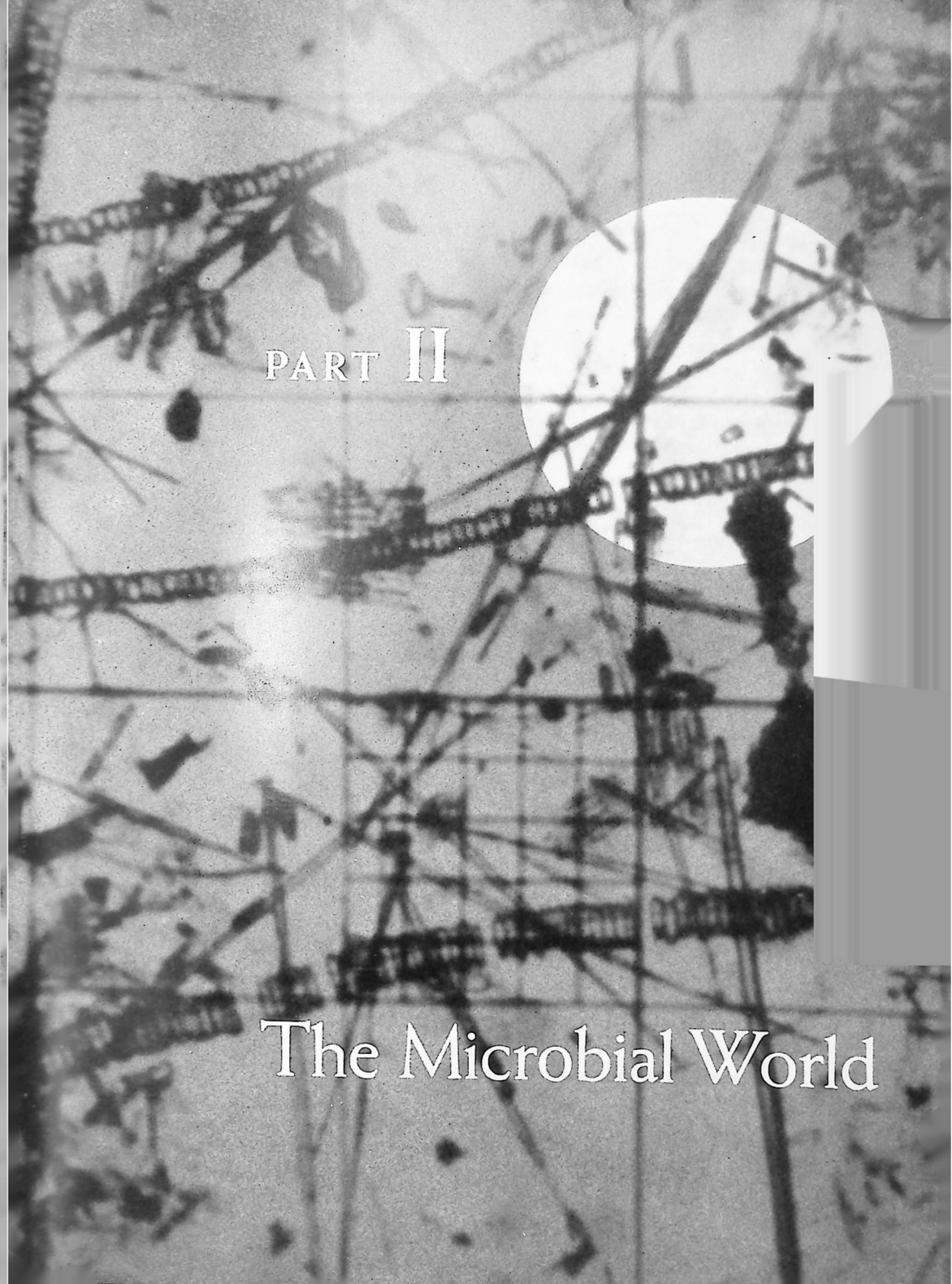
QUESTIONS

1. Define and identify:
(We have not listed the names of the antibiotics discussed.)

antibiotic
chemotherapy
families of antibiotics
pain on injection
circular peptides
delayed toxicity
neurotoxicity
sensitive
resistant
penicillinase
bacterial spectrum
broad spectrum
narrow spectrum
antibiotic dependence
mode of action
glycocitramalic acid phosphate
systemic infection
screening

2. You are the research director of a pharmaceutical company. Your bacteriolgist tells you that he has found a streptomycete that will inhibit the growth of gram-negative organisms such as *Escherichia coli*, *Salmonella typhosa*, etc. What further tests would you require before you would invest the time of many people for its development?
3. Suppose that in question 2 the bacteriologist had said that the streptomycete inhibited growth of sarcoma 180 (a tumor) in mice. What would you do then?
4. An antibiotic agent requires a dose of 30 g per day, but it is cheap and effective and there are few undesirable side effects. Would you search for one which required 0.1 g per day?
5. Antibiotic 3X2, derived from a mold, is a yellow powder with a "chemical" taste. In the laboratory it acts on several organisms, causing them to leak materials. It has been tried in mice, but was not very satisfactory. What are the arguments for and against trying it in man? What would you try it against? How would you use it?

Facing page, filter-clogging algae from water: *Scenedesmus, Fragilaria, Synedra, Coelastrum*. [Courtesy Millipore Filter Corp., Bedford, Massachusetts.]



PART II

The Microbial World

BACTERIA, which have been the main concern of Part I, are the smallest cells. In one sense they have dispensed with the apparatus of digestion, with the complexities of root and leaf. These small cells are smaller by a real order of magnitude than the amoeba. Morphologically, they are simpler, structurally simpler and organizationally simpler. But in another sense, higher forms of life developed more structure and more organization, rather than that the bacteria dispensed with these added attributes. The bacteria probably never had them.

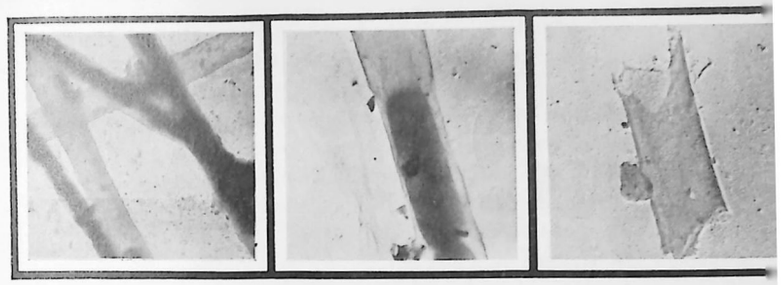
Bacteria are the smallest cells. Is there then no organized life smaller than bacteria? There are certainly some things having a kinship to life which are smaller and simpler—the viruses and the rickettsiae; but they are not cellular. Bacteria are the point where we first find cellular organization, and it is upon this cellular organization that higher and more complex forms have developed. We have thus two paths to follow, one above and one below. The noncellular "living molecules" of a lower degree of organization, the viruses and the rickettsiae, do have kinship to life. They are not necessarily smaller than the smallest bacteria, but characteristically, they have no cells of their own; they live and "grow" and show the manifestation of life only inside other living cells. They make use of cellular organization, but of the organization of somebody else's cell. This is not to say that such pseudocreatures do not have an organization but only that they are dependent for their growth—their increase in number—upon other living cells.

The viruses and rickettsiae were originally recognized because they produced

disease, and it is more convenient to discuss them later in connection with the diseases produced. We therefore, in this section, recognize that viruses (Chapter 29) and rickettsiae (Chapter 30) are part of the microbial world but defer their discussion. This Part will concentrate on the microorganisms having cellular organization which have developed more organization, more structure, and more versatility in the world.

In Part I we pointed out that modern microbiology was characterized by its chemical approaches and by its flagrant disregard of taxonomic conceptions and descriptions of organisms—in short, by its emphasis on biochemistry, not morphology. It was also pointed out that there was a kind of microbiologist who disliked the chemistry but loved the morphology, and who represented the extension of classical biology into the microscale. This Part, dealing as it does with higher forms of microorganisms, is still written and organized from the viewpoint of the chemical approach to cells, and not from the viewpoint of their taxonomy or morphology.

Chapter 15



THE HIGHER BACTERIA

"While all knowledge is valuable, one kind may be more valuable than another and surely we would place biology well in the front rank. Its aim is to grasp and understand the essential character of life.

"However, to attain any assured understanding of life is one of the most difficult things in the world. One might suppose that there was some single method of study which would be adequate. If so, we should naturally be well trained in this method.

"But suppose that there is no single or general method applicable to all the problems of biology. In this case, with what facts shall we begin our inquiry? For the facts which form the starting points in different subjects must be different. And the kind of training we undertake must likewise be different."

ARISTOTLE, *Essay on the Soul*

WE ARE CONCERNED in this chapter with organisms more complex than bacteria. While we are not concerned with formal classification schemes, these are of help in understanding the kinds of complexities which may be added to the bacterial cell and the kinds of organisms thus produced. Bacteria are single-celled microscopic "plants" devoid of chlorophyll, reproducing by binary fission. But a modern microbiologist is concerned with more microorganisms than just the bacteria. It is important that one understand the nature of organisms coming within the domain of microbiology. In the classical sense, microbiology is the study of any organism one may see with a microscope. Unicellular algae and protozoa come within its

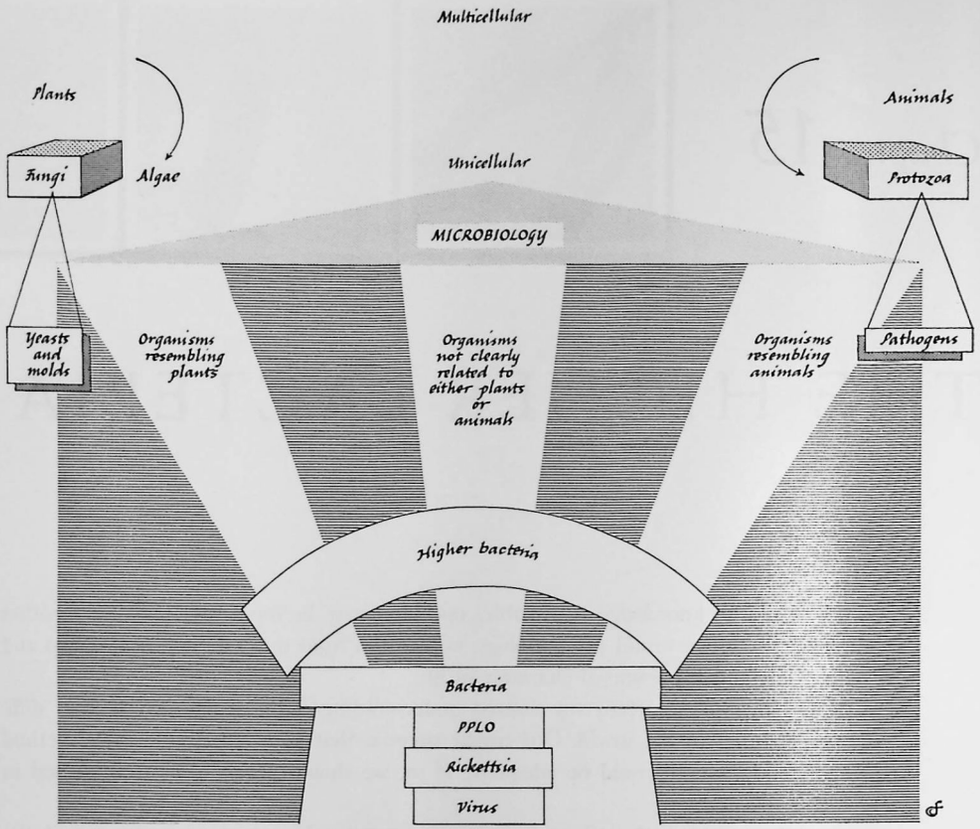


FIG. 15-1. *The organisms studied in the field of microbiology.*

ken, as indeed they do, if only superficially. But as biological science has developed, the study of algae has become a characteristic feature of botany, while the study of protozoology, as its name implies, has become part of zoology. The approach, the methodology, and even the philosophy of these sciences is somewhat different from the comparable approach, methodology, or philosophy of the modern microbiologist. But there still exists a wide array of organisms, clearly not bacteria, clearly not algae, clearly not protozoa, and it is with these that the modern microbiologist is legitimately concerned.

It is supposed by many that there is a consistent and continuing line of development from simpler creatures to more complex. If so, we should expect fairly clear lines of development, paths of increasing degree of organism complexity, somewhat as expressed in Fig. 15-1. But if we look for clear evidence of such a progressive path, it is not readily apparent. We have therefore left some blanks in the pathways of evolution which future study may supply. The modern microbiologist is concerned with the area within the "house" shown in Fig. 15-1, and by the nature of his work he also has contact with the yeasts and molds

and the pathogenic protozoa. But except for these, the modern microbiologist normally confines his attention to the class Schizomycetes, the fission fungi. The Schizomycetes are separated into ten orders:

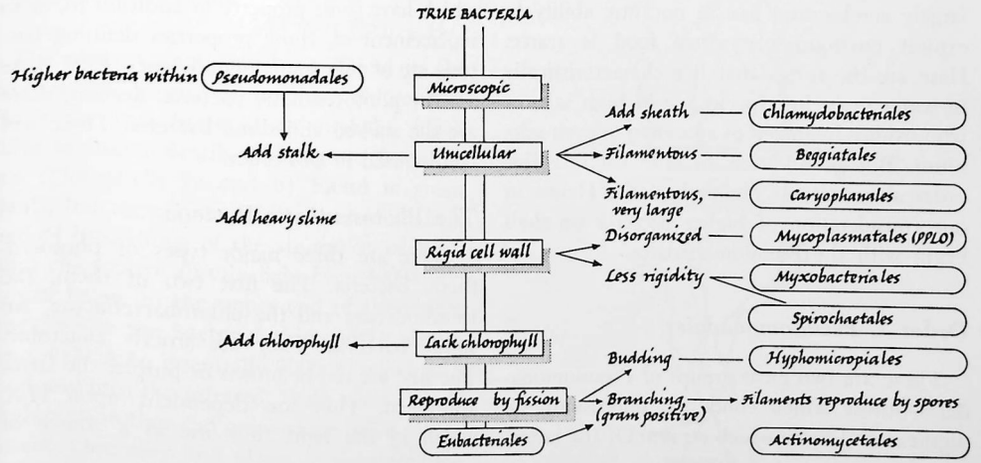
I. Pseudomonadales
   bacteria motile via polar flagella
II. Chlamydobacteriales
   the sheathed bacteria
III. Hyphomicrobiales
   the budding bacteria
IV. Eubacteriales
   the remainder of the bacteria
V. Actinomycetales
   the branching bacteria
VI. Caryophanales
   multicellular filaments in slime
VII. Beggiatoales
   the filamentous gliding bacteria
VIII. Myxobacteriales
   single cells with gliding movement
IX. Spirochaetales
   slender flexuous cells
X. Mycoplasmatales
   pleomorphic organisms with peculiar reproduction

The numbers used are those of Bergey (7th ed.). The relations of these orders are illustrated in Fig. 15-2.

All those organisms which correspond to the definition of bacteria have been placed in two orders—the Pseudomonadales and the Eubacteriales. Pseudomonadales contains some higher bacteria as well. It is therefore of some importance to look at the kinds of bacteria we may find in these orders.

The motile forms of the organisms placed in the order Pseudomonadales are motile because they possess polar flagella; the motile forms of those placed in Eubacteriales are put there because of peritrichous flagella. While there seems at first glance to be a rather meaningless sort of a separation, it happens that most of the bacteria we have been discussing in previous chapters—the cocci, the gram-positive cylinders, most of the gram-negative rods—are members of the Eubacteriales. Among the cylinders, it is only the oxidative gram-negative rods which produce little gum that are motile by means of polar flagella and thus belong to the Pseudomonadales. The spirals and the vibrios are also motile in this same fashion and hence they are placed here. It further happens

FIG. 15-2. *The orders of higher bacteria.*



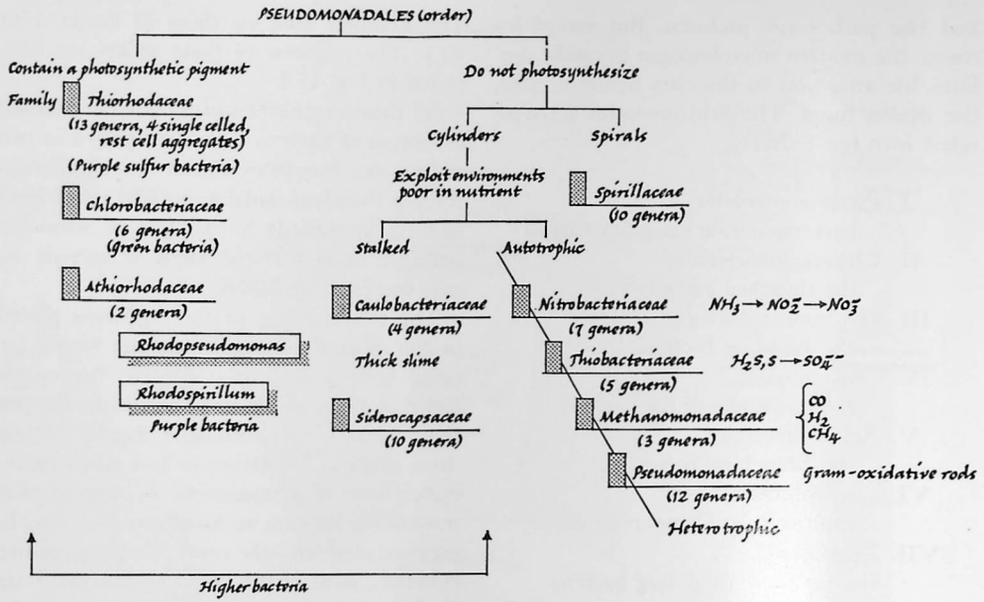


FIG. 15-3. *The order Pseudomonadales.*

that the organisms so placed in this order have several characteristics in common. There are few animal or human pathogens. *Vibrio comma* is the only really vigorous pathogen in the entire order. But plant pathogens are present. As a group, the order Pseudomonadales is largely aerobic and has an uncanny ability to exploit environments where food is scarce. Here are the forms that live characteristically in water or in hot springs, or in high salt, or who oxidize hydrogen or ammonia or even selenium. But in addition to true bacteria this order also contains higher bacteria. Hence in our consideration of higher bacteria we shall begin with the Pseudomonadales.

Order I. *Pseudomonadales*

There are two great groups of Pseudomonadales—those which contain a photosynthetic pigment and those which do not. Of the latter, there are several groups with unusual properties. The organisms within this order are illustrated in Fig. 15-3.

Note that the autotrophic bacteria are present. The order contains both true and higher bacteria. But the higher bacteria, i.e., those which have some property in addition to, or in replacement of, those properties defining bacteria are of two fundamental types. First there are the photosynthetic bacteria. Second, there are the stalked and slime bacteria. These will be discussed in this order.

The Photosynthetic Bacteria

There are three major types of photosynthetic bacteria. The first two of them, the thiorhodaceae and the chlorobacteriaceae, are **sulfur bacteria.** They are strictly anaerobic. The first are red or brown or purple; the latter are green. They are dependent upon H_2S, which in the light they use as a source of

hydrogen to reduce CO_2 to cell substance, forming S or later SO_4. They are mostly unable to use organic substances but are dependent upon H_2S and light. The third group, the athiorhodaceae, are **nonsulfur** bacteria; they do not use H_2S but organic substances. They are purple.

In green plants, including the algae, the overall photosynthetic process is

$$H_2O + CO_2 \xrightarrow{\text{light}} O_2 + \underset{\text{carbohydrate}}{(CH_2O)}$$

In bacterial photosynthesis there is also a light-driven reduction of carbon dioxide, but *oxygen is never produced.* Instead, the hydrogen used to reduce the CO_2 to $[CH_2O]$ comes from some externally supplied donor rather than from water.

The photosynthetic bacteria, then, include the sulfur bacteria, which obtain hydrogen from H_2S—(1) green sulfur bacteria and (2) purple sulfur bacteria. Their photosynthetic reaction is

$$2H_2S + CO_2 \longrightarrow S + CH_2O + H_2O$$

The third photosynthetic group is composed of (3) the nonsulfur purple bacteria, whose reaction is

$$2H_2A \text{ (organic substance)} + CO_2 \longrightarrow 2A + (CH_2O) + H_2O$$

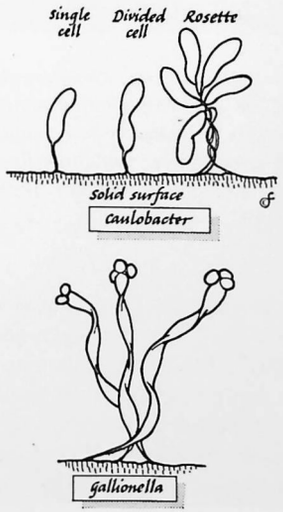
The photosynthetic pigments found in the bacteria are green, as is plant chlorophyll. There are two bacterial chlorophylls which differ in minor details of structure from the two chlorophylls (a and b) found in green plants, but these minor differences result in a marked broadening of the absorption of light. Plant chlorophyll absorbs light from 4000–7000 Å, just below to the upper end of the visible range, while the bacterial chlorophylls absorb from 4000 Å to essentially 9000 Å, which is a good way into the infrared. With such a range of absorption they can grow where green plants cannot. One may find places in nature where the green and the purple bacteria are growing photosynthetically beneath a layer of algae, using the infrared which the algae had not absorbed.

The red or purple color of the purple bacteria is caused by the presence of red or orange carotenoid pigments, which pigments may function indirectly in photosynthesis. But the green chlorophyll is always present, even if masked by the others. Both kinds of pigments occur as small granules in the cell, thousands per cell, and these grana are the functional units of photosynthesis.

Stalked Bacteria

Still within the order Pseudomonadales there exist **stalked bacteria,** placed in the family Caulobacteriaceae, in which there are four genera. There is, of course, nothing in the definition of bacteria which indicates that they must be free-swimming, yet the addition of a stalk is certainly an unusual and noteworthy matter. The stalked bacteria possess a fine stalk or "holdfast" with which they are able to attach

FIG. 15-4. *Caulobacter and Gallionella.*



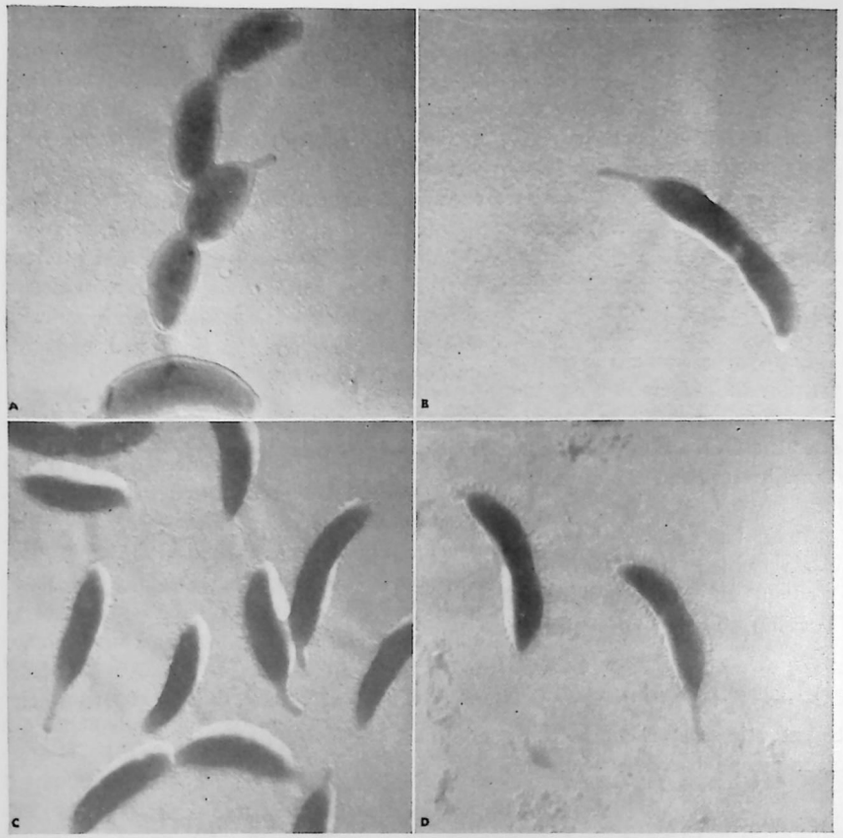


FIG. 15-5. *Caulobacter. These are electron micrographs which show the stalks. In addition, these photographs are useful in showing something about the gram stain (the organisms are gram-negative). A contains untreated cells. B contains cells after staining with crystal violet; the cell appears to have shrunk somewhat. C contains cells after treatment with crystal violet, iodine, and alcohol. D is after counterstaining with safranine.* [*From Grula and Rowe,* J. Bacteriol., **79,** 760 (1960). *Courtsey of Dr. Grula.*]

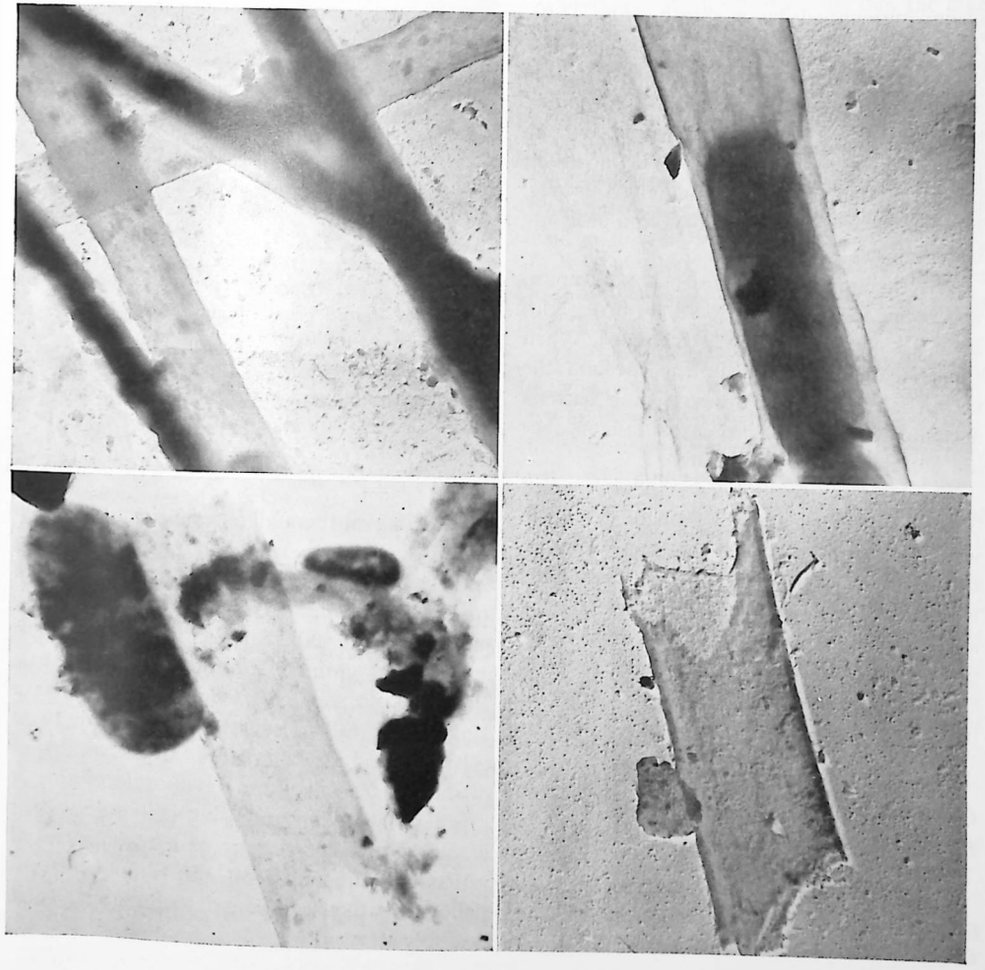
themselves to a surface. They occur in water or on surfaces washed by water, and especially in flowing water where their stalk can protect them from washing away. With some genera iron can be deposited in the stalks and as such, they may cause the precipitation of iron, with subsequent clogging of pipes. They are sometimes considered members of the **iron bacteria**—organisms that precipitate iron, but do not necessarily live on it. There are two main types of stalked bacteria (Fig. 15-4):

Caulobacter sp. (Fig. 15-5) has its stalk emerging from the end of the cell. Frequently the organisms occur in rosettes. They divide by binary fission, have a rigid cell wall, and, except for the stalk, are otherwise like the true bacteria. They seldom deposit iron in the stalk.

Gallionella sp. has the stalk emerging from the side of the cell in the form of a thin, flat ribbon which may twist and turn—and, of course, become entangled with other ribbons. These kinds of cells frequently deposit iron in the ribbon.

So little is known about the Siderocapsaceae

FIG. 15-6. Sphaerotilus. *Upper left shows strands of* Sphaerotilus, *some empty and some with cells (original 11,570 ×). Upper right shows an individual cell contained within the sheath (37,000 ×). Lower left shows the individual cell outside of its sheath (26,000 ×). Lower right shows the empty sheath (37,000 ×).* [*Courtesy of Dr. N. C. Dondero, Rutgers University. The prints were made by Dr. S. Weinreb.*]



that it is of no benefit to discuss them. They are single cells embedded in a heavy slime. The remainder—of the "higher bacteria"—belong to other orders, being regarded as too far away from the true bacteria to be considered real bacteria.

Order II. *Chlamydobacteriales*, the Sheathed Bacteria

These organisms are composed of cells in chains surrounded by a sheath (Fig. 15-6). They proliferate abundantly in flowing water with the production of masses of cells that absorb and entrap other materials. The aggregations caused by these filaments cause contamination of waters, corrosion and obstruction of pipes, interference with commercial fishing, interference with waste disposal, and unsightly water courses.

The chemistry of the sheath is not known, and because it is difficult to see on living cells, little is known about how it is formed. When there is abundant iron in the medium the sheath takes up iron. Although these bacteria accumulate iron in nature in this fashion, they are not able to live on the iron.

The principal genus known today is called *Sphaerotilus*. Earlier it was thought that there were two main sheathed bacteria—*Sphaerotilus*, without iron deposits, and *Leptothrix*, with iron. But it turns out that these are the same organisms; whether or not iron is deposited depends on the environmental conditions, not the least of which is the supply of iron.

Order III. *Hyphomicrobiales*, the Budding Bacteria

There are four genera, with one species recognized in each. Since on occasion one will see the names of these genera used, we list them here but regard them all as varieties of *Hyphomicrobium vulgare*, which has been more extensively studied (Fig. 15-7). The four genera are *Hyphomycrobium*, in which the buds are borne on a little stalk; *Rhodomicrobium*, which is its photosynthetic counterpart; *Pasteuria*, which has no stalk; *Blastocaulis*, which has a long slender stalk. The last two have not been cultivated and are known only by examination of slides placed in water. *Hyphomicrobium* is capable of attacking, and using as its sole source of carbon and energy, any of a most unusual variety of compounds—oil, tar, phenols; essentially any carbon compound so far given to it is readily attacked.

FIG. 15-7. A *typical* Hyphomicrobium. *This culture was found growing in distilled water, an example of its ability to get along on traces of organic matter. The cells, on the end of long stalks, became elongated and put out a small bud, which eventually becomes the daughter cell.* (*Photograph courtesy of Dr. N. Dondero, Rutgers University.*)

Order IV. *The Eubacteriales*

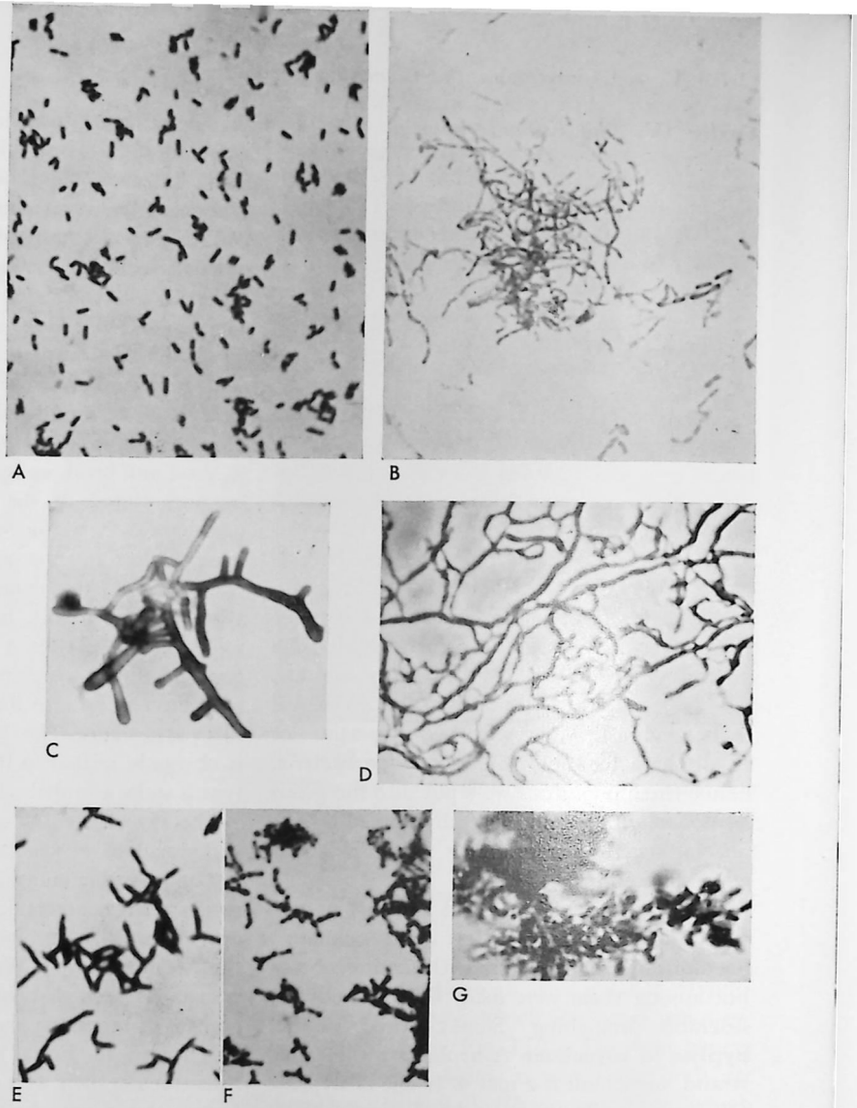
These are the true bacteria and for the most part their separation is discussed in Chapter 7 on bacterial taxonomy. There are no higher bacteria in this order.

Order V. *Actinomycetales*, the Branching Bacteria

The true bacteria did not show branching; if an occasional branching form was found, it was generally regarded as a cell in which the cell wall was in some measure incomplete and protoplasm had extruded thereby. However, organisms were discovered, early in bacteriology, which did show branching with considerable regularity; so often indeed that it could not be regarded as inadequate cell wall. The organism causing tuberculosis was one of these, and during its study by Koch it was also found to be acid-fast. These were two properties not at all characteristic of more common bacteria, hence these organisms were put into the genus *Mycobacterium*; while generally studied by bacteriologists, they were always felt to be somewhat different.

Later bacteriologists found acid-fast or partially acid-fast forms—the latter organisms decolorized more quickly with acid alcohol—but among them were many that showed considerable branching. Some indeed showed **hyphae** or **mycelium.** A hypha is a threadlike strand, mycelium is a mat or tangle of hyphae. Of course cells in chains had been known before but these hyphae were long things; further, there seemed to be no cross-walls—the "chain" was really a single cell. These long cells ranged all the way from two to ten times the length of a single *Lactobacillus* to cells a thousand times as long, but otherwise as thin as lactobacilli. These very long cell filaments could be very stable, hardly ever breaking up into individual cells. There was a continual shading of forms from short unstable hyphae—not more than two to three cells in length and easily broken up by shaking—to those which were very long and could hardly be broken with an inoculating needle. These forms were originally called **proactinomyces** and could be separated into the short, unstable hyphae (the α type) and the long, stable hyphae (the β type). There is a practical method of easily distinguishing between these types. When we have the short, unstable mycelial type of organisms growing in liquid culture, the hyphae are so short and break up so readily that the fluid becomes turbid. If the long, stable type of mycelium is grown in liquid culture the fluid remains clear and the organisms grow in little balls, almost like colonies. In addition, the α type produces a soft, bacteria-like colony on solid media while the β type produces a hard, compact colony which may even, on occasion, send mycelia up into the air, and thus have a fuzzy appearance. The short α type of organism is obviously related to the bacteria, but the β type is really a hyphal cell—a long filament.

The *Mycobacterium* was obviously different, perhaps close to the true bacteria but acid-fast and possibly branching. But the proactinomyces were even more different. Therefore they were eventually put into a separate order—the Actinomycetales. We have in the previous paragraphs been describing the gradual development of branching and mycelial forms, illustrated also in Fig. 15-8. However, in the Eubacteriales there were also some pleomorphic forms—the *Corynebacterium*—somewhat related to *Lactobacillus* but more aerobic. These formed club-shaped cells, and their mode of division was uncertain; certainly it was some modification of binary fission. It was possible to see something that looked like branching; indeed, electron microscope photographs seemed to demonstrate branching adequately. However, it was argued that these were "really" merely osmotically imbalanced cells and that



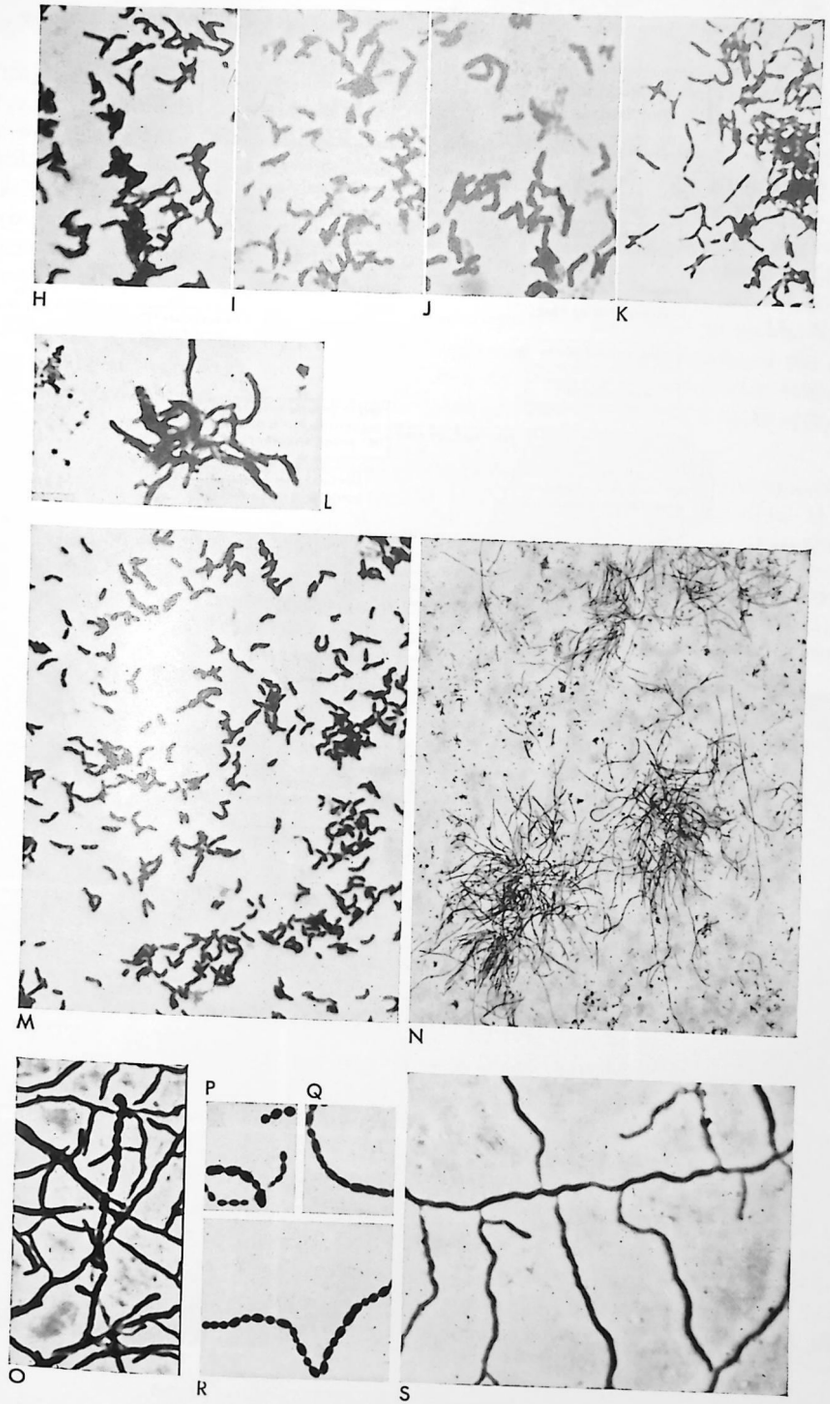


FIG. 15-8. *The Development of the Mycelial State from* Corynebacterium *to* Streptomyces. *A and B are photographs of a* Corynebacterium *causing a subacute bacterial endocarditis. A is the form usually seen; B is the form which develops on antibiotic treatment. C is* Nocardia, *the aerobic mycelial forming type which forms no spores. E–L are gram stains of* Actinomyces, *the anaerobic forms without spores with branching and a rudimentary mycelium. M and N are the same* Actinomyces, *M growing in culture where it is primarily bacterial, N growing in the hamster where it is in the mycelial form. D, O, P, and Q are of* Streptomyces, *D and Q being the mycelium. D is a 40 hour culture; Q is younger. O shows the sporulating body on the aerial mycelium while P shows the spores at different stages of maturation. [A and B from Wittler, Malizia, Kramer, Tuckett, Pritchard, and Baker.* J. Gen. Microbiol., **23,** *315 (1960). Courtesy Dr. Ruth G. Wittler. C from Davis and Freer,* J. Gen. Microbiol., **23,** *163 (1960). Courtesy Drs. Davis and Freer. E–L from Pine, Howell, and Watson,* J. Gen. Microbiol., **23,** *403 (1960). M and N from Pine and Harden,* J. Bact., **78,** *164 (1959). Courtesy Dr. L. Pine. D, O, P, Q from Hopwood,* J. Gen. Microbiol., **22,** *295 (1960). Courtesy Dr. D. A. Hopwood.]*

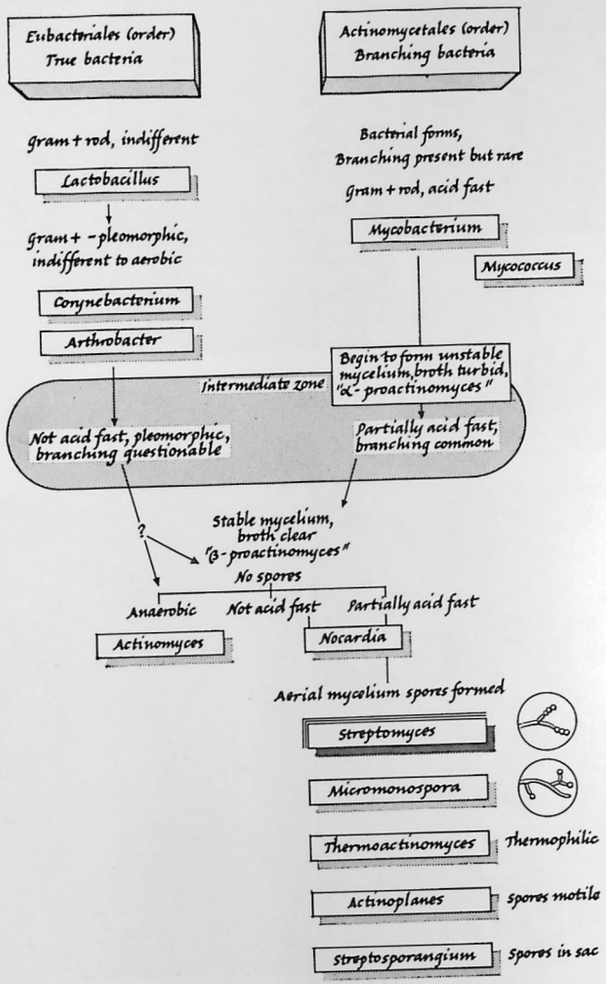


FIG. 15-9. *The development of branching in progressing to the streptomyces.*

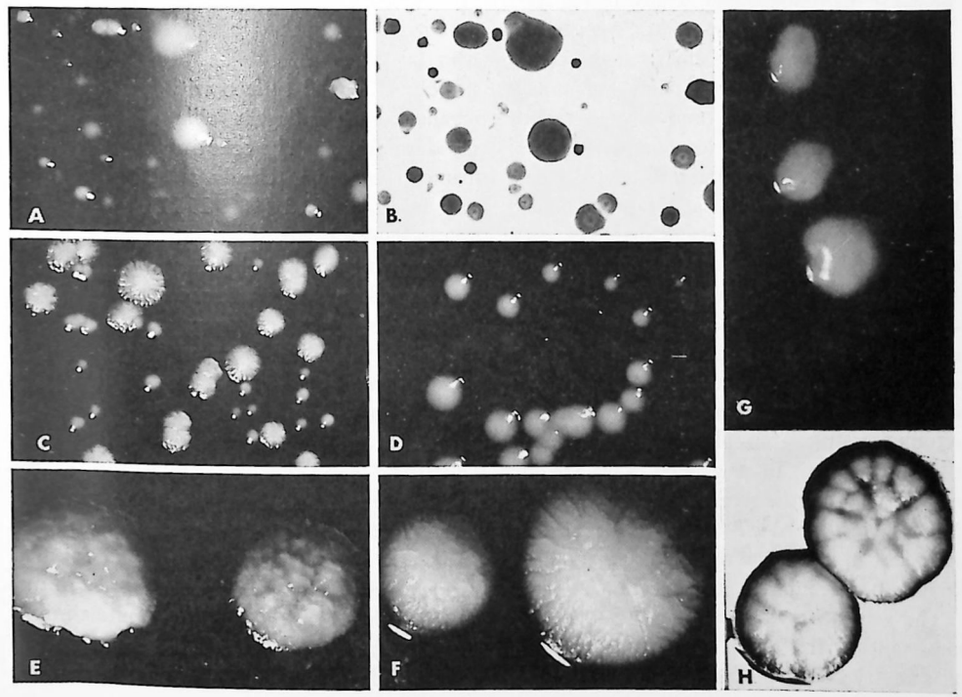
no "true" branching existed. The *Corynebacterium* genus, then, remained within the true bacteria. There were good practical reasons for doing this. *Corynebacterium diphtheriae* is the cause of diphtheria, at one time a most dangerous disease indeed. There were some "diphtheroid" (i.e., diptheria-like) organisms found in soil, of the genus *Arthrobacter*, and some of these showed branching. Further there were some non-acid-fast α proactinomyces and some of these were clearly related to *Arthrobacter*. There were also some non-acid-fast β proactinomyces, but whether they developed from acid-fast mycobacteria or non-acid-fast α proactinomyces is not clear. These relationships are shown in Fig. 15-9.

With the β protoactinomyces we have arrived at a hyphal cellular condition with a long stable mycelium. The α proactinomyces were, and still are, in too confused a state to designate as genera, but there is good reason to set up two genera in the β proactinomyces. The first genus assembles the anaerobic forms into *Actinomyces*; the second assembles the aerobic forms—most but not all being partially acid-fast—into the genus *Nocardia*. Both genera contain pathogens.

The genus *Actinomyces* contains the organism *Actinomyces bovis*, the cause of lumpy jaw in cattle and rarely occurring lung infections in man. The organisms are difficult to maintain in culture; the hyphae are scant and relatively little is known about the genus.

The genus *Nocardia* contains organisms causing tumors—mycetoma or madura foot—tumors in which a filamentous form can be found, but which are not caused by molds. *Nocardia* organisms also cause infections of the respiratory tract sometimes resembling tuberculosis (pseudotuberculosis). The colonies of *Nocardia*

FIG. 15-10. *Colonies of non-spore-forming mycelial forms.* A *through* F *are* Actinomyces bovis, *while the photo at top right is* Actinomyces israeliis; *both are anaerobic mycelial forms.* H, *lower right, is the aerobic type,* Nocardia. *Note how some of the colonies look like those of bacteria, and some have a more varnished appearance. They are all hard to the touch and can be penetrated only with a stiff needle.* [A *through* F *from Pine, Howell, and Watson,* J. Gen. Microbiol., **23**, *403 (1960), and top right from Pine and Harden,* J. Bacteriol., **78**, *164 (1959); courtesy of Dr. L. Pine.* H *is from Davis and Freer,* J. Gen. Microbiol., **23**, *163 (1960); courtesy of Drs. Davis and Freer.*]



may be soft and waxy if the organism is acid-fast or they may be hard and glossy (Fig. 15-10). The hard colonies frequently cannot be penetrated by the usual bacteriological needle; a small scalpel is needed to cut into them. Essentially the same is true for all the hyphal forms. The colonies do not generally have any aerial mycelium and do not form spores. However, it is not unusual for the hyphae to break up into segments, each of which is capable of forming an independent colony.

But there remain still other organisms bearing an obvious relation to those previously discussed. They are characterized by possessing an extensive mycelium, a "hard" colony, although it might be "fuzzy." This fuzziness is due to the development of aerial hyphae. Further study shows that the hyphae does not break up into fragments, as does *Nocardia*; rather, spores are formed. There are several modes of spore formation, shown in Fig. 15-9, but in the most important genus, *Streptomyces*, the spores are formed in chains at the end of the aerial hyphae. The spores are usually larger than the hyphae and have a slightly higher heat resistance. None are acid-fast. As a group the *Streptomyces* and its related genera occur in soil and are capable of growing on and decomposing a wide variety of materials, especially materials very resistant to decomposition. As a group the whole order Actinomycetales is capable of attacking resistant materials—oil, gasoline, wood, plastics, tar, lignin, asphalt; while the growth may be slow, eventually the carbon these resistant materials contained is returned to the atmosphere by the work of these organisms. In a general way, *Streptomyces* and *Nocardia* seem to be soil forms and *Micromonospora* and *Actinoplanes* seem most abundant in water; the remainder are too little known to warrant any generalization.

The genus *Streptomyces* is by far the most important of the group. The organisms give off a musty odor reminiscent of newly plowed soil, and the smell of the "good earth" is most probably due to them. Further, among the *Streptomyces* are most of the organisms producing antibiotics (see Chapter 14). Few if any cause animal disease but one, *Streptomyces scabies*, is the cause of potato scab.

Order VI. *Caryophanales*

These organisms are generally very large plump filaments containing as many as ten to twenty cells, and each cell is exceedingly large, sometimes as much as 3–4 μ in diameter. They reproduce by binary fission of the filament, which yields two filaments, each composed of fewer cells. They seem to be intestinal inhabitants but not much more is known of them.

Order VII. *Beggiatoales*, the Filamentous Gliding Bacteria

It is difficult to describe gliding to someone who has never seen it, but these organisms move over a solid surface without the aid of organelles such as flagella, cilia, etc. How they do so is unknown. The cells are usually in long filaments but some clearly related organisms may be single-celled (Fig. 15-11). Multiplication in most is by transverse rather than binary fission. If they are motile, they are usually of the genus *Beggiatoa*, whose members also have the property of containing granules of sulfur within the cell when hydrogen sulfide is present. They do not contain chlorophyll. There are some spiral forms (genus *Thiospirillopsis*), some in which the filament is braided (genus *Thoploca*), and some in which the entire thread is not motile although there may be short segments which are (genus *Thiothrix*). There is a similar group of morphologically distinct forms which *do not* deposit sulfur granules. There is a further group, quite similar to the *Beggiatoa*, in which there is no motility or sulfur deposits inside the cell, although there may be sulfur outside the cell; they are called *Leucothrix*.

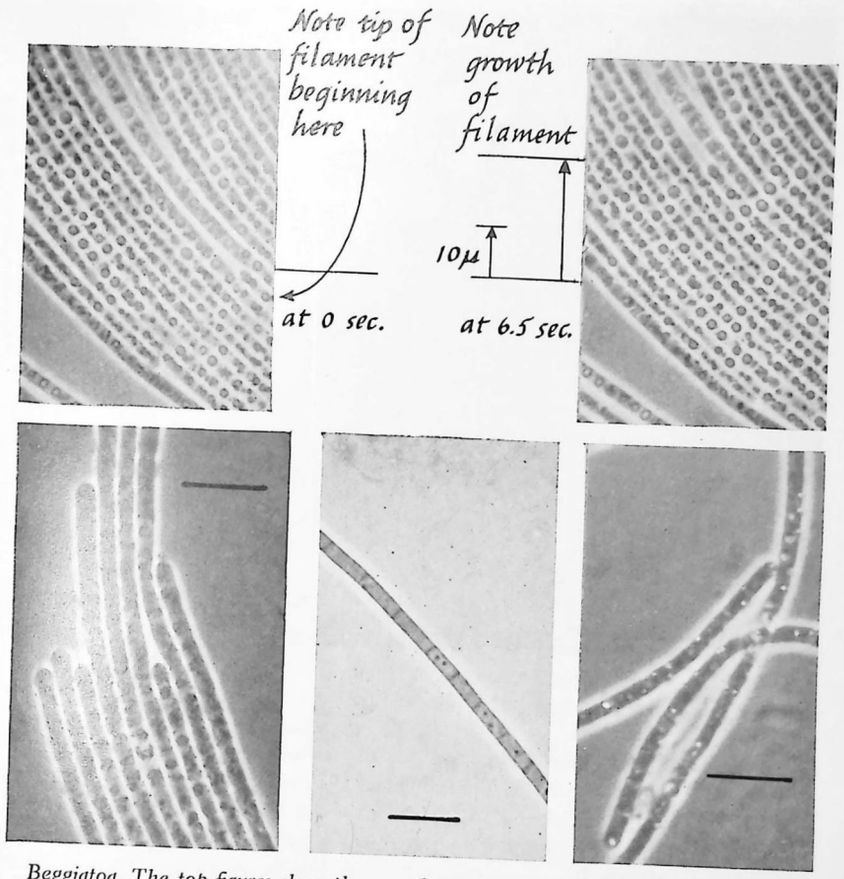


FIG. 15-11. *Beggiatoa. The top figures show the growth of a culture of* Beggiatoa alba. *Note the strand indicated by the arrow at the left figure and the same strand, 6.5 seconds later, in the right figure. In this interval it has grown approximately 20 μ. The lower left photo is a dark-contrast phase microscope picture of the edge of a colony (the line in each case represents 10 μ). The cells contain many inclusions which obscure the cross walls. The center photo shows a dying strand with few cell inclusions, where the cross walls are now visible. The lower right photo shows young cells exposed to H_2S in the medium; note the deposit of the light-colored sulfur granules in the cells. [From Faust and Wolfe,* J. Bacteriol., **81**, 99 *(1961). Courtesy of Dr. Wolfe.]*

Order VIII. *Myxobacteriales*

These are organisms which reproduce by transverse fission, have some characteristics of the bacteria, but do not have a rigid cell wall —at least not in the same sense that bacteria do. The vegetative cells are flexible. In general they are called myxobacteria, being thought to resemble in some aspects the Myxomycetes, the slime molds. The myxobacteria are usually

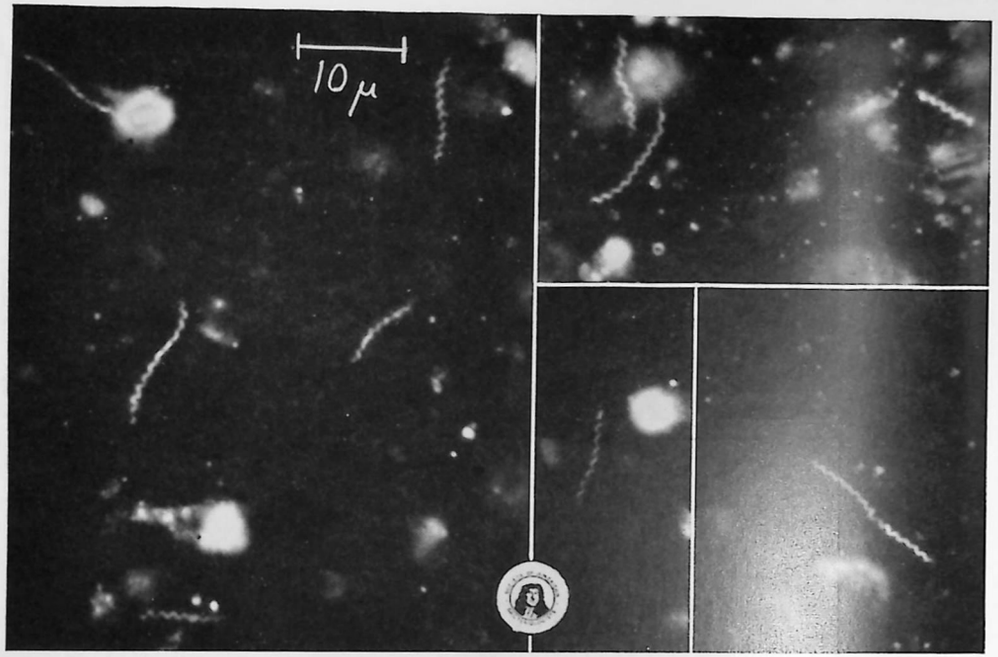


FIG. 15-12. *Spirochetes;* Treponema pallidum. *These are dark-field photographs of ground preparations from rabbit testes, prepared by Dr. T. Rosebury.* [Am. Soc. Microbiol. *LS-327.*]

embedded in a heavy slime. Colonies of myxobacteria are shown in Fig. 2-18D and individual cells are shown in Figs. 5-4 and 18-7. Some of these forms produce, in addition to the thin rod characteristic of the vegetative cell, an oval sporelike cell. Further, there seems to be the possibility of organization of many independent cells to form a fruiting body. There are three important genera, among twelve genera at present recognized:

Cytophaga sp. attacks cellulose, but forms no sporelike cells.

Sporocytophaga sp. also attacks cellulose, but forms sporelike cells; it has no fruiting bodies.

Myxococcus sp. forms fruiting bodies which may be a large droplet a thousand times the size of the cell. Some of these bodies show a high degree of organization.

These genera are discussed, especially the *Cytophaga* group, in connection with cellulose decomposition (p. 337), but a word should be said about **fruiting body.** At the edge of a colony the rod-shaped cells are actively motile and, in groups of a few to many hundreds, may extend out as tonguelike projections. These moving cells coat the substrate with a thin layer of slime. With a degree of organization above that of *Cytophaga* certain of the cells take on a thicker coat and become rounded and refractile. These sporelike cells are sometimes called **cysts.** Above *Sporacytophaga,* before such cysts are formed, a fruiting body develops. This may be no more than a uniform mass of cysts held together by slime, or it may consist of cysts which are raised above the surface on stalks. These raised fruiting bodies are usually colored and may be large enough to be seen with the naked eye.

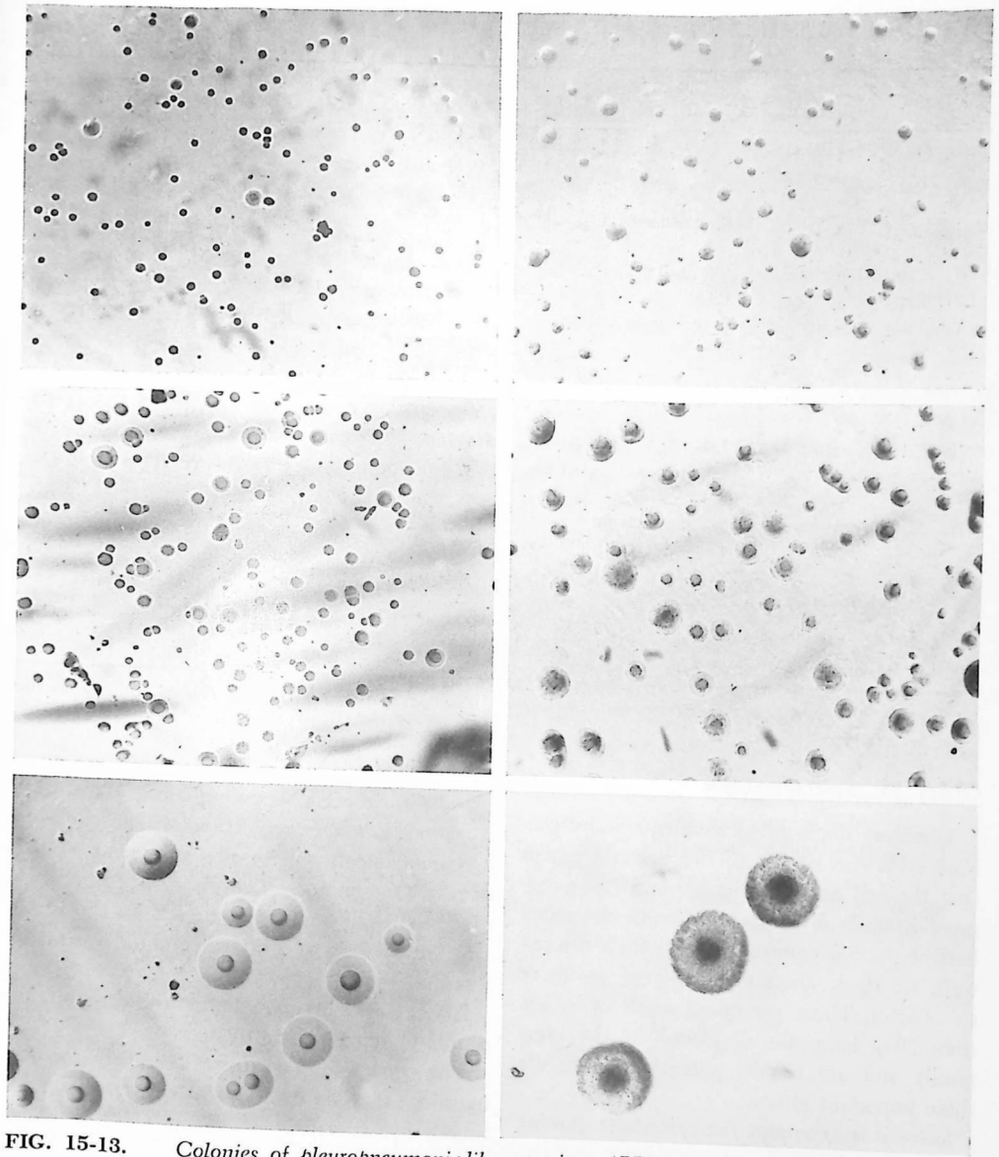


FIG. 15-13. *Colonies of pleuropneumonia-like organisms (PPLO). These are two related organisms of the PPLO group,* Mycoplasma *and the L form of* Streptobacillus. *The colonies take on a "fried-egg" look which seems to be due to the penetration of the capillaries in the agar by these somewhat nonrigid organisms. They then grow in the interstices of the agar, forming a ball-like colony which, when the organisms reach the surface, spreads out into a thin film.* [*From Razin and Oliver,* J. Gen. Microbiol., **24,** 225 *(1961). Courtesy of Dr. S. Razin.*]

TABLE 15-1. *The Higher Bacteria*

Order	Common Name	Important Groups or Genera	How Different from True Bacteria
I. Pseudomonadales	Photosynthetic bacteria	Thiorhodaceae Chlorobacteriaceae Athiorhodaceae	Photosynthetic
	Stalked bacteria	*Caulobacter* *Gallionella*	Stalk
II. Chlamydobacteriales	Sheathed bacteria	*Sphaerotilus*	Sheath
III. Hyphomicrobiales	Budding bacteria	*Hyphomicrobium*	Budding
V.* Actinomycetales	Branching bacteria	*Mycobacterium* *Actinomyces* *Nocardia* *Streptomyces* *Micromonospora*	Branching to filaments to spores borne on filaments
VI. Caryophanales	Large filamentous bacteria	—	Large filaments
VII. Beggiatoales	Filamentous bacteria	*Beggiatoa* *Leucothrix*	Cells in filaments
VIII. Myxobacteriales	Gliding bacteria	*Cytophaga* *Sporocytophaga* *Myxococcus*	Flexible cell wall
IX. Spirochaetales	Spirochetes	*Borrelia* *Treponema* *Leptospira*	Flexible cell wall, spirals
X. Mycoplasmatales	PPLO		No cell wall

* Order IV, the Eubacteriales, comprises the true bacteria. Pseudomonadales has true bacteria in addition to the higher bacteria shown above.

Order IX. *Spirochaetales*

These are single-celled, slender, spiral organisms otherwise similar to the bacteria except that the cell walls are flexible and the movement is easily distinguishable from the members of the *Spirillum* genus, in which the cell walls are rigid. There are two large groups of spirochetes. Those possessing small cells, less than 20 μ long, are all placed in the same family and are usually parasitic. There are three important groups:

Borrelia sp. contains the spirochete causing African and American relapsing fever and also the organism associated with trench mouth (Vincent's angina).

Treponema. Treponema pallidum is the cause of syphilis (Fig. 15-12); *Treponema pertenue* is the cause of yaws.

Leptospira. Members of this genus are aerobic. One is especially remembered, if only for its name, *L. icterohaemorrhagiae*, the cause of a type of infectious jaundice known as Weil's disease. One harmless *Leptospira* (*L. buccalis*) can usually be seen in scrapings from the teeth and gums; is is a common organism in the mouth.

All these short spirochetes are difficult to stain and difficult to cultivate.

The other group of spirochetes are mostly harmless; they are from 30–50 μ in length, and usually live in water.

Order X. *Mycoplasmatales*, the Pleuropneumonia Group (PPLO)

These organisms are related not only to the bacteria, but also to the gliding bacteria, and are characterized by a lack of a rigid cell wall. They are **pleomorphic** and break up into coccoid forms of varied size, some small enough to pass through a filter, where, after such passage, they form colonies (Fig. 15-13). Some of these pleuropneumonia-like organisms (called PPLO for short) cause a type of respiratory disease. Other true bacteria, especially *Salmonella* and *Proteus*, can, when grown under proper conditions, also form something which looks much like PPLO. Some organisms earlier called L forms also belong here. The entire group appear to have lost the ability to form a rigid or complete cell wall, and yet are able to live under conditions that true bacteria, deprived of their cell wall, find impossible. One may apparently convert some true bacteria to L forms and to PPLO(?) by growing them in the presence of penicillin, thus preventing proper cell wall development, but whether these converted forms are precisely the same as the L or PPLO is not known.

Summary

In Table 15-1 we have attempted to summarize the information on higher bacteria. It appears that here is a wealth of microbial organisms whose study is in its infancy. Doubtless there are many more living things of unique and fascinating properties had we but the wit to search for them or to see them when they appear to us.

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QUESTIONS

1. Define or identify

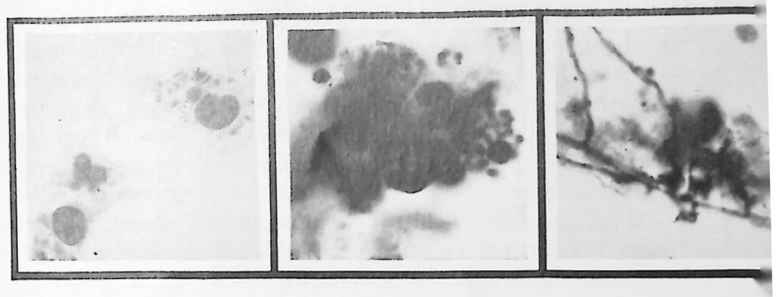
Shizomycetes	*Hyphomicrobium*	iron bacteria	*Beggiatoa*
Pseudomonales	actinomyces	sheath	*Cytophaga*
Eubacteriales	streptomyces	*Sphaerotilus*	PPLO
polar flagella	proactinomyces	*Caulobacter*	spirochete
stalked bacteria	lumpy jaw	*Gallionella*	
gliding bacteria	stable mycelium		
holdfast	hyphae		

2. You are called in as an expert consultant to a paper mill which has a problem which they

think is due to algae. The paper as finally rolled and pressed has occasional small spots in it which turn brown and the centers fall out with time. On examining the pulp beds you find them overgrown with *Sphaerotilus*. What could you do to prevent the trouble?

3. What would you call a gram-positive filamentous organism which shows branching but no spores? The filaments are several microns long but only about 0.5 μ wide.
4. What would you call a flexible rod-shaped organism which was motile and in which you could sometimes find a large sporelike body?
5. Why do we separate those organisms conforming to the definition of bacteria into two families?

Chapter 16



HIGHER MICROORGANISMS

"What are the most successful individuals which Life has to show? The multi-cellular. And what has gone to their making? The multi-cellular organism is in itself a variant from the perennial antagonism of cell and cell. Instead of that eternal antagonism it is a making use of relatedness to bind cell to cell for co-operation. The multi-cellular organism stood for a change, in so far, from conflict between cell and cell to harmony between cell and cell. Its coming was, we know now, pregnant with an immense advance for the whole future of life upon the globe. It was potential of the present success of living forms upon the planet. Implicit in it was for one thing the emergence of recognizable mind. It is surely more than mere analogy to liken to those small beginnings of multi-cellular life of millions of years ago the slender beginnings of altruism today."

SHERRINGTON, *Man on His Nature*

IN THE PRECEDING CHAPTERS we have discussed those living creatures clearly within the area of microbiology. Perhaps a narrow interpretation of "bacteriology" would be somewhat restrictive, but certainly all would agree that the study of the bacteria and that broad area we have called "higher bacteria," including *Streptomyces*, are the area of a distinct science, microbiology. Here the microbiologist holds sway and is not challenged by other sciences of different viewpoints. For creatures smaller than bacteria, those living within other cells, while still within the border of microbiology, there has grown a distinct and separate science of

virology, related, indeed, but different in its methods, its approach, its philosophy. There are many eminent microbiologists who are not virologists and who make no pretense of knowing this subject. There are also many virologists who make no pretense of knowing other areas of microbiology.

When we progress to higher organisms, those more differentiated, we enter an area of knowledge where there are several sciences. First there is *protozoology*, the study of protozoa, a division of zoology. Second, there is the study of the chlorophyll-containing plants without root system or leaf, the algae; certainly this is a division of botany. Third, there is mycology, the study of the fungi or molds, those plants which lack chlorophyll and lack also root, stem, or leaf. This is also a part of botany and may also be found as a division of plant pathology. Mycology also includes the study of yeasts, the single-celled fungi, although there are so many persons devoting their entire attention to yeasts that this study might also be regarded as a separate science.

At one time it was possible to retain within the broad area of microbiology a somewhat detailed knowledge of each of these kinds of creatures. But it seems apparent today that the modern microbiologist, chemically oriented and practically minded, cannot hope to do more than glance at these creatures. Or perhaps we should say that we should abandon the pretense that any one book can adequately cover all of these areas. We shall therefore devote only a very general discussion to each. The relations among the four groups of organisms are shown in Fig. 16-1.

The Algae

These organisms are the chlorophyll-containing plants without root, stem, or leaf. They may of course be single-celled or many-celled, and the latter may grow to several yards in length or longer. They are, by and large, aquatic creatures and have been called "the grass of many waters." None cause disease but some, on growth, produce poisonous substances. Sometimes they grow so vigorously as to produce poisonous tides, which may be green, red, yellow, or brown. As a matter of interest, this kind of a growth of algae may well have been one of the plagues visited upon the Egyptians.[1] Further, they may grow and cover the surface of ponds and other bodies of water and sometimes create a nuisance. They may contribute odor and taste to water supplies. But in general their growth is more valuable and benign. They serve, of course, as food for aquatic animals—from small minnows to whales. The amounts growing in waters, while normally too dispersed to be harvested profitably, are enormous. An inland lake such as Lake Winnebago in central Wisconsin—about 12 miles wide and 25–30 miles long—pours about 130 tons of algae a day into its outlet.

It is possible that they may be used as food for man in the future—they are rich in protein, fat, carbohydrate, and vitamins, and make a suitable fodder for animals. It is possible to make allegedly palatable soups and noodles from them. Experimentally they are grown for this purpose in large polyethylene bags on a pilot-plant scale. When algae photosynthesize they use CO_2 and produce O_2, as do green plants. Apparatus using them is being studied for space travel. To supply the oxygen for a man, an area of algae of 5 square meters would be required; under these circumstances about 600 g of algae would be produced, enough to satisfy the minimum daily requirement of vitamins and some of the energy. At present the food chain is somewhat inefficient—roughly 100,000 pounds of algae feed 10,000 pounds of small floating animals, which feed 1,000 pounds of crustaceans, which feed 100 pounds of small fish, which feed 1 pound of large fish.

[1] Exodus, 8:17–21; verses 20 and 21 say in part, "the waters of the river were turned to blood. And the fish that was in the river died; and the river stank, and the Egyptians could not drink of the water of the river . . ."

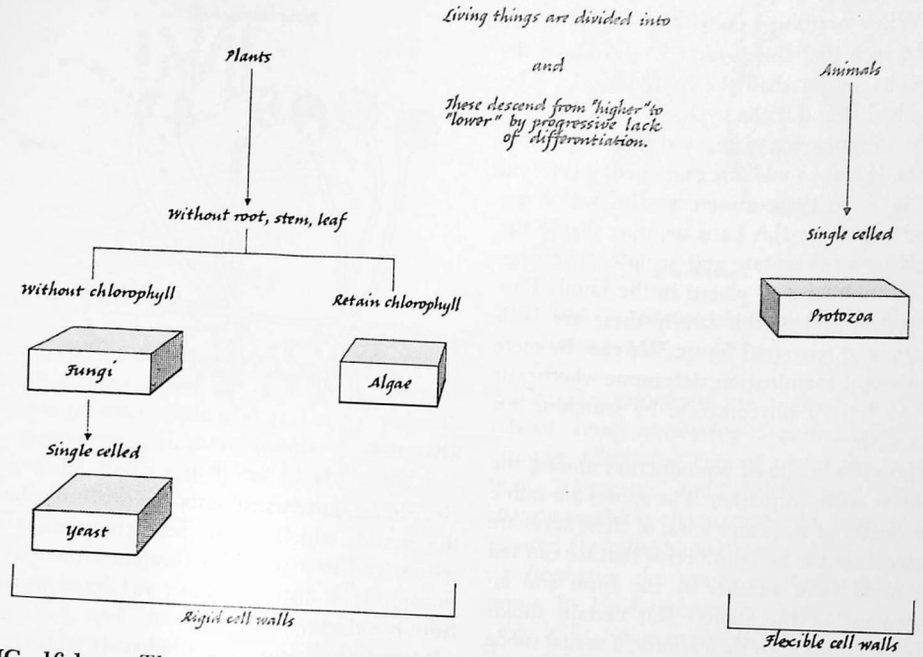


FIG. 16-1. *The relationships between the higher microorganisms.*

An interesting biological form is the lichen—an incrustation on rocks, trees, soil. Lichens occupy some of the most forbidding environments on earth and some of the most extreme; they are found on the sun-baked desert rock, too hot to place a hand on, to the rocks just below the snow line on the highest mountains. The lichen is not a plant—it is a colony; two entirely different organisms, a fungus and an alga, associate to form it. The fungus seems to live off the alga and the biological relationship is a stable one. Some lichen colonies seem to be over 2,000 years old. The alga involved can live without the fungus but so far as is known the fungus must live in this association, at least in nature, although they can be cultvated in the laboratory.

The Fungi: Molds

Without a precise definition, these creatures are the plants, devoid of seed, stem, leaf, or root and without chlorophyll, which reproduce by methods other than fission. There are three main types: (1) The Basidiomycetes" (family), the mushrooms or "higher fungi," whose consideration we omit entirely. (2) The fungi, with long mycelium (= the molds). (3) The lower fungi, with short or nonexistent mycelium (= the yeasts).

The Molds

To the naked eye the growth is fuzzy or cottony, sometimes colored black, blue, green, or

red. When examined under the microscope it will be seen that they consist of strands of tissue wider by far than the bacterial cells. The individual strand is the hypha; the mat formed by the interweaving of many strands is the mycelium. If many molds are examined, it is found that in some there are cross-walls, which are called **septa;** on this basis we may divide the molds into nonseptate and septate. The nonseptate molds are all placed in the family Phycomycetes, and in this family there are both aquatic and terrestrial forms. We can, by mere microscopic examination, determine whether or not we have a phycomycete by searching for cross-walls.

The main mode of reproduction among the molds is spore formation. The spores are borne in a variety of ways and some of these ways are so characteristic and distinctive that we can tell one mold from another by the form and arrangement of the spores. But certain molds have, in addition to their spores, a sexual mode of reproduction wherein two mycelia strands fuse or otherwise intermingle and form a variety of sexual spores. In virtually all cases the sexual spore formation is a rather rare process and usually requires very special conditions. Hence, the spores you will see in casually examining cultures are all nonsexual spores.

As we have mentioned, the classification of molds is a matter for experts and is based upon details of morphology of the hyphae, the asexual spores, and the sexual spores. The modern microbiologist, unless he is specifically a mycologist, does not normally have such taxonomic details at his fingertips. Instead, then, of outlining any taxonomic scheme, we shall describe and list a few common genera which have some special importance in microbiology.

Rhizopus sp. (nonseptate, i.e., Phycomycetes). This is the common "black mold" of bread but on most bacterial culture media, upon which it grows readily, it has a light airy appearance. The hyphae strands (Fig. 16-2)

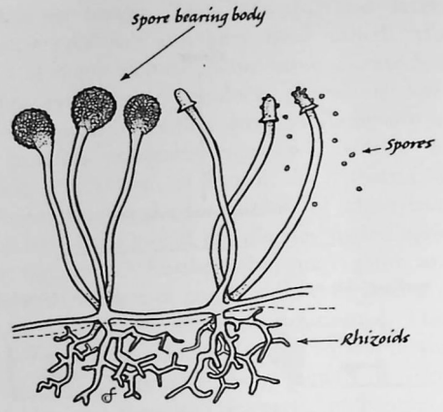


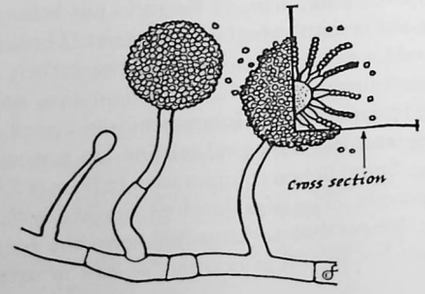
FIG. 16-2. *Rhizopus.*

give rise at particular points to **fertile hyphae,** the strands which are to bear the nonsexual spores, and to a sort of a rootlike structure, a rhizoid. It is usually found in decomposing fruit, bread, etc.

Mucor sp. (nonseptate, Phycomycetes) is very similar in appearance to *Rhizopus* but has no rhizoids.

Aspergillus (Fig. 16-3). Like most others described here, this is a septate mold. The spores may be black or green and sometimes red or yellow and thus the mass of growth may have this color. There are a few pathogenic species, especially in birds (aspergillosis), but members of this genus are found mostly on spoiled foods,

FIG. 16-3. *Aspergillus.*



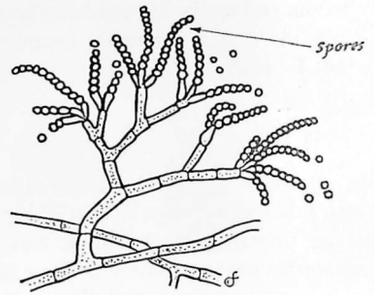


FIG. 16-4. *Penicillium.*

paper, etc. In man these molds sometimes cause ear infections. Some are pathogenic to plants. Others produce citric acid and other materials of commerce. The asexual spores are borne on a fruiting body usually raised above the medium, and these bodies have the appearance of a "spiked war club."

Penicilium sp. In this septate fungus the spores have the "brush" structure shown in Fig. 16-4, and are commonly found on spoiled fruit. They are normally greenish, but may be white, blue, or black. Some are responsible for particular cheeses—Roquefort and Camembert. Some produce penicillin and other antibiotics. The green mold commonly seen on spoiling citrus fruits is usually penicillium. They are not pathogenic.

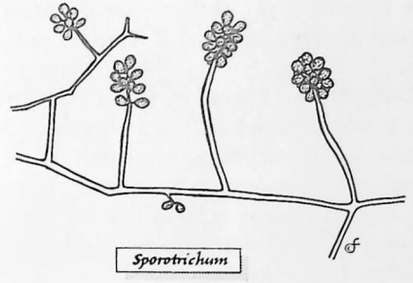
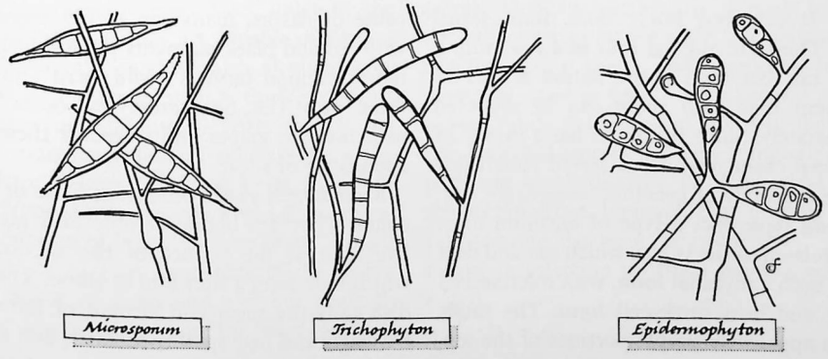


FIG. 16-6. *Sporotrichum is involved in more deep-seated fungus infections.*

The *Dermatophytes* are a group of closely related fungi producing superficial mycoses (fungus infections) coming under the general terms "ringworm" and "athlete's foot." *Microsporum* produces characteristically large, rough, pointed (spindle-shaped) spores (Fig. 16-5); in *Trichophyton* the spores are longer and narrower; in *Epidermophyton* they are rounded (Fig. 16-5). Some invade the hair follicle; some do not. They grow readily on common laboratory media and unfortunately the spores may live for years in wood floors, dressing cabins, etc. Hairs infected with *Microsporum* fluoresce under ultraviolet light.

Sporotrichum (Fig. 16-6) also causes an infection of the skin, especially the hands. In cul-

FIG. 16-5. *Pathogenic fungi, Dermatophytes, involved in ringworm. Trichophyton is also involved in "athlete's foot."*



ture it shows a mycelial growth; in tissues it appears as single cells and is difficult to recognize.

Trichoderma (Fig. 16-7) is not pathogenic but is widely found in soil. It is very active, metabolically attacking proteins with the rapid liberation of ammonia. It is also exceedingly active in decomposing cellulose. It has a green powdery appearance and on first sight may be mistaken for *Penicillium*.

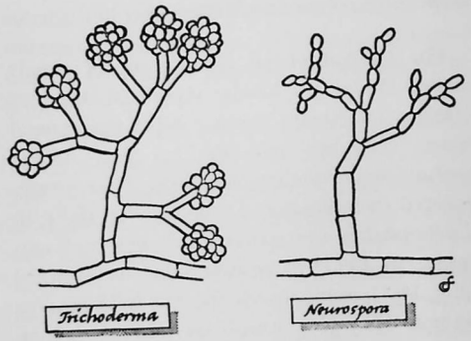


FIG. 16-7. *Trichoderma and Neurospora.*

Neurospora (Fig. 16-7) is not perhaps of itself quite as important in the economy of nature as man as made it. It is the "red bread mold." Its septate mycelium may break up into single cells or small chains of cells. It has a loose network of fluffy aerial mycelium bearing pink or orange-red spores. These are its asexual spores. It can, but rarely does, form sexual spores. These are aligned four in a row, within a little sac, but such is the nature of this arrangement that each spore can be dissected out separately. Since this mold has a variety of easily seen characters, it has served remarkably well for the study of genetics.

Oidium represents a type of organism more closely related to the yeasts, which can and does exist in both a mycelial form, with intermeshed hyphae, and in a single-cell form. The single cells are apparently simple portions of the septate mycelium and can be formed from any part of it. The whitish mold frequently found growing on milk is usually an *Oidium*.

The Yeasts

The yeasts are single-celled nucleated microorganisms, spherical or more or less elongated, reproducing primarily by budding, although some employ fission. In a sense yeasts are molds that produce no mycelium, and the gradation between *Oidium* and yeasts is very gradual. The single cells of yeasts might be regarded as a septate mycelium, if they formed mycelium. The important genera are given here.

Saccharomyces; nonsexual reproduction by budding. Here are the common beer, ale, and wine yeasts.

Schizosaccharomyces; nonsexual reproduction by fission. The fission differs from bacteria in that first the nucleus divides, followed by the rest of the cell. These yeasts are not very common.

Zygosaccharomyces; the honey yeasts. They divide primarily by budding, but are characterized by their ability to stand high osmotic pressure due to sugar. They can and do grow in honey, fermenting it to an alcoholic beverage known as mead.

Torula; much like *Saccharomyces* except for the lack of sexual spores. Many of these yeasts produce little or no alcohol, but sometimes they cause off-flavors; many are highly pigmented, with red and black pigments predominating. A more common term is "wild yeast" although "true" yeast (i.e., *Saccharomyces*) occur "wild" in nature, on grapes and wherever there is an abundance of sugar.

Mycoderma; yeasts producing little or no alcohol. They are highly aerobic and generally live right at the surface of the media, over which they form a thin film or slime. They oxidize away the sugar and alcohol of fermented beverages and may spoil their flavor and aroma.

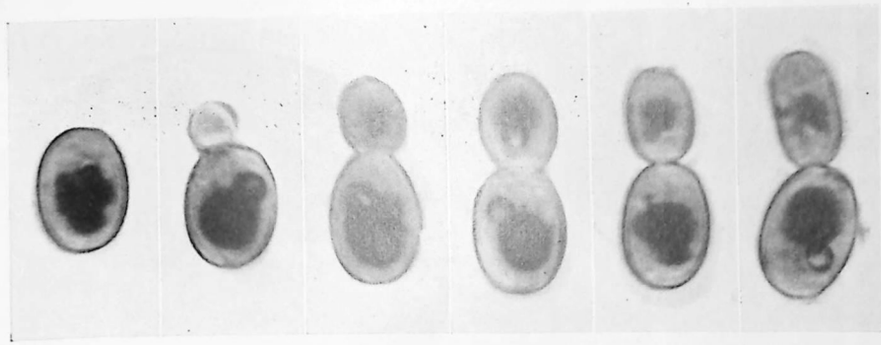
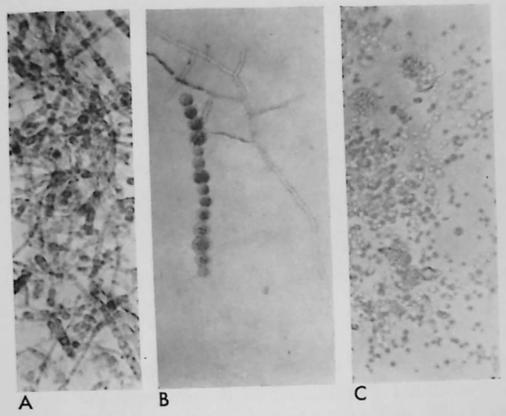
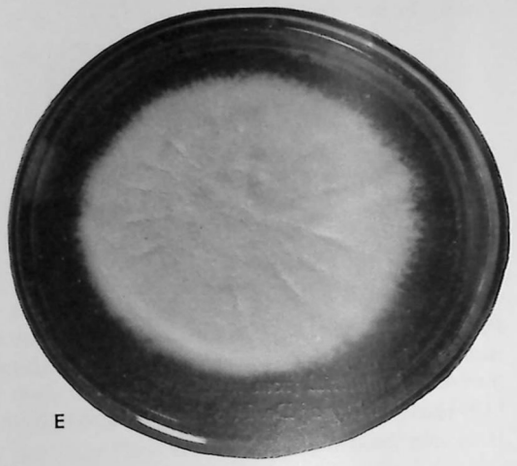


FIG. 16-8. *Details of bud formation in a yeast,* Candida utilis. *These photographs employed ultraviolet photography and an "optical tracing substance," S-adenosyl methionine, to demonstrate the transfer of material from mother cell to the bud.* [*From Svihla and Schlenk,* J. Bacteriol., **79**, *841 (1961). Courtesy of Dr. F. Schlenk; Argonne National Laboratory photograph.*]

In the vast majority of cases, when we say yeast we mean the *Saccharomyces*. The structure is illustrated in Fig. 16-8, where a closely related yeast is shown. They are much larger than bacteria, they possess a definite nucleus, sometimes difficult to see unless we stain for it, and they reproduce by budding. A cell may bud from a bud not yet severed from the mother cell and thus form a temporary mycelium. They occur in nature whenever sugar is present in high concentration, thus occurring commonly in orchards and vineyards. Various strains of yeast have varied properties. Many particular strains, generally classed as *Saccharomyces ellipsoideus*, produce particular wines. They differ in the flavors and aromas they are capable of producing and generally produce high alcoholic content (16–20%). Some, such as those producing champagne and burgundy, settle rapidly and form a compact sediment; others remain suspended for a long time and settle with difficulty. Other strains are concerned primarily in the production of beer ("bottom yeasts") or ale ("top yeasts"). They contribute to the characteristic flavor of beers and ales but generally produce less alcohol than the wine yeast. A beer mash fermented by a wine yeast or a grape juice fermented by a beer yeast does not yield desirable products. Still other yeasts produce high alcohol but little flavor and are used for industrial alcohol production. Cells of yeasts in old cultures develop a thickened cell wall which becomes filled with granular reserve material, and the cells go into an apparent resting stage, in which they are more resistant to changes in the external environment than usual, and from which the vegetative cell can regerminate.

Pathogenic yeasts. There are a variety of infections, usually deep-seated systemic diseases, which are caused by certain yeasts or yeastlike agents. They are particularly difficult to treat since there are so far no really adequate agents effective against them. The organisms, for the most part, exist in both a mycelial and a yeast phase. While it is not our intent to delineate the medicinal aspects, we have summarized the pertinent yeast diseases in Table 16-1. We should point out, however, that in most medical literature the term "blastomycosis" simply means an infection in which budding cells are found.





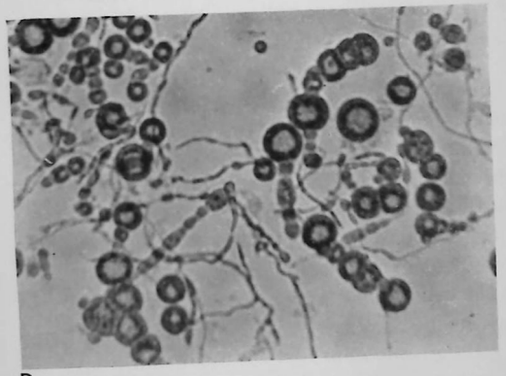


FIG. 16-9. *Morphology of* Coccidiodies immitis. *Various forms which may be found under different conditions. The large spheres break down to liberate spores into the medium.* [*Condensed from Converse and Bessemer,* J. Bacteriol., **78,** 231 (1959) *and* **79,** 480 (1960). *Courtesy of Drs. Converse and Bessemer. U. S. Army photographs.*] *Morphology of two-week old culture on petri plate and a wet mount (lactophenol-cotton blue of the mycelium.* (E, F) [*From Foley, Berman, and Smith,* J. Bacteriol., **79,** 480 (1960). *Courtesy of Drs. Foley and Smith.*]

Protozoa

The protozoa are single-celled animals. While one or two possess chlorophyll, and are thus intermediates between plant and animal, most are devoid of chlorophyll. Just as the ultimate character of the plant was the rigid cell wall, the protozoa exhibit the ultimate character of the animal—the nonrigid cell wall. In fact this animal cell wall has the osmotic properties of the animal cell and corresponds to the cell membrane of the plant or bacterial cell. There are many thousands of species of protozoa, some almost as simple as bacteria, some with exceedingly complex structures and life cycles. The study of protozoology is itself so large a science that it has a subdivision, parasitology or medical parasitology, which is concerned with those animals parasitic in or on man. This, of course, includes not only the single-celled animals, but, by general custom, also the worms which infect man. We shall not include the worms.

Classification of the protozoa is a matter for experts and it is not our purpose to classify these organisms except in a very general way. For most protozoa there is a life cycle consisting of an active phase, in which the animal feeds and multiplies, and a resting stage, sometimes an encystment. The majority of protozoa either absorb food from solution or feed directly on microbial cells. Reproduction is generally by fission, either longitudinal or transverse; a few protozoa have sexual cycles. For the general microbiologist it is the pathogenic protozoa with which he has concern, but before we pass to a consideration of the protozoal parasites, however brief that may be, two other matters will be of interest.

First, protozoa play a curious and little understood role in the population of the soil. Their presence tends to increase its fertility, but how this is brought about is not at all clear. Second, termites have only a limited ability to digest the cellulose (food) that they live on, and if the protozoan inhabitants of their intestines are removed they usually starve. Hence a large part of the action of termites in destroying wooden structures is due to protozoa living in the termite gut.

TABLE 16-1. *The Major Diseases Caused by Yeastlike Fungi*

Disease	Organism	Remarks
Cryptococcosis (European Blastomycosis) (Torula Meningitis)	*Cryptococcus neoformans*	The older term for this organism was *Torula histolytica.* Generally ulcers on skin, spreading to blood stream or nervous system. Organism heavily encapsulated.
Blastomycosis (American blastomycosis) (Gilchrist's disease)	*Blastomyces dermatitidis*	May become chronic, but systemic form rapidly fatal. Chronic state derives from infection in skin; systemic form from lungs (Fig. 16-10).
Moniliasis	*Candida albicans* (*Monilia albicans*)	Disease has many aspects depending on invasion site; skin, nails, mucous membranes, lungs, menges. In throat may be mistaken for diphtheria. When the disease invades the mouth it is called "thrush."
Coccidiomycosis	*Coccidioides immitis*	Disease has two forms, an acute self-limiting respiratory infection or a rapidly fatal fever-ridden disease otherwise resembling tuberculosis. In tissue, the organism does not bud but forms small spheres; in culture it appears like a mold. Disease first thought to be caused by a protozoan, hence its name (Fig. 16-9).
Histoplasmosis	*Histoplasma capsulatum*	This is an air-born infection, especially from chicken coops. The disease exists in four forms, from chronic skin lesion to systemic infection; the latter is usually fatal. Organism originally thought to be a protozoan (Fig. 16-10).

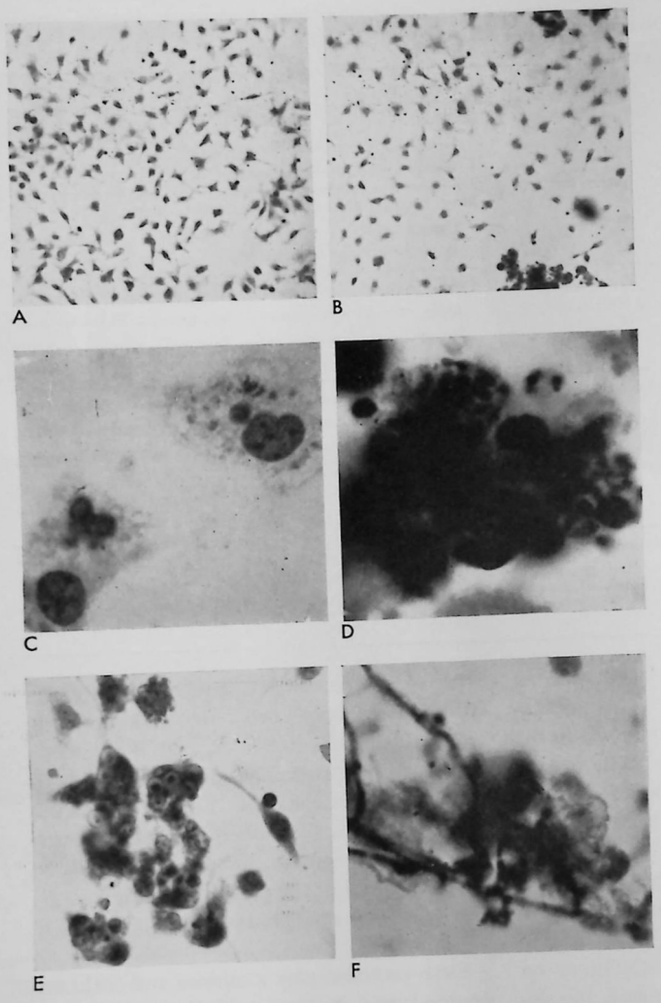


FIG. 16-10. Histoplasma *and* Blastomyces. *A is an uninfected tissue culture of mouse peritoneal exudate. B has been infected with* Blastomyces dermitidis. *C is an enlargement of one of the areas of parasitization shown in B. D and E are enlargements of cells infected with* Histoplasma capsulatum. *F shows the development of fungus like mycelium in the tissue culture.* [*A, B and C from Howard and Herndon,* J. Bacteriol., **80**, 522 (1960), *and D, E, and F from Howard* J. Bacteriol., **78**, 69 (1959). *Courtesy of Dr. Howard.*]

Pathogenic Protozoa

1. Amebiasis or amebic dysentery: *Endamoeba histolytica.* This disease takes many forms and clinical descriptions are varied for the apparent reason that the amoeba alone appears unable to initiate the disease but requires the collaboration of moderately pathogenic bacteria. Germ-free animals inoculated with bacterial-free protozoa show no disease. In man the protozoan penetrates the intestine, causing inflammation of it, and may penetrate to the liver and lungs. In the intestine the amoeba form cysts which are most resistant to chemical disinfectants, especially chlorine, and its control is a matter of preventing contamination.

2. African sleeping sickness: *Trypanosoma gambiense.* The organism, shown in Fig. 16-11 may be seen wiggling among the red blood cells. It is transmitted from one host to another by the tsetse fly. The organisms are picked up in the blood and travel through the stomach of the insect to the salivary gland, where they are injected into the new host. The disease is distributed in animals but in Africa man is the usual source. There are only two species of flies involved as carriers and they are confined to Africa and South America, which is also the limit of the human disease. There are other pathogenic trypanosomes, some causing widespread disease in animals as well as man. All are insect-borne.

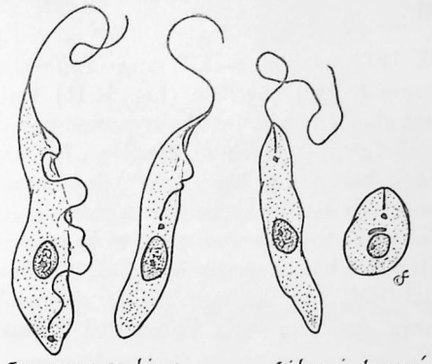
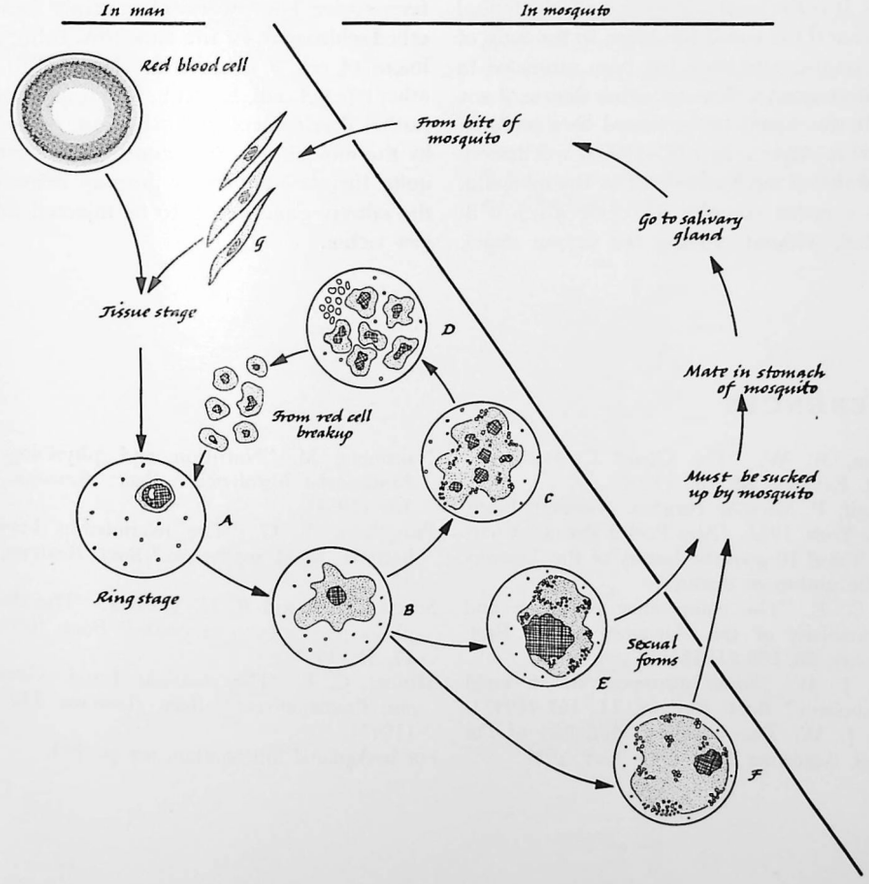


FIG. 16-11. *Pathogenic flagellates.*

FIG. 16-12. *The transformations undergone by the malaria parasite. Drawn for* Plasmodium vivax; *other forms differ slightly. (See text.)*



3. Leishmaniasis or kala azar: *Leishmania donovani*. This flagellate (Fig. 16-11) has a stage closely resembling the trypanosomes and another stage in which the flagella are lost. The disease kala azar ("black fever") is a chronic one which later may become a skin disease. Closely related organisms seem to be involved in the skin lesions usually associated with "oriental sore," as leishmaniasis is commonly known. All seem to be transmitted by small flies.

4. Malaria: *Plasmodium vivax*. While the other protozoal diseases are largely confined to the tropics, malaria (there are several kinds) has been known in Europe and the United States. It is the most widespread of any tropical disease and the toll it has taken in the form of death or incapacitation has been estimated to have been greater than any other disease of any sort. It was found to be caused by a protozoa in 1880 in Algeria. In 1897–1898 it was demonstrated that it was transmitted by the mosquito. It has a rather complex life cycle which is illustrated, without naming the various stages, in Fig. 16-12. The uninfected red blood cell may become infected either by a long slender form (G) from the salivary gland of the mosquito or from the very small celled form of the parasite generated when a red cell bursts. The initial uninfected cell has a characteristic ring on it (A), which is converted into an irregular body with only one nucleus (B). From B, the nonsexual form becomes enlarged and filled with minute red granules (C). The single nucleus now divides into 12 to 24 (usually 16) nuclei (D). The cytoplasm now divides into the same number of cells or nuclei and the red cell bursts, sending the small malarial cells into the blood stream. It is at this point that the fever recurs. This process of multiple fission is called **schizogony.** At the same time other specimens of cell B may be developed into two other types of cells, E and F, which undergo no further development until they are picked up by the mosquito. In the stomach of the mosquito they fuse and their progeny migrate to the salivary gland, ready to be injected into a new victim.

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For background information, see p. 494.

QUESTIONS

1. Define or identify:

algae
fungi
yeasts
plankton
lichen
molds
fertile hyphae
rhizoid
hyphae
mycelium
blastomycoses
life cycle
amebic dysentery
African sleeping sickness
oriental sore
septa
phycomycetes
Rhizopus
Mucor
Aspergillus
Penicillium
dermatophytes
Sporotrichum
Trichoderma
Trichophyton
Microsporum
Oidium
Neurospora
aerial mycelium
Schizosaccharomyces
Zyosaccharomyces
Torula
wild yeast
mycoderma
bottom yeast
top yeast
malaria
schizogony

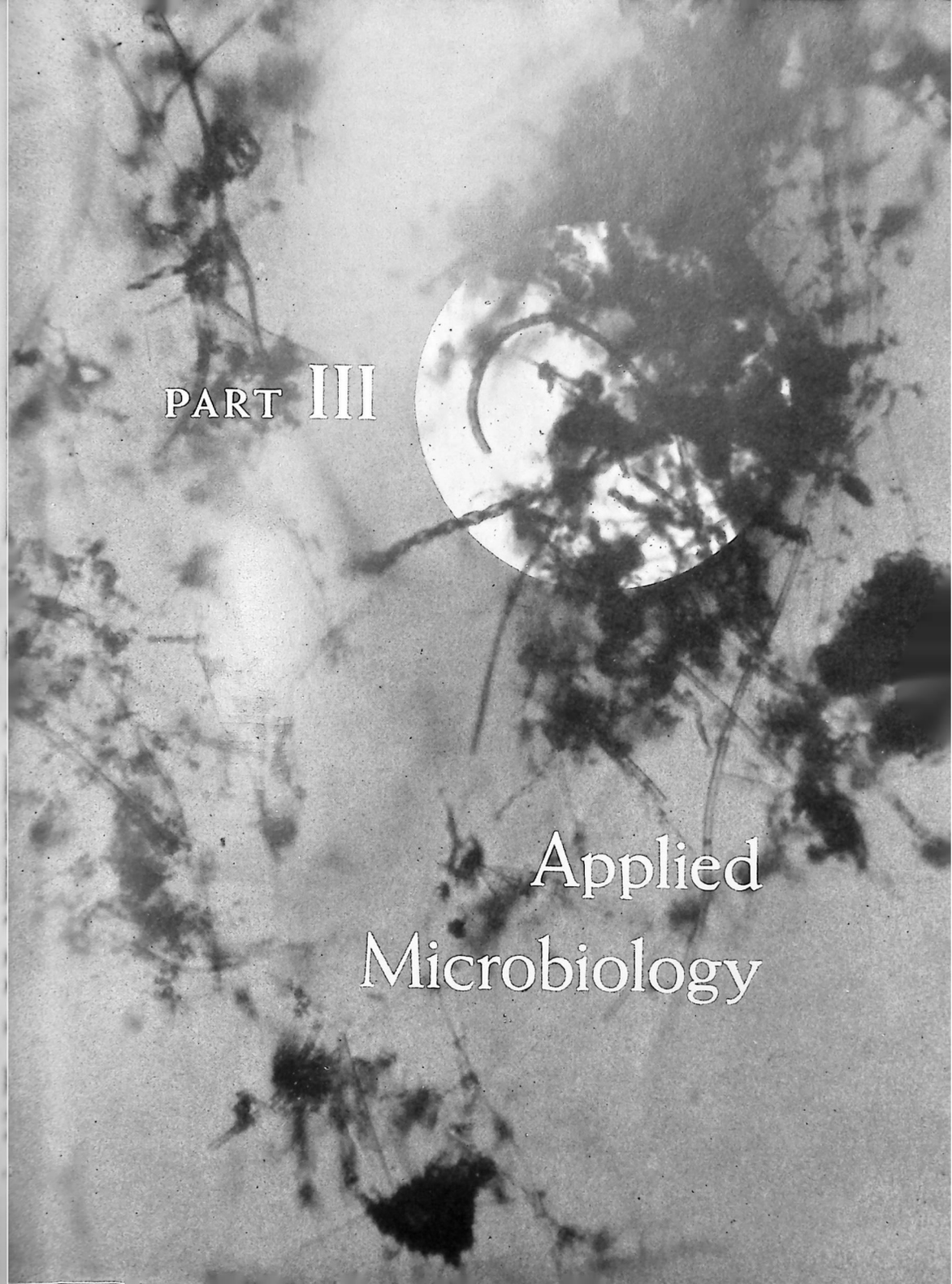
2. In 1726 Sir John Floyer wrote of a "remarkable instance of an asthmatic who fell into a violent fit by going into a wine cellar where must was fermenting." This is presumably the first case of an allergy to molds or yeasts. The spores of *Alternaria* and *Hermesdendrum* are second only to pollen as wind-borne allergic reactions. How would you attempt to count these in the air?

3. The growth of algae is frequently responsible for the scum on water, for "dog days," and for various other difficulties. Among these is the development of bitterness in water. How would you defend a client accused of permitting the growth of the bitter algae?

4. The anopheles mosquito, the carrier of malaria, is present in several areas of the United States. Why don't we have recurring malaria epidemics?

5. What causes ringworm? Why is the disease called by this name?

Facing page, higher Bacteria found in water: *Spirophlyum, Leptothrix, Crenothrix.* [Courtesy P. M. Borick, Bristol-Myers Company.]

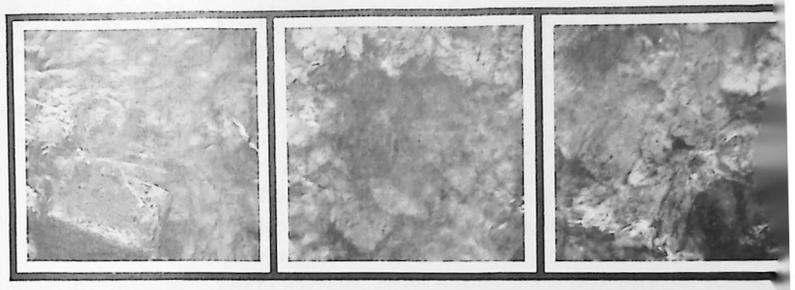


PART III

Applied Microbiology

WE HAVE EARLIER mentioned that one of the characteristics of the modern microbiologist which distinguishes him from the more classical type is that he is familar with and contributing to a vast application of microbiology, not only as it applies to disease, but as it applies to our daily life, to our commerce, to our agriculture. And in this regard modern microbiology is not the handmaiden to some other previously established art or science, as it tended to be to medicine, but rather, microbiology is frequently the driving force, the reason that the art or science was established in the first place. The modern microbiologist is therefore an integral part of the modern world. He lives in no ivory tower separated from the needs and wants of man, but is in the thick of life. It is perhaps for this reason that microbiology is close to our daily living, that it has attracted a particular type of vital person, namely yourself, to its study.

Chapter 17



THE MICROBIOLOGY OF WATER, SEWAGE, AIR

"When the Persian king goes to war, he is always well provided not only with victuals from home and his own cattle, but also with water from the Choaspes, a river which flows past Susa. No Persian king ever drinks water of any other stream, and a supply of it, *ready boiled for use* is brought along in *silver* jars carried in a long train of four-wheeled mule wagons wherever the king goes." (Italics ours.)

HERODOTUS, *On Cyrus the Persian*, Book I, 189

THE CURIOUS and unusual properties of water play so important a role in living systems that it is commonly said, "No life without water." But it is almost equally true to say, "No water without life," for sterile water in nature is hard to come by. Of course we can sterilize water and there are a few places in nature where the water is essentially sterile—snow on high mountains, for example. But in the more usual water, even that from deep wells, microorganisms may be found. Much the same may be said for air.

This chapter considers several aspects of the microbiology of water and air, especially the detection of the contamination of water and the disposal of sewage; in these the microbiologist plays an important role. The chapter is divided into these sections: The microbiology of natural waters (p. 310), Detection of fecal contamination of water (p. 313), Purification

of water (p. 316), Sewage disposal (p. 317), Microbiology of air (p. 321), and other related aspects (p. 323).

Microbiology of Natural Waters

One would suppose that water in clouds and in rain would contain no living things and thus be sterile, at least until it struck the earth. But this is not the case; rain water collected in sterile containers and protected from contamination generally contains 1 to 10 organisms per milliliter. Over an average city in the temperate zones, rainfall brings down 5 million microorganisms per year. Snow similarly contains living bacteria and, surprisingly, hail stones usually contain many more. The first rain or snow that falls contains more organisms than that falling later, so that it is apparent that air is the origin of the bacteria and that the rain and snow are washing out suspended organisms.

Rivers, lakes, wells, underground streams, ponds, and indeed, wherever water occurs in nature, there too will be found living microbes —mostly bacteria, but sometimes molds and streptomyces. Their numbers will vary tremendously, depending primarily upon what they may find to live on. Of course, various food materials—leaves, stems, animals, and what not —are continually falling into water. There are water plants, algae, fish, and thousands of things—a feast indeed for any of many kinds of microbes. How many can feed depends on how much there is to live on, the kinds of organisms controlled by the physical environment (*p*H, temperature, etc. of the water), and the nature of the material to be decomposed.

Decomposition in Nature

We must here digress to explain some basic principles of decomposition, which will be applied in several places later. These principles are almost obvious once you know them, but it is just possible that no one has yet taken the trouble to explain them—too much of knowledge is taken for granted.

Most natural materials—leaves and grass and "shoes and ships and sealing wax and cabbages and kings"—are chemically composed of many substances. They are made up of fats, proteins, carbohydrates, cellulose, tannins, lignins, but rarely of just one substance. In a very general way, the materials that are soluble in water are the most easily decomposed—sugars, alcohols, amino acids—and of course they will be attacked first and most rapidly. In a general way, too, more organisms can attack the easily decomposed substances than can attack the more difficult; practically every microbe can attack glucose but fewer can attack fat.

We can arrange materials in an "order of decomposability":

	Animal	*Plant*		
I	40%	20%	Simple sugars Amino acids Water-soluble substances	Easily decomposed by many organisms
II	40%	20%	Water insoluble Not too complex—proteins, hemicellulose, simple starches, etc. Simple fats	Readily converted into water-soluble materials and then readily decomposed
III	0	40%	Cellulose	Resistant to most organisms but readily decomposed by some
IV	10%	20%	Ring structures—phenolic substances, lignin, tars, oils, etc.	Difficult to decompose, somewhat specialized organisms

The rough percentages given for plant material and animal tissues will of course vary somewhat with the material, just as pine needles, oak leaves, and wheat straw differ, but the differences are not too great. From an analytical point of view one can, by the use of very simple crude methods, roughly separate an unknown material into the four general classes.

Class I. Determine how much is soluble in water.

Class II. Of that remaining, determine how much can be dissolved by boiling for a short while in dilute acid.

Class III. Of that remaining, determine how much can be dissolved by boiling in concentrated acid.

Class IV. The residue which is not inorganic (ash) is the part difficult to decompose.

Most organisms can attack substances of Class I, and it makes but little difference where the soluble material came from—a fish, a skunk, an old log. While the soluble material will differ, of course, it is still readily attacked by a wide variety of organisms and many such substances are attacked by the same organisms; hence the soluble material is soon all decomposed. Class II, of which starch is an example, is somewhat restricted. Not all bacteria can attack starch. Hence only those that can will grow. Class III is still more restricted. There are relatively few bacteria that attack cellulose, and these are of a restricted type; but many molds and streptomyces do so; hence we would expect these to begin to appear as the major portion of the microbial population after the materials of Classes I and II have been used up. Class IV is even more restricted. Its substances will be attacked by some few bacteria, some molds (especially higher molds), but many streptomyces. We can almost tell by looking at the kinds of microorganisms predominant in a given situation—assuming all organisms have access to it, which is the case in nature—the state of its decomposition.

The situation in water differs in two major aspects from that at other sites in nature where decomposition occurs. The water dilutes out the soluble organic material so greatly that its concentration is generally very low. And, because of the depth of the water, oxygen may be scarce, especially in the lower layers.

The first factor, that of dilution, thus favors organisms which are able to grow at low concentrations. In a sense they have to pump a great deal of water through their cells to get a little food; only those that use the food efficiently, that oxidize it all the way, can live. It would be a further advantage to be able to live on a wide variety of different substances. There is a major group of organisms (*Pseudomonas*) that possess just these properties and they are a prominent characteristic of water flora. We may easily recognize typical species such as *Ps. fluorescens* by its fluorescent pigment formed on most media. Plates made from natural waters show a predominance of this type of colony.

Since, in very low concentration of nutrient, an organism has to pump much water to get a little food, anything which will help concentrate the food will promote the growth of more organisms. Added food will do so, of course. But there is another way to concentrate food, usually forgotten; this is adsorption. If a plate of glass is suspended in water, it soon collects a very thin film of materials previously in solution but now adsorbed on the glass surface. Organic matter collects on rocks, piles, ship bottoms—the so-called fouling of ships. Just at their surface there are many more organisms because, for a limited space at the surface, food is more plentiful. One aspect of this puzzled the early water bacteriologists. If water was plated directly from a lake it had a particular bacterial count, but if collected in a sterile bottle, transported back to a laboratory, and plated the next day after vigorous shaking, it would

generally show a much higher count. The reason is that the glass from the bottle adsorbed nutrients from the water, permitting more growth on the glass surface than was possible when this same amount of nutrient was distributed throughout the water.

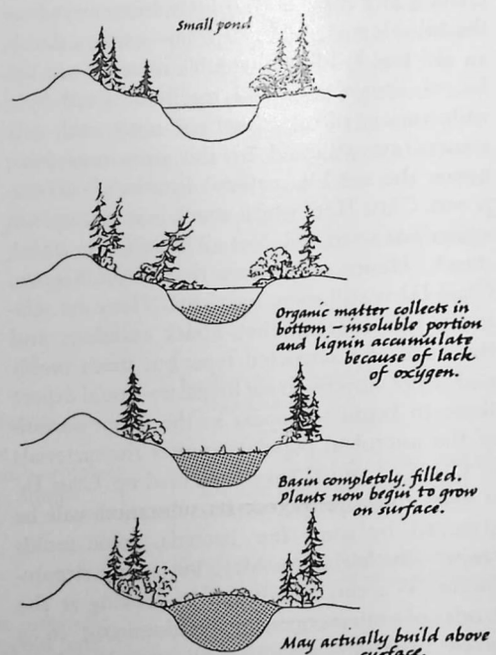
This aspect of adsorption has also been taken advantage of in another way. There are organisms which firmly attach themselves to solid surfaces. They can therefore live in running water and run less risk of being washed away. At the same time the surface adsorbs the nutrient from a large volume of water flowing over it. Such bacteria are sometimes called epiphilic (surface lovers) and usually stick to the surface by way of a gummy capsule. There are some that have modified their mode of growth sufficiently so that this capsule has become a stalk. Such stalked bacteria (Chapter 15) may take several shapes. They have not been adequately cultured so that little is known about their physiology. Some deposit ion in their stalks. Some, indeed, grow to such an extent and deposit (adsorb) so much extraneous material in their stalks that in some rare cases they have clogged water pipes.

The second aspect peculiar to decomposition in water is the relative lack of oxygen in water. Of course, rivers and streams, especially if they have rapids and waterfalls, are kept aerated by their turbulence. They generally are clear and have low populations of bacteria; added organic materials are usually rapidly oxidized away. But a large lake is a different story. True, there are waves at the surface, but generally they do not cause turbulence, nor incorporate air, much below two or three feet. Below this level oxygen penetrates slowly and is generally lacking. In some cases there may be algae which give off oxygen in light, but this does not penetrate too far, either. At the bottom af a fairly deep lake there is therefore little oxygen. This is also true of shallow water—even so shallow a body as a marsh—if its organic matter is highly concentrated.

So far as soluble materials go, and even insoluble ones like cellulose, this is not too serious a bar to decomposition. There are quite a few anaerobic bacteria capable of attacking cellulose, which bacteria frequently give off a great deal of gas—hydrogen, CO_2, methane, or "marsh gas"—but class IV materials, almost without exception, require the oxygen of the air.

How do organisms get the oxygen? Sometimes they don't, and the organic matter accumulates. These accumulations can be seen as black layers in mud or on the bottom; sometimes they fill the entire lake; at times they build up above the lake. Some we call marshes, some peat bogs. But they are nothing more or less than the accumulated organic material dif-

FIG. 17-1. *The natural history of a peat bog.*



ficult to decompose. Given air, they will be decomposed. If a bog or marsh is drained, or the bottom material dredged up and spread on the soil, it will be decomposed in a year or two, even though it may have been accumulating for centuries. We find, on examination, that the organisms growing on it are mostly molds (if it is acid) or streptomyces (if it is largely neutral). Figure 17-1 shows the natural history of such a bog.

At other times there is a turnover of the water which brings a certain amount of oxygen to the bottom. This turnover is caused by a difference in temperature between the upper and lower layers, usually helped by a suitable wind. If this is frequent enough, sufficient oxygen may be carried to the lower layers.

Before consideration of the sea, which has slightly different but comparable relations, it is sensible to inquire whether there is a characteristic flora of fresh water. We can find a wide variety of answers, but the sensible answer is that there is a characteristic flora of an environment. So far as waters possess a similar environment, they will possess a similar flora characteristic of that environment. Waters possessing other environments—a marsh compared to a lake—will possess different floras. The flora characteristic of the sea has one factor in its environmental pattern which differs from fresh water, for the sea is salty. There is a great deal of salt—roughly 3.5%, of which over 75% is NaCl. Many bacteria from the domain of fresh water can live in this concentration of salt, yet they tend to disappear when carried to the sea and to be replaced by a "marine flora." Why they should disappear is not readily apparent since sea water is not particularly toxic nor unusual as a medium. It has been supposed that marine protozoa may be responsible for the reduction in numbers and that the "more typically marine forms" are better able to survive their depredations. We have used quotation marks here because we have in mind bacteria which on isolation require for growth a medium using sea water as the diluent. We are not completely sure that these are absolutely "typical," but certainly this type occurs in large numbers in the ocean. This requirement for sea water can be frequently replaced by equivalent salt concentrations and appears to depend upon a requirement for sodium, although there are some investigators who claim that only sea water will suffice.

At any rate, these bacteria requiring sea water are considered to be "marine" forms. Generally they are bacteria that resemble in other respects the forms found in fresh water, being generally highly oxidative. But in the sea there is another most interesting group. In the depths of the sea, at tremendous hydrostatic pressures up to 1000 atmospheres, there live bacteria which when brought to the surface require not only sea water but high pressure in order to grow; pressures, indeed, which are toxic to forms normally living at one atmosphere at the surface. Not much, understandably, is known about these, but they appear to be similar in their characteristics to the organisms of surface waters, except for the requirement for hydrostatic pressure.

Detection of Fecal Contamination of Water

Chemically pure water is more difficult to find than uranium; what we normally drink as "pure water" has minerals, gases, and other chemical substances dissolved in it. It has been known for many years that water can transmit disease. By "pure water" we mean water that is "fit to drink," water that will neither cause illness nor offend us esthetically; we do not knowingly drink water in which we have washed anything, even though it may not cause us to become ill. Under primitive conditions contaminated water comes primarily from water contaminated with fecal matter from animals.

Under more crowded conditions contamination is from sewage, which, while it may contain a variety of "used water," also contains human fecal matter. We could thus determine the possibility of contamination of water if we could determine the presence of fecal matter.

It happens that fecal matter has, as do so many particular environments in nature, a particular and quite distinctive flora of microorganisms. So if we could determine the presence of bacteria which are characteristic of feces but are not found abundantly elsewhere, we could suppose that the water may well be polluted with fecal matter or sewage and thus would not be fit to drink. In the intestinal tract of man and animal there exists a quite characteristic, easily recognizable group of closely related organisms which bacteriologists place in the genus *Escherichia*, the most common species being *E. coli*. These are gram-negative rods, highly fermentative, which ferment lactose with the production of acid and gas. These characters serve to detect the presence of this organism or this group, since if we inoculate water into a lactose medium and find that acid and gas are produced, we may presume that *E. coli* is present; thus we may presume that the water has been contaminated. When done under certain standard conditions, this is called the **presumptive test.** We presume that the water is contaminated and do not drink it until we know for certain.

A simple thing like acid and gas production from the fermentation of lactose would seem to be a property so widely found among bacteria as to be useless for the purpose of detecting contamination. It happens, however, that there are relatively few organisms having all the properties outlined—i.e., gram-negative and fermenting lactose, forming acid and gas—which occur anywhere except in the intestinal tract. There is one major group, however, which is widely distributed in nature, living primarily upon grasses and plants, which does have these properties and which is placed in the genus *Aerobacter*. Since, on occasion, these organisms do get into water and since they do not necessarily indicate intestinal contamination, one must be sure that the acid and gas observed really come from bacteria of intestinal origin. This resolves itself into a problem of distinguishing between *Escherichia* and *Aerobacter* species. If *Escherichia* (particularly *E. coli*) is present, then the acid and gas from lactose means sewage or fecal contamination; if *Aerobacter* (particularly *A. aerogenes*) is present, the acid and gas from lactose has no sanitary significance. The water may have passed through a field of wheat, for example, or a leaf or branch may have fallen in.

The principal organisms of both genera are small gram-negative rods, fermenting lactose, forming acid and gas, and fermenting a variety of other sugars. However, they differ in other quite characteristic ways, as shown in Table 17-1.

TABLE 17-1. *Distinguishing Characteristics of E. coli and A. aerogenes*

	E. coli (Fecal type)	*A. aerogenes* (Plant organism)
Indole test	Indole is produced from tryptophan	None produced
Methyl red test	Methyl red is turned red (positive), which means that a *p*H of below 4.5 is produced in 2% glucose broth	Methyl red remains yellow (negative), which means less acid is produced from glucose
Voges-Proskauer test	This test measures acetylmethyl carbinol production; none is produced by *E. coli*	Acetyl methyl carbinol is formed
Citrate fermentation	*E. coli* does not use citrate	Growth on citrate media

These four tests go by the names of their first letters, i.e., IMViC tests. A fecal *E. coli* is ++−−; a nonfecal *A. aerogenes* is −−++.

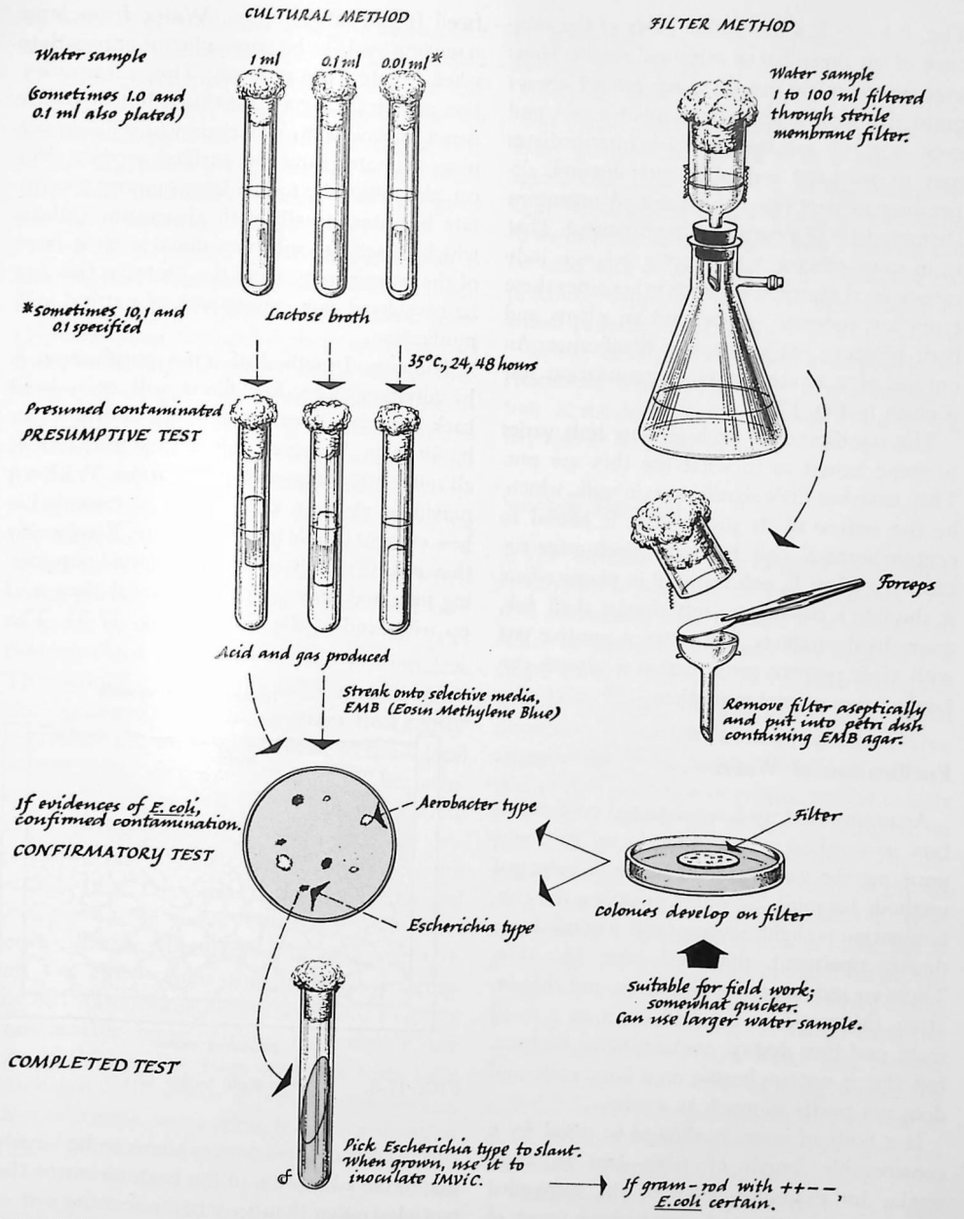


FIG. 17-2. *Outline of a typical water contamination test.*

The ++−− is a reasonable index of the presence of an organism of intestinal origin. However, as one might expect, things are not always quite this simple and strains of +++− and even ++++ are known. Such intermediates may or may not indicate contamination, depending in part upon the kinds of organisms characteristic of a particular environment. That is, in some cities a +++− or a +−++ indicates a fecal source, whereas in other cities these types are common in soil and on plants and their presence has no sanitary significance. An outline of a typical water contamination test is given in Fig. 17-2.

The significance of such sanitary tests varies to some extent as to what use they are put. They have but little significance in milk, which, by the nature of its production, is bound to contain some *E. coli*, but have much more significance when *E. coli* is found in places where it shouldn't be—water, soft drinks, shell fish, many food products. For water, a positive test with clear positive confirmation is almost certainly a sign of contamination.

Purification of Water

Assuming that we have detected contamination in drinking water, how do we go about purifying the water? There are three principal methods for purifying water, assuming the contamination is slight; sewage itself requires more drastic treatment, described later (p. 317). These are sedimentation, filtration, and chlorination. Of course we can boil water on a small scale, and thus destroy contaminating bacteria, but this is not applicable on a large scale and does not purify so much as sterilize.

If a body of water is allowed to stand for a considerable length of time—two to three weeks, for example—much of the suspended matter in it settles and carries down most of the bacteria. Chemical and biological oxidations take place and the water is gradually freed from contamination. Water from large reservoirs tends to be purer when it leaves than when it enters the reservoir. This self-purification of water is a very real thing, but of course is not applicable to situations where large volumes of water must be purified rapidly. We can also artificially form a large gummy precipitate in water, usually with aluminum sulfate, which on settling will carry down with it most of the organic matter and the bacteria; this can be considered as a counterpart of natural self-purification.

The second method of water purification is by filtration. Very fine filters will even hold back bacteria, so we can produce sterile water by filtration. However, this is slow and not at all suitable for large-scale purification. Without providing absolute sterility, it is remarkable how efficient simple filters really are. Frequently they consist merely of a bed of gravel supporting finer and finer sized stones until they end up with sand at the surface (Fig. 17-3). The

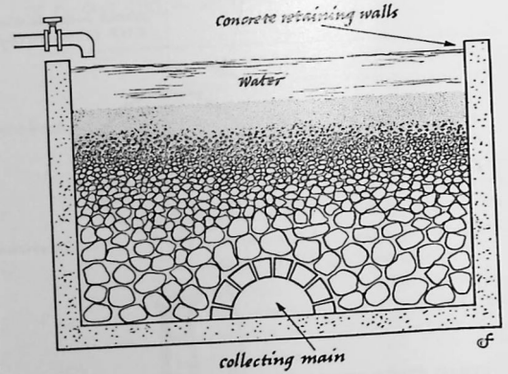


FIG. 17-3. *Large-scale water filter.*

effectiveness of these devices seems to be largely due to the adsorption of the bacteria on to the particles, rather than an actual screening out of the organisms. Soil itself is an excellent filter and underground water such as is brought up by wells is generally safe and pure unless there has been a channel into the well that avoided filtration. Even under somewhat less than the best of conditions, these methods, and the coagulant, too, will remove easily from 95 to 99% of the organisms present, as well as colloidally suspended matter and even much of the organic matter in solution.

The third process for water purification is chlorination. The amount of chlorine required depends on the organic matter present, more chlorine being required if there are more bacteria, more organic matter, and a shorter time to act. Two parts of chlorine per million is suitable for almost anything but taste, but it is used in swimming pools. For drinking water 0.02 to 2.0 parts per million (usually 0.3) are used, the operator judging the load by plating for bacteria and adjusting the chlorine to be added to meet it. This process will kill waterborne pathogens, with some loss of taste; most of us are so used to some chlorine in our water that we think that water without it is tasteless. This would seem, on the surface, to be much like boiling, i.e., sterilizing rather than purifying. After all, we wouldn't want to drink fecal matter, even if it wouldn't hurt us. But actually, chlorination is a method of purification. The chlorine reacts with water to produce oxygen in a highly active state, which serves to oxidize away the offending organic matter and thus purify the water. This is why it requires more chlorine when there is more organic matter. The effectiveness of chlorination in stamping out water-borne disease is attested by a very considerable body of data. For example, the city of Milwaukee takes its water from Lake Michigan, into which it puts its own sewage, after adequate purification; but it is a situation which permits some contamination. The city had formerly used only a filtration system and in five years had 24 deaths from typhoid per 100,000 population. Since the addition of chlorination to the filtration process no deaths have occurred from water contamination for many years.

Because chlorine does have a taste which some people find objectionable, there have been many attempts to replace it as a purification agent. One such attempt is to use ultraviolet light, which generates active oxygen in addition to its intrinsic lethal action. Such a process can be used with bottled waters, but it is not too practical—suppose a bacterium were shaded by a dust particle; and it is as yet quite expensive.

Water once purified must be stored under conditions which protect it from contamination. It should be tested not only at its source, but also at its point of use.

Sewage Disposal

The method of waste disposal in Western civilization is primarily by way of sewage. Yet it is remarkable how difficult it is actually to define sewage and sometimes how difficult it is to get rid of it. It is over 99.9% pure water; the rest, being largely organic matter in colloidal dispersion, is best left to the imagination. One aspect of its decomposition which differs from that of decomposition of organic matter in soils or water is that it has plenty of nitrogen, so that most of it will decompose readily. The object of sewage treatment is primarily to get rid of it, which sometimes proves difficult. It is easy enough when there is only a little; but it is sometimes a very difficult problem for all the sewage of a large city. In more formal terms, we wish to remove the organic matter from the sewage and have the pure water left, prevent the pollution of the water supplies, render the material esthetically acceptable, and of course do all this as economically as possible, since no one wants to spend a lot of money on it.

This can all be done if the material in it can all be oxidized away; most of the substances in it can readily be attacked by many bacteria, and those substances that cannot be oxidized

tend to clump and settle out. If we can let enough bacteria work on sewage long enough with enough oxygen, purification is quite possible. This is, of course, what happens in nature when sewage in relatively small quantity is turned into streams, rivers, or oceans; the bacteria—and sewage itself contains 3–10 million per milliliter—oxidize it all away. Providing that not too much is put in, the oxygen dissolved in the water can be used by the bacteria to decompose the material and the stream or ocean is no worse for it. But this is the catch. Today, every person uses on the average 100–150 gallons of water a day. For a small-sized city of 30,000 this means 3 million gallons a day. And this amount requires quite a bit of oxygen—as much as is dissolved in a lake 10 miles square. If more sewage is put in than can be handled, all the oxygen in the water will be used up, the fish die, incomplete decomposition obtains, and odors develop. The canals of Holland or Venice are fine examples of this process. In many places the drinking water is taken from the same source, or the water provides a recreational area. In some places there just is no body of water large enough to dispose of the sewage produced by the population. An early idea was to pour the sewage out onto the land. Here there would be air enough, providing again one did not put on too much sewage; but the area of land required for a city like Paris, where this system was tried, was so large that sewage had to be piped for miles outside of the city. The system was really in trouble if there was an exceptionally wet spell of weather. If, therefore, man was to exist in large communities, some process had to be devised for sewage disposal. Western civilization is based on water. Note that the Orient has not yet solved its waste problem, and note also how it has suffered for this.

We may look at the solutions from two viewpoints; that of the small-scale operation of the individual home, as for example a farm or a suburban community without sewage disposal, or that of the metropolis. The approach is essentially the same, but the scale of operation and kind of machinery used is different. There are the further problems of special types of waste (paper mill sulfite, industrial sewage), which we only point out.

On the small scale, the solution has been the **septic tank.** This consists in essence of an arrangement such as that shown in Fig. 17-4.

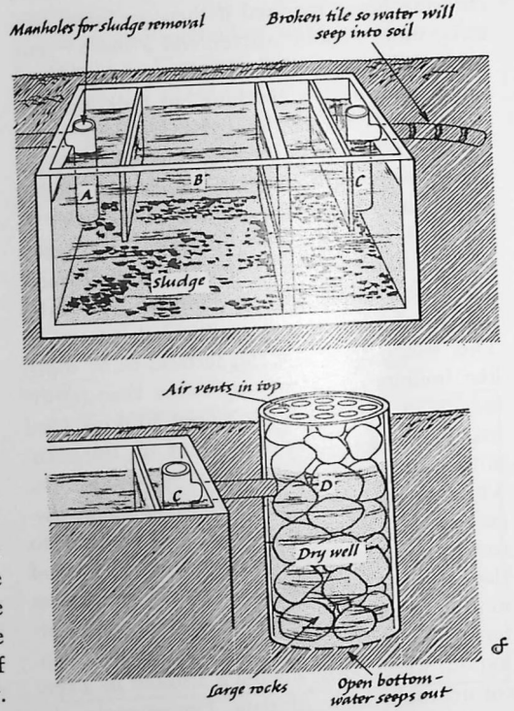


FIG. 17-4. *Septic tanks and dry well.*

Part A is called a grease trap and tends to collect waste fat which floats on top; it is sometimes necessary to skim it off and let it decompose in the air. In the main chamber (B) there is no dissolved oxygen, but anaerobic bacterial decomposition goes on rapidly. Complex carbohydrates, proteins, and other materials are broken down by fermentation—not to CO_2 and water, but to soluble breakdown products such as sugars, alcohols, organic acids, glycerol, fatty acids. When these spill over at C, which occurs intermittently and only when material is added at A, the fluid trickles down the rocks or tile in the "dry well" (D) which contains a good deal of air. The bacteria living on these rocks oxidize the products of breakdown completely to CO_2 and water. In chamber B, however, there are some materials which are quite resistant to decomposition. These include the lignins; see p. 334 for a description. The materials in B tend, however, to floc and to precipitate out. Hence they settle to the bottom as a sludge, which has to be taken out occasionally, perhaps once every few years, by draining, dipping, or even pumping out as a slurry. It can be put on soil, where it contributes to the humus. The size of tank required depends upon the amount of sewage being put into it. Roughly, a 750 gallon tank (5 ft by 5 ft by 5 ft) will handle the average domestic sewage of a family of four. A certain amount of care must be used with such a tank; for example, too much mercuric chloride down the drain would kill off all the bacteria. But such systems have been in operation for years in many places and have proved most satisfactory, even though the householder has no idea as to what happens in the system.

In large-scale operation, there are two types of processes employed. One, called the **Imhoff tank** process, is closely related to the septic tank but is on a larger scale. The other, called the **activated sludge** process, is particularly designed for handling large quantities of sewage in a rather small space.

The Imhoff tank is shown diagrammatically in Fig. 17-5. A tank 20 ft deep, 30 ft wide, and 100 ft long will handle 2 million gallons a day. The flow is adjusted so that it requires from 12–24 hours for the sewage to pass from one end of the tank to the other. At this very slow

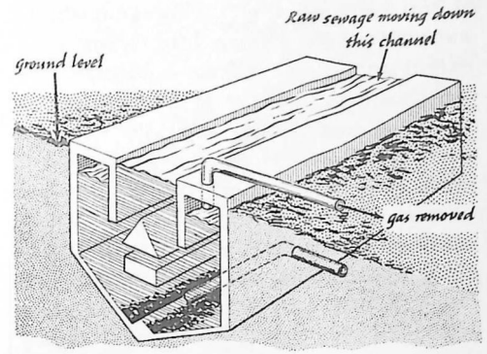


FIG. 17-5. *Imhoff tank system.*

rate of flow many things settle out, whence they fall into the large tank and are digested. Gases produced from the digestion in the main chamber are sometimes allowed to escape, sometimes capped and used to fire boilers to operate pumps, and sometimes even connected to the city gas mains. Gas production is slower in winter than in summer so that such a tank may put gas into the city supply in summer and withdraw it from the city supply in winter to operate the pumps. The sludge at the bottom slowly digests and is tapped off at intervals, dried, and put on the soil. The runoff or **effluent** from such a tank is usually sprayed into the air, to get oxygen dissolved into it, and it falls over a trickling filter which consists mostly of a rock bed 8–12 ft deep (Fig. 17-6). The spraying is intermittent so that as a layer of water goes down the filter it draws a layer of air above it down through the filter. Under these highly aerobic conditions, the last stages of oxidation are completed. Generally at the end the effluent is chlorinated, not only to be extra certain that no possible pathogens have survived, but also to provide an extra supply of oxygen, since one effect of putting chlorine into water is to generate active oxygen. Obviously the Imhoff tank process is the septic tank process adapted to a large scale, and employs an anaerobic hydrolysis followed by an aerobic oxidation. But it still

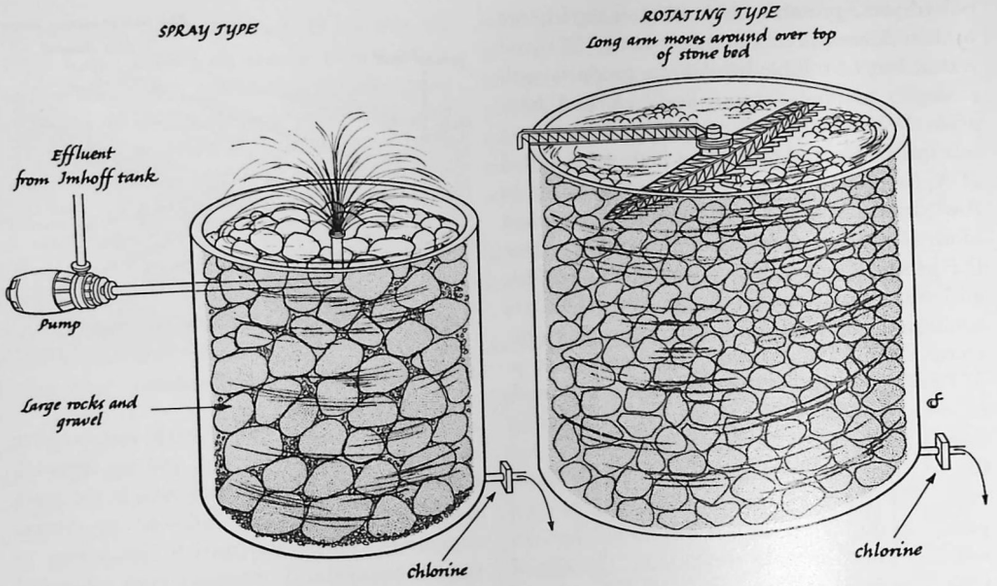
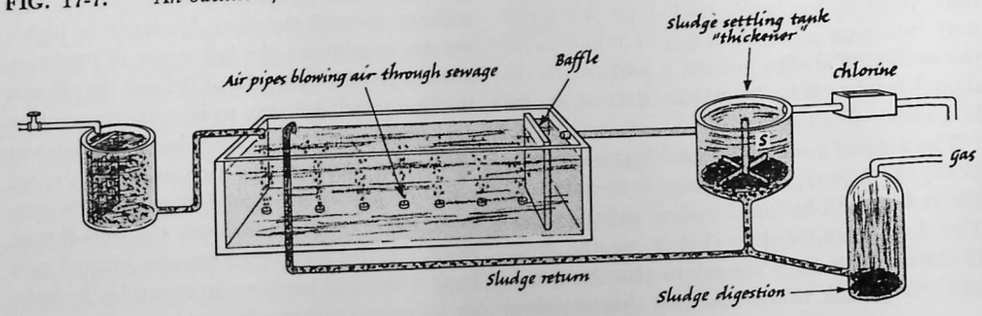


FIG. 17-6. *Trickling filter system.*

takes a considerable space: 3000 sq ft of tank plus at least as much for the trickling filter for every 20,000 people. Generally this is not too bad, but many cities, close to a lake or sea, just do not have this much space on their shoreline —Manhattan island in New York City, for example. It is also too expensive to pump all the sewage miles inland, treat it, and then pump it back again.

For situations such as New York's, another process has been developed. After all, with enough bacteria and enough air and enough time, we can decompose sewage. We can get enough air by forcing air through the sewage. The more air and the more bacteria, the less time; and the less time, the less space it takes to handle the sewage. We can get more bacteria —what better place than the sludge that just

FIG. 17-7. *An outline of the activated sludge process.*



came out of the previous batch of sewage? The system is outlined in Fig. 17-7. After a screen to take out large particles, and a grease trap, the sewage is pumped into a big vat togther with about ⅕ to ⅓ of sludge from the settling tank (S). Air is blown in vigorously and with all the air, and all the bacteria, decomposition goes on very rapidly; by the time the sewage reaches the effluent end, most of the oxidizable materials have been decomposed. It then pours off into a settling tank, the sludge flocs and settles out, the pure water overflows, is chlorinated, and sent to the river, lake, or other body of water. The sludge is continuously removed, about ⅓ to ⅕ of it being added to the tank for recirculation, the remainder being put into anaerobic digestion tanks. Here it decomposes extremely fast, since it is really loaded with bacteria, giving off gases such as hydrogen and methane, which can be used to run the pumps that blow the air. This process is efficient and requires a small space. There must be, however, the right bacterial flora in the sludge—one that actively decomposes the sewage in the allotted time and then leaves the residue of such a character that it settles out readily. This is sometimes hard to obtain and a good working sludge from one plant is frequently used as an inoculum to start others. It requires more control and more skill to operate and costs more to run, but where space is expensive it may be cheaper to operate than other systems. Synthetic detergents, being not only difficult to decompose biologically, but also capable of generating foam with aeration, are a serious problem (Fig. 17-8).

Biological Oxygen Demand (BOD)

This is a test used to determine how much oxygen the baceria will require to decompose adequately the organic matter in a given sewage. As a laboratory measure the sample of sewage is given a known (and excess) amount of oxygen, is tightly stoppered, and incubated at 20°C for 5 days. At the end of this time the oxygen remaining is measured. The sewage load is usually expressed in terms of BOD.

Microbiology of Air

From time immemorial, air has been associated with disease. Night air or swamp air

FIG. 17-8. *Suds developed in stream due to inadequate removal of detergents in sewage treatment.* [*Copyright, 1961, Time, Inc. Frank Scherschel,* Life, *12/22/61.*]

brought malaria. The fogs and miasmas brought plague and epidemics. The stench of water and of offal and the foulness of swamp air and sewer gas—all of these were associated with corruption and disease. But while there is a certain relation of these matters to disease, the "night air" was generally malarial because it permitted the mosquito to bite people. The "air of corruption," while mostly noticeable because of bad odor, generally reflected grossly unsanitary conditions which provided for the easy spread of enteric diseases. Yet the air could be a harbinger of death, even if indirectly.

Yet air cannot serve as a medium for growth; the organisms found in it came from other materials and have only a transient survival in air. The organisms come from soil, from the organic wastes of animals and man, from the oral, nasal, and rectal passages of animals and man, and from the lungs, through coughs and sneezes. Obviously, most of the organisms come from the soil as dust. Most are not capable of causing disease and may well be ignored in this matter, but they are capable of growth in bacteriological media and must therefore be kept from such materials. With modern life, the problem of air-borne infection is of increasing importance. Hospital beds should not be too close together. Crowds in trains, subways, and even automobiles encourage more organisms in the air and more chance of the passage of disease. While the problem of smog and chemical air pollutants does not include bacterial dispersion as such, many of the same factors which contribute to such air pollution also tend to increase the microbial populations, especially the formation of aerosols and fine dust particles which serve not as dispersants but as shelter to the organisms in the air.

Obviously, for an organism to survive in air for any period, it must be able to stand drying and sunlight; thus relatively few pathogens can survive for very long in the air. With "natural air," the air above an open field, we find mostly the spores of bacteria, molds, and streptomyces, together with a few other organisms, usually pigmented; staphylococci and micrococci are common. It is probable that the pigment protects the organism against the sunlight. Organisms are present as free cells or spores, attached to dust particles, in droplets of moisture, or on or in the bodies of insects.

Knowledge of the microorganisms in air is of importance in two connections. First, there are several "air-borne" infections of man, hence there is a medical aspect to air bacteriology. Second, since air is used by many organisms during their growth, it becomes a considerable problem to supply sterile air, free from contaminating organisms, for the growth of those organisms we wish to grow; hence there is an industrial aspect of air bacteriology.

Medical Aspects of Air Bacteriology

The tabulation below lists the major diseases transmitted via the air. Since air enters the body through the respiratory tract and since such diseases frequently localize in the nose and throat, as a group they are called respiratory disease. The main diseases are discussed later (Chapter 25), but diphtheria, "strep throat" or septic sore throat, pneumonia, and whooping cough are the air-borne diseases par excellence. Yet the following are also transmitted in this fashion: tuberculosis, smallpox, chicken pox, measles, mumps, influenza, the common cold, and poliomyelitis, together with some mycoses (fungal infections).

MAJOR DISEASES TRANSMITTED BY AIR

1. Bacterial	2. Viral
Diphtheria	Smallpox
Septic sore throat	Chicken pox
Scarlet fever	Measles
Rheumatic fever	German measles
Tuberculosis	Mumps
Pneumonia	Influenza
Meningitis	Common cold
Whooping cough	Psittacosis
	3. Fungi, actinomyces
	Systemic mycoses

In most of these cases the diseased individual spews out into the air the pathogenic organism, which survives for a time and travels until it reaches a susceptible individual, this person breathes it in and thereby sets up the possibility of another case of the disease. The methods of distribution into the air are many, but obviously coughs and sneezes—usually characteristic of respiratory disease—are effective methods. However, simple breathing also releases organisms. Their survival in air depends, of course, on many factors, but it is usually surprisingly short. This is of considerable importance in hospital practice. In military hospitals it was found that most respiratory disease, such as sore throat, did not spread from bed to bed easily when the beds were 4 ft or more apart, but did spread readily when they were only 2 ft apart. We attempt to prevent the spread of air-borne disease also by elimination of the pathogenic bacteria from the air. We approach this problem in two ways. One is to prevent the entry of organisms into the air—control of dust, isolation by hoods or chambers of infected individuals, face masks, etc. The other approach is to remove the organisms in the air by UV light, chemicals (triethylene glycol, for example), filters, and precipitators.

The number of organisms in the air varies tremendously, of course, but under clean conditions is about less than 10 per cubic foot. In the usual office or laboratory it may be from 30–100 organisms per cubic foot. Under some circumstances—bed-making in a hospital, for example, the count may go as high as 2000. Obviously the fewer organisms in the air, the fewer chances of disease transmission or wound infection.

Industrial Aspects of Air Bacteriology

Aside from its ability to carry disease, air always contains microorganisms, but normally these do not cause disease and are generally of little concern. Where these few organisms are really of importance is in industrial fermentations. Submerged aerobic processes, such as those for the production of antibiotics or vitamin B_{12}, require a continuous supply of large quantities of sterile air. This is accomplished by filtration through beds of fibrous or granular material. Industrially this material is charcoal or glass wool; in the laboratory, sterile glass wool or sterile cotton. The cotton plug or the plastic plug on the bacteriological test tube serves as a filter. Such filters remove the organisms in the air and permit the large-scale growth of the inoculated organism.

Other Related Aspects

While these aspects of microbiology are not strictly part of either water or air, it is convenient to consider them here. They do not fit as well elsewhere and they are important enough that you should be aware of them.

Microbiological Warfare

It seems likely that one of the plagues inflicted upon the Egyptians by the Israelites was anthrax, and that the disease was knowingly promoted (Exodus 2:3–11) by the latter. Whether or not this was the first example of biological warfare (which we shall abbreviate to BW) is not certain, but surely disease has played important historical roles and influenced the destiny of nations as of men. When, through the study of microbiology, man came to have some control over infectious disease, he also, for such is the nature of man, began to consider whether or not, and under what conditions, this control could be used as a weapon. BW has not had any large-scale use, but the

potential is present and real. While one may debate whether it is morally right to use BW against any enemy, there is no question but that it is morally right, necessary, and humane to protect our children, our families, our neighbors, and ourselves against those who would attack us with these weapons. We think of the atom bomb as a truly destructive weapon. Table 17-2 compares the effect of one bomber dropping a 20 megaton thermonuclear bomb or 450 lb of a BW agent. We see no reason to dwell upon the potential power of the BW approach.

TABLE 17-2. *Thermonuclear Destruction versus Bacterial Warfare*°

	One B-52 Bomber Could Disperse 450 lb of Agent	Atom Bomb
Effective area	34,000 sq miles	100 sq miles
Morbidity	25–75% morbidity; not necessarily lethal	98% lethal
Residual	Possible epidemic spread elsewhere	6 months
Time	Requires a few days to a week	Seconds
Property damage	None	36 sq miles totally destroyed
Area safe for invasion	After incubation period	3–6 months
Covert application	Great possibility	Little
Capital cost to produce agent	Low	Very high

° *Chem. Eng. News Ed.*, **38**, No. 16, p. 37, April 18, 1960.

The military exploitation of BW is a form of strategic warfare which could be critically important in reducing a nation's will and ability to fight. The eventual outcome may be far more serious than the immediate response. There are three large classes of antipersonnel materials possible First, agents foreign to or rigorously excluded from the United States—i.e., certain virus diseases, especially Rift Valley fever, Japanese B encephalitis, and Venezuelan equine encephalitis. Second, agents largely eliminated from the United States in which, therefore, population immunity is low—cholera, yellow fever, smallpox. Third, agents generally endemic due to limited human contact—plague, tularema, tuberculosis. Further, there are similar classes of antiplant agents and antianimal agents, with most of which we are already waging a contest. Antipersonnel agents do not necessarily cause death, and may in fact, be more valuable if they cause only illness. This would remove not only the sick individual from the military effectiveness of the nation, but also those required to take care of him.

Upon the assumption that such weapons will be used against us, if it is convenient for our enemies to do so, we should point out that we are not totally unprepared; we have successfully dealt with similar kinds of outbreaks, as the Asiatic influenza of 1957–1958. Two problems are critical: the early detection and early warning that such a BW agent has been used, and the rapid identification of the agent employed. These problems are never "solved," but even a partial solution is helpful. We now possess sampling devices for **biological particulates,** which are prototypes of what may eventually be a solution to air-borne dissemination. Microbiologists will obviously play an important role in these developments.

Germ-free Animals and Plants

Since microorganisms are so intimately associated with all living plants and animals, it is of considerable interest to attempt to grow animals and plants in the absence of any bacteria at all. This has been done. Rats and mice have been successfully reared free from bacteria through several generations. Chicks have been hatched from eggs laid by a germ-free hen mated with a germ-free rooster. It is not certain that such animals are free from viruses. Most of the rats and mice die of asphyxia, but some develop lung disease, pneumonia, and liver diseases—all free from microorganisms. The intestinal tract has thinner walls and is somewhat underdeveloped. They have no dental caries; when *Endamoeba histolytica* is administered to such germ-free animals, amebic dysentery does not result; it only appears when *E. coli* or *A. aerogenes* is given simultaneously with *E. histolytica*. Biotin must be supplied; in animals with an adequate bacterial flora enough biotin is synthesized by bacteria to make its addition unnecessary.

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For background information, see p. 495.

QUESTIONS

1. Define or identify:

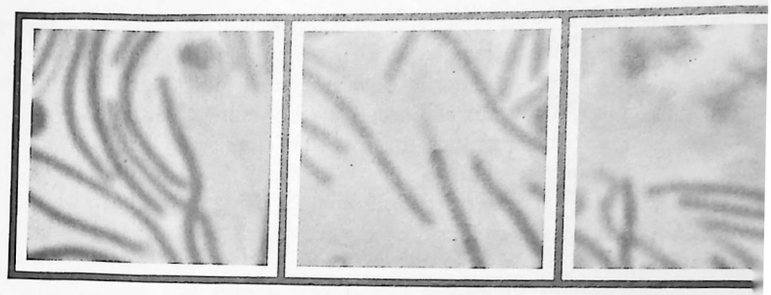
lignin	chlorination
epiphilic	sewage
marine flora	sludge
pure water	septic tank
presumptive test	Imhoff tank
confirmatory test	activated sludge
IMViC	trickling filter
Voges-Proskauer test	effluent
	BOD

2. A housing development comprising 300 homes consults you for advice. This neighborhood is to be a wealthy one and each plot is about an acre. What kind of a sewage system would you recommend?

3. You are given the following data on a series of water samples. Which would you condemn as contaminated?

LACTOSE	IMVIC	REMARKS
1. AG	++−−	
2. AG	+++−	Gram-positive
3. AG	−−++	
4. A	++−−	
5. AG	+++−	Gram-negative
6. AG	Unknown	Gram-negative
7. A	−−++	
8. AG	++−+	Gram-negative
9. AG	−−++	
10. AG	++++	

4. A hot political campaign is being waged in your town over water fluoridation. Someone discovers that you have been sampling the water and testing it for *E. coli*. Since this organism is rarely found you are asked to testify that the water is pure and does not need fluoride. What would you do? When the campaign wages hotter, someone discovers that *E. coli* is not a pathogen and tries to make you look incompetent for testing for it. How would you justify testing for *E. coli* rather than a disease-producing organism?
5. What would you recommend as the most suitable sewage system for a city of 40,000 located on the bank of a small river?
6. *Fortune Magazine*, in vol. 58 (1958), p. 102, describes sewage disposal as the number one public health problem of the next decade. Why?
7. It has been determined that the mold-producing penicillin requires a liter of air per hour per liter of medium. You have a penicillin fermentations tank holding 50,000 gallons of both. This would require 50,000 gallons of air per hour. What kind of a system could you use to sterilize the air being pumped into this tank?
8. The Acme Laundry is accused of putting so much detergent into the sewage system that the activated sludge tank became a batch of suds. How would you defend Acme if they were your client?
9. You are employed as a microbiologist with a company making ultraviolet lamps. List as many cases as you can where they would be useful in cutting down air-borne infection.
10. You are engaged by the government to make a survey of bacteria in the upper atmosphere and they have assigned you a jet fighter, a stratosphere balloon, and a propeller plane for this survey. What system for sampling the air could you devise for each situation?



Chapter 18

SOIL MICROBIOLOGY

"Oh Man
Whosoever thou art
And whensoever thou comest
For come I know thou wilt;
I am Cyrus
The Founder of the Persian Empire
Envy me not
The little earth that covers my body."

From the tomb of Cyrus,
as reported by ALEXANDER THE GREAT

The Nature of Soil

Soil is the upper layer of most of the earth's surface and varies in depth from inches to over twenty feet. The soil possesses certain characteristics which distinguish it from rock. Soil is not dirt. Dirt is "matter out of place" and soil is precisely where it should be—where, indeed, the forces of nature formed it. Soils vary among themselves, in addition to alternations from the underlying rock, in the following properties:

1. Mechanical changes. The structure and texture of soil varies, certainly clearly from that of rock.
2. Physical properties. Soils have "pore spaces," a water-holding capacity, and a colloidal fraction.
3. Chemical properties. Soils differ in com-

position from underlying rock and among themselves, depending on the composition of the rock, the extent of weathering, the colloidal content of the soil, and its composition; sometimes this is called "base exchange level."
4. Biological properties. Soils contain living creatures as well as dead organic remains.

It is almost impossible to talk of soil as a single unit; there are different kinds of soil. The red soils of the south and the red soils of the north even differ markedly because the chemical composition, their origin, and even the forces which made them differed. The lush tropical forest grows on a "soil of sand" while the pine forests have layers of black organic matter, sometimes to a depth of several feet. Think for a moment of the corn fields or wheat fields of Illinois and the black, sticky, gumbo soil of the Mississippi Valley and compare it with the wheat fields of Montana and the gray or red, powdery, dustlike soil of the plains. These are different; even the wheat they grow is different. In the north look at oak and pine, and in the south look at pine and cypress; here too the soils beneath them differ. Does oak grow here and cypress there because the soil is different? or did the growth of pine make the soil different? The answer is both, plus something else.

The type of soil which develops from the underlying rock is a result of the interaction of four factors.

1. The parent material—the rock itself from which the soil formed. Granite gives rise to different soils than marble. This factor includes not only the composition of the rock, but its topography, location, and relief. These last control to an extent the nature of further forces—winds and rains and flowing water which may act upon it.
2. The climate—the rain, the march of seasons, the heights and depths of temperature, the winds, the frost, the ice, and the burning sun.
3. The age—the time, measured in centuries, since it takes 300 years to make an inch of soil, in which these forces of nature have had a chance to act upon the rock.
4. The vegetation—controlled by climate, composition, and topography, but modifying, altering, and finally dominating the soil as well.

In a real sense, soil is produced from weathered rock. The sun, the rain and ice, and wind and water have changed the rock greatly. These forces occur on mountain tops—and they weather the rock indeed—but they, of themselves, do not produce soil. For soil to be formed from weathered rock requires biological forces—the plants that grow in it, the microorganisms which feed on and transform it, and are themselves "turned over"; these are the forces that make soil. And it is these biological forces which give to soil and to landscape those characteristics familiar and necessary to man. In the past there were those who could boast, "Tell me the soil from where you came—and I will tell you your songs, your beliefs, and your very character." This was possible to a large extent when people were close to the land and had little mobility, although it is certainly less true in a day of the drugstore cowboy and the city culture of our time. For centuries, however, it was clear that the type of soil, and the vegetation that grew thereon, determined the type of agriculture possible, and determined the character of the culture which develops on it. We have only recently emerged from the era in which most of the people in any civilization were engaged in agriculture. In a very real sense, the soil determined the stature of its people, their mental habits, their economic condition, their folk songs, their disposition, even their speech. The transplanted New Englander acquired a western twang, or a Texas drawl, or a slurred speech or clipped northern pitch—something different indeed from his native soil. But in any such influence of soil upon culture it is obviously not the plants growing on it now that influence the picture—they could be corn, or oats, or tomatoes, or what not. No, it is the accumulated forces of the past, the remains of life, which are important. Man, at least a whole man, is not merely a "parasite upon vegetable" but is molded by the remains of many "vegetables" from years and years past. These vegetable remains become what they do become, something indeed different from and more important than the plants they started from, by the agency of soil microorganisms. And it is precisely here that microorganisms exert a profound and controlling effect upon all of us. In fact, soil is the beginning of life and the end of it.

These somewhat mystical approaches to soil are introduced to give you, so far as it is possible, some of the flavor, some of the character, some of the emotion that the study of the soil has generated in its students. Indeed, to make you see that this is not a dull dry area of interest to only a few farmers, but that it is worthy of your attention no matter how sophisticated

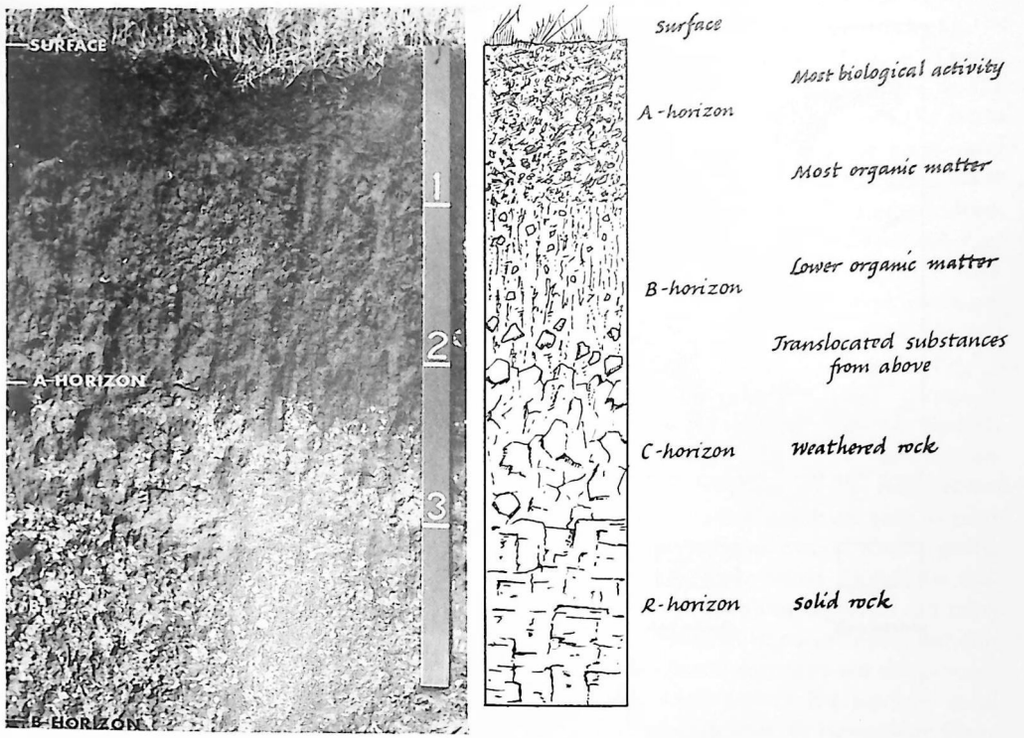


FIG. 18-1. *The Soil profile. This is a profile of the soil developing under grass in a subhumid, cool, but temperate climate. The numbers on the scale represent feet. (Photograph courtesy of Dr. R. W. Simonson, Soil Survey, U.S.D.A., Beltsville, Md.)*

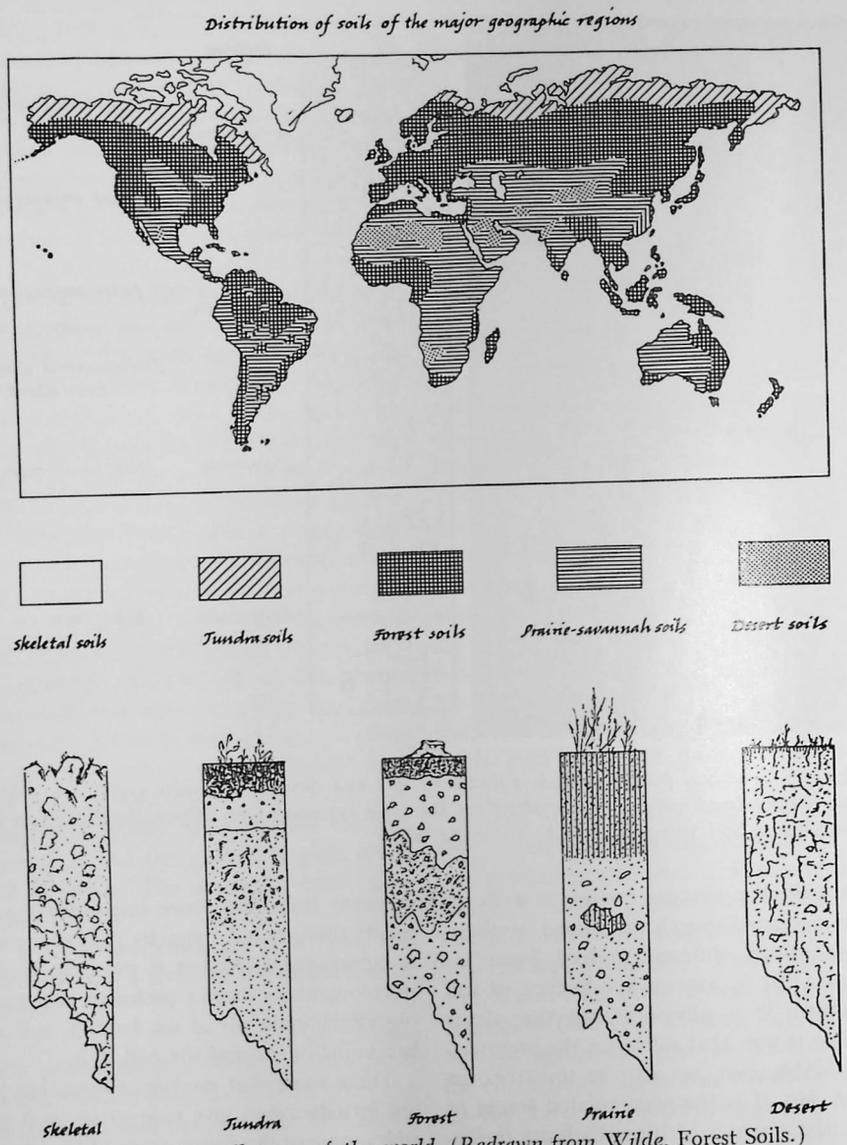


FIG. 18-2. *The major soil types of the world.* (*Redrawn from* Wilde, Forest Soils.)

you may be—for you will not understand man until you understand soil and you may as well begin here.

Soil originated from the underlying rock. As one finds undisturbed soil it consists of essentially three layers—the top soil, the B zone, and weathered rock. This profile, whose nature and character varies, is characteristic of different soils, and soils are classified primarily by the character of this profile into soil groups. (See Fig. 18-1 for a "typical" soil profile.) Figure 18-2 shows the major types of soil found in the world. Without detailed reference to varied soil types, which is, after all, soil science, and not microbiology, we shall have to content ourselves with a general description, a sort of average soil. The top soil is the area exposed to the air, in which most of the biological activity takes place. Here the leaves and grasses which fall are decomposed and penetrate into the soil, here are the roots of shallow-rooted plants, here are most of the creatures which inhabit the soil. Most of these organisms are listed in Table 18-1. While the bacteria are most abundant, they are so much smaller than the other organisms as to actually represent less living tissue than others. Yet neither numbers nor weight is a real index of importance. That index is activity, both in the sense of how much is done and in the sense of what is done. Which organisms one will find, and how many of each type, will depend upon the local conditions: the available food, the amount and the kind; the moisture, temperature, and air, which are interrelated; the *p*H; the heterogeneity—you will see later what this means; and (here is a rather new factor) antagonisms and synergies which may develop.

TABLE 18-1. *Organisms in Soil*

Microorganisms	Numbers per gram	Pounds per acre
Bacteria	1,000,000	2
Streptomyces	100,000	5
Protozoa	10,000	15
Algae	10,000	5
Molds	10,000	50
		77
Small worms	—	150
		227

Think of all the different kinds of soil—granite and sandstone and limestone—as the base, and think of evergreen and oak, and grassland and corn and cactus and cotton as the plants, and the southern bayou and the butte and the badlands as the moisture areas—and you will sense that throughout the world there will be wide ranges of soil conditions. Indeed, somewhere or other every organism will find conditions just right for their growth, with the possible exception of the strict parasites. With so many different kinds and species and genera of organisms, and with such an overlap of possible conditions in soil, it is, of course, in few cases that one can attribute the activity going on in a soil to a single organism. In essentially all cases we are dealing with several forms or several species, all of which are able to carry on the function to some extent. Koch's postulates make little sense when applied to soil. We can prove that a given organism *can* carry out a process observed to occur in soil, but this does not show that it *does* carry out the process.

The most active part of the top soil is, of course, the very top layer. A convenient figure to remember is that the "plowed layer" of soil is, by convention, thought to consist of the top six inches. An acre of soil, six inches in depth, weighs two million pounds (1,000 ton). Below this layer of top soil is the B zone. Here biological activity is less; there is less food, but it contains the leachings from the top layer and some of the deeper penetrating roots. In general, it serves as a source of minerals which the roots bring up to the top layer. The bottommost layer is primarily weathered rock, slowly being transformed into soil.

Decomposition of Organic Matter

It seems obvious that the microorganisms are in the soil because food is there, and their function is to decompose this organic matter. The organic matter comes from leaves and

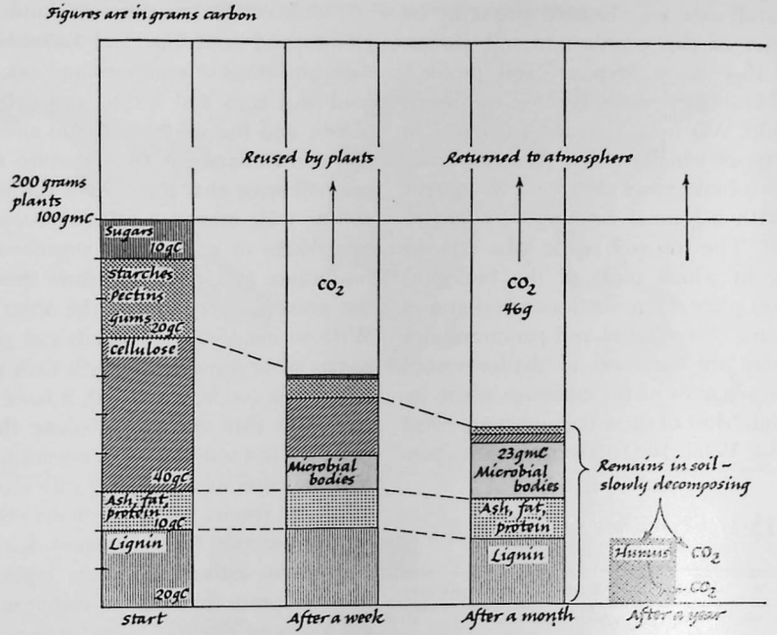


FIG. 18-3. *The course of decomposition in soil.*

straw and refuse of various sorts. Virtually all types of organic materials eventually find their way to the soil or to the sea. The shoes, the ships, the sealing wax, the cabbages and kings, and all else besides—this is the organic matter returned to the soil. It is composed literally of everything under the sun. It is so diverse that at first sight it would seem impossible to describe such decomposition in anything but very general and nonspecific terms. However, this is really not the case, and the decomposition of organic matter in soils can be described in rather precise terms. A time chart of decomposition is illustrated in Fig. 18-3.

In the discussion of decomposition in water, it was pointed out that the vast majority of the material added to soil was plant material composed of 20% soluble, 20% hemicellulose, and 40% cellulose, all of which was readily decomposable in soil. A material does not have to be soluble to be readily decomposed. Thus, 80% of most materials are readily decomposed. These materials are roughly half carbon, so any plant material contains roughly 50% carbon, 80% of which is readily available for decomposition. The remainder of the plant material, the 20% that is not readily decomposed, is made up of about ⅔ lignin and about ⅓ ash, protein, fat, etc. All of the 80% will have been decomposed before much of the 20% will have been even started. These may be illustrated by four problems, each of increasing complexity; the third and fourth are very close to the truth of the matter. By progressing from one problem to the next, however, it is easy to understand the most complex one. If you started with the complex problem first, it might be more difficult.

Problem 1. We have 100 g (or tons or pounds) of a plant material free from nitrogen. If it decomposes in soil (i.e., with access to mixed population), how much nitrogen will have to be added, or how much nitrogen will it take up from the soil?

Answer: The 100 g contains about 80% of readily decomposable material, hence we really only have 80 g of material to deal with. This 80 g is about half carbon, so we have 40 g of carbon to decompose. Of this, two-thirds will go to CO_2, and one-third (13 g) will be incorporated into the bacterial, fungal, or streptomyces cells which are engaged in the decomposition. Living cells of microorganisms contain about 1 part of nitrogen for every 10 parts of carbon—since they are 50% carbon, they have about 5% nitrogen; hence to build 13 g of carbon into cells making about 26 g dry weight of cells, we would require 1.3 g of nitrogen. Thus, to decompose 100 g of nitrogen-free plant material would require the addition of 1.3 g of nitrogen.

Problem 2. How much nitrogen would be required to decompose 100 g of rye straw which contains 0.24% nitrogen, assuming that all the nitrogen is available?

Answer: From problem 1, we know that 100 g of material would require 1.3 g of nitrogen. The 100 g already has 0.24 g of nitrogen, so it requires the further addition of 1.30 − 0.24 or 1.06 g of nitrogen to decompose.

Problem 3. How much nitrogen would be required to decompose 100 g of cornstalks containing 0.66% nitrogen?

Answer: This is much like problem 2, except it requires the further piece of information that only some of the nitrogen is available. After all, not all of the nitrogen in a plant is easily used; some of it is tied with the less readily decomposable materials. This amount is roughly the same as the carbon which is resistant to decomposition, i.e., 20%; hence in most natural materials, roughly 80% of the nitrogen is available, ready to be used by the microorganisms, either in an inorganic form or as an amino acid readily decomposed.

From problem 1, 1.3 g of nitrogen are required. The corn stalks have 80% of 0.66 g = 0.528 g nitrogen available; hence 1.30 − 0.528 = 0.77 g of nitrogen must be added.

TABLE 18-2. *Approximate Composition of Plant Materials as Per Cent of Dry Material* *

	Grass	Corn-stalks	Oak Leaves	Alfalfa
Ether-soluble	1.8	1.80	3.7	2.7
Water-soluble	6.2	14.2	13.9	17.2
Hemicellulose	21.1	17.6	12.9	8.5
Cellulose	38.6	29.7	13.8	26.7
Lignin	14.6	11.3	30.3	10.8
Total nitrogen	0.24	0.66	0.77	2.58
Ash	4.2	7.5	5.1	10.3
Total	8′.7	82.8	80.5	78.8

* Adapted from F. G. Tenny and S. A. Waksman, *Soil Science*, **28**, 55 (1929).

Problem 4. Given the composition of a material, determine how much nitrogen is required to permit its decomposition.

Answer: Suppose we chose oak leaves from Table 18-2. You will note that this is comparatively high in lignin. Further, the analysis is not especially good (only 80.5% recovery) but we will assume that the analytical errors are about equally distributed in all fractions, i.e., the material contained 30.8/0.8 = 38% lignin. The ash, of course, is not decomposed and we will assume that its determination is correct. Thus, for 100 g of dry leaves, we have 94.9 g of organic material. As an approximation we shall call this 95 g. This 95 g contains 38% lignin (slowly decomposed), so 62% × 95 g = 58.9 g of decomposable material; the 62% came from 100% − 38% lignin. The readily decomposed material is 50% carbon, or there are 58.9/2 =

29.5 g of carbon to be decomposed. One-third of this remains in cells, so $29.5/3 = 9.8\%$ g of carbon will be found in the microbial cells, and this will require 0.98 g of nitrogen. The leaves contain 0.77 g of nitrogen but not all of this is available, roughly only as much is available as is the carbon; i.e., 62% is available, and $62\% \times 0.77 = 0.47$ g. Hence, $0.98 - 0.47 = 0.51$ g of nitrogen which would have to be added.

This problem, therefore, corrects for ash, for analytical error, and for the fact that there is higher lignin than usual in oak leaves.

Suppose we repeat this for the alfalfa. Assume that ash is correct and the other errors are evenly distributed. Then per 100 g we have 89.7 g of organic material containing $10.8\%/78.8\% = 13.7\%$ lignin, or 86.3% is decomposable; $89.7 \times 86.3 = 77.4$ g of dry material which is decomposable and, since this is half carbon (= 38.7 g) and one-third of the carbon goes into cells ($38.7/3 = 12.6$ g), 1.26 g of nitrogen are required. The material contains 2.58 g of nitrogen of which 86.3% is readily decomposable, so 2.22 g of nitrogen is available. Hence in this case no nitrogen has to be added, since on decomposition $2.22 - 1.26 = .96$ g will be liberated.

Let us recalculate problem 3 more precisely, on the basis of composition listed in Table 18-2 rather than on just general averages. According to Table 18-2 100 g dry cornstalks would have 92.5 g organic matter and would be close to 85% decomposable, since 11.3/82.8% is close to 15% lignin; hence, $92.5 \times 0.85 \times 0.5 \times 0.33 \times 0.1 = 1.31$ g of nitrogen which will be required for decomposition. The cornstalks contain 0.66 g of nitrogen of which 85% is available; hence $1.36 - 0.66 \times 0.85 = 0.80$ g of nitrogen will have to be added per 100 g of cornstalk; compare this to 0.77 g based on the general assumptions of problem 3. It has been determined empirically that for most conditions and most substances, if a material contains more than 1.5 to 1.7% nitrogen, it will decompose to liberate ammonia, but that if it contains less than this amount, it may tie up available nitrogen in the soil.

Humus

A precise definition of **humus** which has a meaning is rather difficult to formulate. Rather, we must really rely upon an explanation of the process to understand what humus is rather than upon a definition. The definitions to follow do have some meaning when you understand the process, but by themselves are not very useful.

Humus is the mass of residual and synthesized organic matter of the soil. Humus is the organic matter of the soil. Humus is the result of decomposition under soil conditions.

If, as indicated in Fig. 18-4, A, we add 100 g of sugar to a mineral salts medium and inoculate it with *E. coli*, we will obtain residual products, never a complete conversion to carbon dioxide. There will always be the cells that grew and their decomposition products. Yet this residue is not humus. In soil it would become humus, but without the conditions prevailing in soil, it is rare for humus to be formed. That is, a mere residue is not humus; something else happens in soil which gives the residue certain distinctive properties which we recognize as humus, as indicated in Fig. 18-4, B. The result of decomposition under soil conditions, i.e. humus, is generally a black, waxy material —the "fat of the land."

Since it consists of all the more or less resistant materials from added plant, animal, and microbial residues, it is obviously not a homogeneous material. But it is something more than just such mixed residues. What is it, then, that happens in soil that converts these residues into humus?

As a prototype of what happens, we can illustrate the process by experiment. If we take a protein, egg albumin for example, and inoculate it with microorganisms, it readily

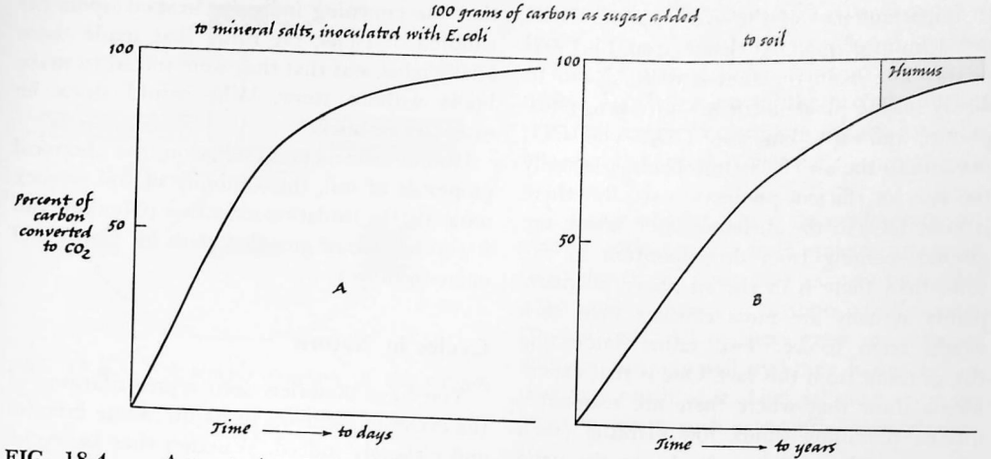


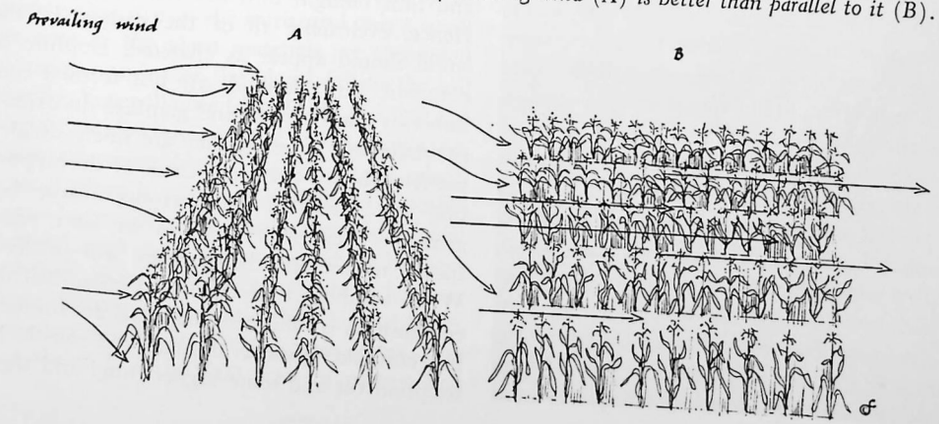
FIG. 18-4. *A comparison between decomposition in a pure culture and in soil.*

decomposes. Lignin slowly decomposes. If we mix a neutral solution of lignin and of egg albumin and precipitate them at their isoelectric point (*p*H 4.0–4.5), they precipitate as a complex, i.e., as a single individual. If this precipitate is kept wet, the egg albumin is readily decomposable; the presence of the lignin in the complex does not influence to any great extent the availability of the egg albumin.

But if this complex of lignin and egg albumin is dried, then wet and redried over several cycles of wet and dry, there appears to be a chemical reaction between the lignin and the protein, so that the egg albumin becomes almost as resistant to decomposition as the lignin. This combination, which for lack of a more precise name has been called ligno-protein, is the dominant character of humus. While humus may be formed in soil in different ways than in the experiment above, the "artificial humus" and the natural one are closely similar.

There are several functions of humus—it is

FIG. 18-5. *Planting perpendicular to the prevailing wind (A) is better than parallel to it (B).*



an important part of the soil system and not just a lump of material that it would be well rid of. The main function is to hold and to slowly release plant nutrients—nitrogen, phosphorus, minerals, but also CO_2. The CO_2 pressure in the air (0.03 atmospheres) is really too low for efficient photosynthesis. But there is more CO_2 in the air immediately above the ground—coming from decomposition in the soil—than there is in the air above, so many plants actually are more efficient than they would seem to be. Two rather interesting things result from this fact. One is that experiments show that where there are reasonably intense prevailing winds, row planting (such as corn) is better perpendicular to the wind (Fig. 18-5, A) since wind parallel to the row (B) tends to sweep out the CO_2 coming from the soil. Some of the old superstitions about planting during certain phases of the moon have to do with the subsequent temperature changes and occurrence of prevailing winds. The other is that the carbon cycle (see later) occurs most intensely in a distance about a foot above and a foot below the soil surface.

The humus has colloidal properties; it is a "base exchange" material, holding not only nitrogen and phosphorus in organic combination but also inorganic ions—much like an ion-exchange resin. This property prevents the leaching out of soluble minerals and makes them more readily available to plants, since many plants seem to feed not entirely upon food in solution, but by way of an ion-exchange mechanism.

A second important property of humus is the alteration of the physical property it gives to soil. It influences color, texture, structure, moisture-holding capacity, and aeration of soil, and is an important factor in the soil's structure —stickiness or resistance to erosion. Perhaps the most curious recognition of this structure-controlling property of humus is given in the Bible (Exodus 5, 6–19) where it is pointed out that the crowning indignity heaped upon the children of Israel, the thing that made them finally rebel, was that they were forced to make bricks without straw. Why would straw be necessary to bricks?

Humus influences, in addition, the chemical properties of soil, the solubility of soil or rock minerals, the oxidation-reduction potential, and is a storehouse of growth factors for plants and microorganisms.

Cycles in Nature

You have doubtless seen representations of the cycles of elements in nature, some fanciful and elaborate indeed. Whether they be cycles of carbon, phosphorus, nitrogen, potassium, or what not, there is always at least one step—usually labeled decomposition or sometimes more accurately microbial decomposition—and it is in this step that the microorganisms play a critical role in nature. If there were but a single carbon-containing substance which was not decomposable, then eventually all of the carbon in the world would appear in this substance, assuming it was continuously formed at a finite rate. Carbon itself (as charcoal, graphite, diamond) is not decomposed by microorganisms probably because, while it is oxidizable, it cannot be dissolved in anything and thus brought into the cell for oxidation. Hence, eventually all of the carbon in the world should appear as charcoal, graphite or diamond. But diamonds are not formed continuously and charcoal and graphite do oxidize spontaneously in air if they are finely divided particles; hence there are cases of decomposition not mediated by microorganisms, but generally these are very slow or have some special requirement before they will proceed. When conditions are such that microbial decomposition is inhibited, materials accumulate. For example, lignin is decomposed mostly by streptomyces and some higher fungi and these

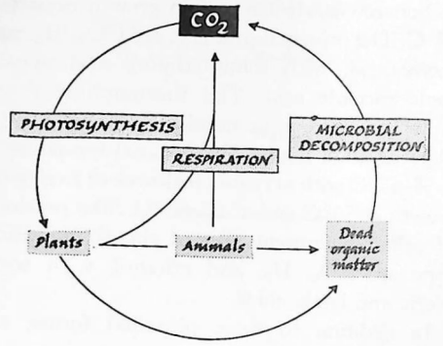


FIG. 18-6. *A simple diagram of the carbon cycle.*

are quite aerobic; hence, lignin is only very slowly decomposed under anaerobic conditions and tends to accumulate in swamp and lake bottoms.

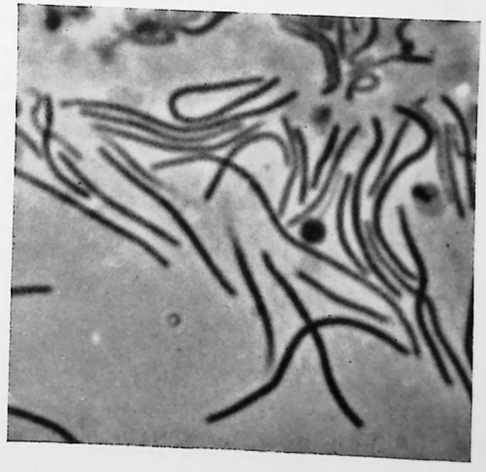
Certain of the cycles of elements in nature have special aspects that make them worth while to examine more closely. We shall use only simplified diagrams for such cycles since more elaborate ones may be left to your imagination. A simple carbon cycle is illustrated in Fig. 18-6. The one point which we wish to call attention to is the following. Considering all of the plants (grasses, trees, shrubs, etc.) which cover the land, certainly the sum total of these ought to weigh many times the weight of animals that live on the earth, and only a small per cent of the plant materials are consumed by animals; most of the plants fall to the soil directly. You will note from Table 18-2 that the greatest single constituent of plants is cellulose, being anywhere from 15–40% of the plant. Second place is occupied by either hemicellulose or lignin, depending upon the type of plant material that is being considered. It happens that these three materials constitute by far most of the carbon in the world. The United States alone has roughly 360,000,000 acres of crop land and about 1,000,000,000 acres of pasture and range land, with about 250,000,000 acres of forest.

The property of decomposing cellulose is not widely distributed among the bacteria. In fact very few aerobic bacteria can decompose cellulose; and they are the following.

1. *Cellulomonas.* These are small, gram-variable or gram-negative rods; spheres are sometimes described. There also seem to be further some other forms; cellvibrio, cellfalcicula-pointed rods. All of these grow on cellulose although not very well, and they do not decompose very much of it. They all grow on many other materials such as pectins, agar, glucose, etc.

2. *Cytophaga* types. These are long slender rods, 3–8 μ long, generally embedded in slime. They were first found about 40 years ago and were described as sinuous rods which later developed a round sporoid body. It proved difficult to separate the long rod from the round

FIG. 18-7. *Cytophaga. The round bodies are not sporoids but involution forms. The strain shown is an anaerobic one which ferments agar. [From Veldkamp, J. Gen. Microbiol., 26, 331 (1961). Courtesy of Dr. H. Veldkamp.]*



sporoid so it was impossible to be sure that one had a pure culture. Old cultures tended to have the sporoid and young cultures only the rod, but the amount of slime present prevented the certain use of single-cell isolation. Further, these did not look like spores of bacteria; see photograph, Fig. 18-7. After some controversy, it appeared that they were not true bacteria, although we earlier placed them among the higher bacteria (p. 287). Apparently they are intermediate forms between the bacteria and the myxobacteria, as follows.

TRUE BACTERIA		
Non-cyst-forming cytophaga	no sporoids, no slime	Most strains grow only on cellulose
Cyst-forming cytophaga	sporoids, slime	
Myxobacteria	plasmodium, slime	

Aside from the cellulomonas types and the cytophaga types, aerobic decomposition of cellulose is possible by streptomyces and fungi. Most streptomyces can decompose cellulose, but, like the aerobic bacteria, they do so relatively slowly, and probably because of this slowness are unable to compete with the more rapidly growing fungi. A surprising number of the common molds—many strains of *Aspergillus niger*, but especially species of the genera *Trichoderma*, *Fusarium*, *Verticillium*, *Monospora*, and even some *Pennicillium*—are particularly active decomposers of cellulose. In general, the Phycomycetes do not decompose cellulose, but the others probably account for most of the cellulose decomposition in aerobic soils. That is, the molds actually decompose the greatest weight of cellulose. Among all of these aerobic forms, there are both mesophilic and thermophilic types.

Anaerobic cellulose decomposition is essentially confined to the bacteria. They are mostly anaerobic spore-formers, thus being members of the genus *Clostridium*. They are of two principal types—mesophilic and thermophilic. The mesophilic forms grow relatively slowly at between 30–45°C but no growth occurs at 55°C. The principal products are CO_2, H_2, and acetic acid, with some ethanol and usually some succinic acid. The thermophilic forms grow, of course, more rapidly; 0.1% cellulose is digested in 24 hours. The optimal temperature is 55–65°C with very much slower or negligible growth at 50°C and at 67–68°C. The products of cellulose fermentation of the thermophilic type are CO_2, H_2, and ethanol, with some acetic and lactic acids.

In addition to these principal forms, the spore-forming anaerobes, there are other non-spore-forming anaerobes decomposing cellulose—both curved rods and pigmented cocci. However, so far as can now be judged, they do not seem to be especially important in soil, even though they may be in the rumen of cattle, and there is more than the suspicion that some may be anaerobic counterparts of the cytophaga.

The microorganisms decomposing hemicellulose are not nearly so specific as those decomposing cellulose; they include a wide variety of aerobic and anaerobic bacteria, fungi, and actinomyces. These are important in retting (p. 391). However, some organisms attack one type of hemicellulose in preference to another and there is a certain amount of specificity in this respect, and some hemicelluloses, like the galactans, are more resistant than others. Still, the property of decomposition is so widespread that hemicellulose can be considered among the readily available materials in plant tissues.

Lignin, on the other hand, is a very resistant material, both chemically and microbiologically. It is decomposed primarily by streptomyces.

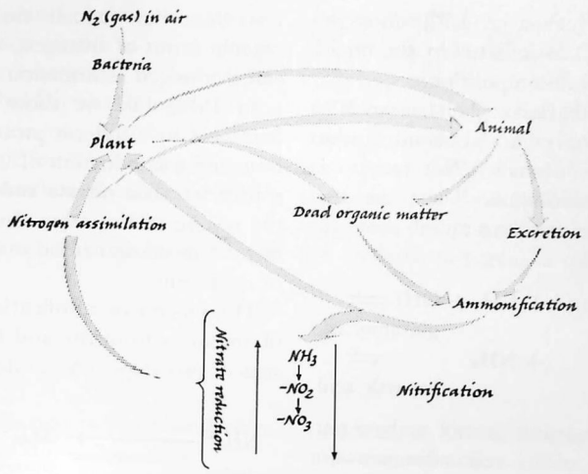


FIG. 18-8. *The nitrogen cycle.*

The Nitrogen Cycle

The nitrogen cycle (Fig. 18-8) contains several distinctive features that warrant discussion; the stepwise processes and the organisms involved are outlined in Table 18-3. One of these, nitrogen fixation, is of such importance that it is discussed separately in Chapter 19. Obviously, there is a vast store of nitrogen as nitrogen gas in the air, but this is inert and cannot be used until it is **fixed**, i.e., converted into other forms—ammonia or proteins. There are relatively few organisms capable of carrying out nitrogen fixation; they are all heterotrophic, some are photosynthetic, and there are both anaerobic and aerobic forms.

Plants and animals contain most of their nitrogen in the form of protein. When they die the protein is broken down to amino acids, and these are deaminated to liberate NH_3; hence this process of liberation of ammonia from organic materials is called **ammonification.** Most organisms are capable of ammonification, although there are a few among the lactic acid bacteria which apparently lack deamination reactions. Whether or not ammonia is released to the surrounding environment depends, as discussed earlier, upon the nitrogen

TABLE 18-3. *Process and organisms of the nitrogen cycle*

Process	Reaction	Type of Organism	Conditions
Ammonification	Organic N $\longrightarrow$ NH_4^+	Many kinds	Wide variety; 1.5–1.7% N
Nitrification	$NH_4^+ \longrightarrow NO_2^-$	Few; autotrophic	Aerobic
Nitrification	$NO_2^- \longrightarrow NO_3^-$	Few; autotrophic	Aerobic
Nitrate reduction	$NO_3 \longrightarrow NO_2 \longrightarrow NH_3$	Many kinds	Wide variety
Nitrogen assimilation	Combined N $\longrightarrow$ protein	All types	Wide variety
Nitrogen fixation	N_2 gas $\longrightarrow$ combined N	Few; heterotrophic	Both aerobic and anaerobic
Denitrification	$NO_3 \longrightarrow N_2$	Usually heterotrophic	Anaerobic

content. If this is less than 1.5–1.7%, all of the ammonia liberated is assimilated by the organisms engaged in the decomposition.

Most organisms—bacteria, plants, animals to some extent—can convert ammonia into amino acids and thus into proteins. This process is called ***nitrogen assimilation.*** There are two great gateways of ammonia to amino acids (p. 185):

1. α-ketoglutaric acid + NH_3 + (2H) $\rightleftharpoons$ glutamic acid
2. Fumaric acid + NH_3 $\rightleftharpoons$ aspartic acid

Sometimes an organism cannot make a particular constituent of its cell substance—for example, tryptophan; thus it will require tryptophan for growth but it can, nevertheless, assimilate the rest of its nitrogen requirement from ammonia. Sometimes, of course, reactions 1 and 2 are missing, in which case several amino acids need to be supplied. It was thought for many years that plants could assimilate only nitrates, which is indeed the preferential form of nitrogen for most plants. Yet it is now clear that most plants can readily assimilate ammonia. (The earlier experiments did not correct for *p*H changes.) Even nitrate when assimilated is reduced to ammonia in the plant, and ammonium ion is the form which enters into the nitrogen constituents of tissues. It is curious that while ammonium ion is necessary for the conversion to amino groups ammonium ion itself is toxic at rather low concentrations to plant and animal tissues, and there are complicated mechanisms for storing ammonia in nontoxic form. In the plant the ammonium ion is generally converted into either glutamine or asparagine; in the animal it tends to be picked up and converted into urea, which is excreted. The process of conversion of inorganic nitrogen such as nitrate or ammonium into organic forms in the cell is called nitrogen assimilation and is obviously carried out by most organisms. For those few which necessarily assimilate organic forms of nitrogen, this process is also called nitrogen assimilation.

In Table 18-3 we show the conversion of ammonia to nitrate, a process called **nitrification,** and the reduction of nitrate to ammonia, a process called **nitrate reduction.** One is *not* the reverse of the other. Instead they are two distinct processes carried out by different kinds of organisms.

The process of nitrification is the oxidation of ammonia to nitrite and to nitrate, and consists of two steps, (A) and (B):

$$\underset{\text{ammonium ion}}{NH_4^+} \xrightarrow{\text{(A) } \textit{Nitrosomonas}} \underset{\text{nitrite ion}}{NO_2^-} \xrightarrow{\text{(B) } \textit{Nitrobacter}} \underset{\text{nitrate ion}}{NO_3^-}$$

These reactions are not widely distributed among organisms in nature but are pretty much restricted to two groups of organisms—which because of this and related properties are put into two separate genera—*Nitrosomonas* and *Nitrobacter.* Both of these organisms are autotrophs, whose properties we shall discuss below. First, however, we must outline what we mean by the reactions being restricted to these organisms. Are there no heterotrophs which carry out ammonia or nitrite oxidation? Apparently there are, but the amounts of nitrite or nitrate formed are always very small; and there seems to be little doubt, from the information available, that the agents of nitrification are autotrophic bacteria capable of converting ammonia to nitrite and nitrite to nitrate, using these reactions as sources of energy.

The organisms involved are very small rods living in a completely inorganic medium and obtaining their carbon from CO_2. Curiously, many kinds of organic matter are toxic to them, especially substances having free amino groups. The *Nitrosomonas* is not capable of attacking nitrite, which is toxic to it, nor is *Nitrobacter* capable of attacking ammonia. At one time it

was supposed that plants could only assimilate nitrate and were quite unable to use ammonia. This proved to be erroneous, but when this belief was strongly held, it was thought that the process of nitrification was the "bottleneck of nature" and that soil fertility could perhaps be measured by the number of nitrifying organisms in the soil.

When nitrate is assimilated by plants or microorganisms, it is reduced to ammonia. Many microorganisms also are capable of reducing nitrate to ammonia. This process is called nitrate reduction. But there are some organisms which, under anaerobic conditions, can use the oxygen combined in NO_3 to carry out their metabolism. They liberate nitrogen gas, and this process, which represents a loss of available nitrogen, is called **denitrification.**

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For background information, see p. 495.

QUESTIONS

1. Define or identify:

soil	cytophaga
soil profile	sporoid
base exchange level	retting
hemicellulose	nitrogen fixation
lignin	ammonification
humus	nitrification
lignoprotein	nitrate reduction
carbon cycle	nitrogen assimilation
cellulomonas	denitrification

2. Professor von Cellulose reports that the addition of Douglas fir sawdust to soil increased its fertility immediately. Why do you not include this in the book you are writing on recent advances in American agriculture?
3. There is the story of the farmer visiting Manhattan who was told that Battery Park was worth more than a million dollars a square yard and who replied, after feeling the soil, that it was not worth over forty dollars an acre. What criteria was he using? How could he judge the soil by feeling it?
4. The farm manager of a large estate writes to you, asking advice as to how to compost several years accumulation of leaves, mostly birch and oak. He expects you to provide him with

a formula which will permit the leaves to decompose since he has already tried to compost them with no success. What would you advise? What should he add? How much?

5. In the manufacture of burlap, sisal is placed in water and the hemicellulose fermented away, leaving the tough cellulose fiber (a type of retting). The ponds in which this is done develop high acidity and are very high in organic matter. Your company has just acquired an old sisal retting area and plans to put up a fancy hotel. How would you go about cleaning up the organic waste so that it could be put to the best use?

6. In addition to the organisms mentioned in the text, there are others that are capable of cellulose decomposition to a lesser extent. Devise a method for searching for these.

7. Dr. Orteyes de Novo reports that in the jungles along the Amazon River the number of bacteria in the soil rarely exceeds 100,000 per gram. He therefore concludes that the bacteria have little to do with organic matter decomposition. Why would he be right or wrong?

8. An agricultural research institute is devoted to a study of the influence of the phase of the moon on crop yields. Over the years they have been able to provide striking evidence that planting in certain phases of the moon gives better yields than others. This effect is apparent in the plains and the tundra, but it is of no effect in forests. What explanation could you give for these effects?

9. In a visit to an institute of soil bacteriology you are shown a series of plates containing mostly streptomyces. These people find few bacteria and few molds in the soil they study. What could you conclude about the state of decomposition of the organic matter in this soil?

10. It has been pointed out that the weight of 5,000 million bacteria per gram of soil (a not unusual figure) would be 4 tons of bacterial substance per acre. What per cent of the acre is bacteria? How many tons of bacteria are there in the United States?

Chapter 19

NITROGEN FIXATION

"The producer of corn and meat, according to the prevailing system, despoils his own land . . . and lastly, large towns, like bottomless pits, gradually swallow up the conditions of fertility of the greatest countries . . . the sewers of the immense metropoles of the ancient world engulfed, in the course of centuries, the prosperity of the Roman peasants, and when the fields of the latter would no longer yield the means of feeding her population, these same sewers devoured the wealth of Sicily, Sardinia and the fertile lands on the coast of Africa."

VON LIEBIG, *Letters on Modern Agriculture* (1859)

IT HAS BEEN KNOWN since antiquity that legumes would "restore" the soil. The nitrogen cycle shows obvious losses of nitrogen. One is by the process of denitrification; the nitrogen gas formed is no longer available to plants. There is loss into the sea which is not recovered. There is loss by explosives; while this may seem minor, except in war, it is actually reasonably large. Blasting of rock, earth, stumps, etc. in the United States alone liberates about 50,000 tons of nitrogen. Losses by these three sources are relatively large. But even if they were small, eventually they would use up all the fixed nitrogen, and plants, animals—in fact, all of us—would soon die. While we may replace soil losses for a time by using nitrogenous deposits such as guano, these too would eventually be used up. It is no comfort to reflect that the world has supported a vegetation and an animal population for many centuries; we may further reflect that never in its history has the world supported so many humans, a group notorious for the losses they encourage in the nitrogen cycle. There are areas of the world in

which precisely this has happened. The human population has outrun the nitrogen supply, and this very fact tends to cause more loss. Two chemical processes—the Haber process and the "Berkland-Eyde process"—are capable of fixing nitrogen, but the amount of nitrogen fixed by these methods is very small compared to the losses sustained.

But all is not lost; while there are always prophets of doom, it happens that the nitrogen losses are mostly replaced by biological nitrogen fixation; indeed, the world's supply of fixed nitrogen may actually be increased. There are two main types of nitrogen-fixing organisms. First, the free-living bacteria, the principal group of which is *Azotobacter; azote* is the French word for nitrogen. Second, the bacteria which live in the roots of legume plants. These are placed in the genus *Rhizobium; rhizo* is the Greek word for root. The combination of the plant with the bacteria is capable of fixing nitrogen. The rhizobia are called the **symbiotic** nitrogen-fixers since there is here a symbiosis, i.e., two organisms living together for the benefit of both. We shall consider each type separately.

Free-living Nitrogen-fixing Bacteria

One may easily obtain bacteria capable of fixing nitrogen by inoculating with soil a medium containing an energy source but free from compounds containing nitrogen. Only those organisms which are capable of fixing nitrogen can grow, and they do. This is precisely what was done when such bacteria were first discovered. We do not know whether the following description is completely accurate, but it is likely that it happened in this way. There were, in 1880, two eminent bacteriologists who were interested in bacteria as bacteria, and not just because they caused disease. These men were Winogradsky in Russia and Beijerinck in Delft, Holland. (Incidentally, the history of both is worth study.) In 1880 they each set out independently to try to isolate the bacteria, if there were any, which fixed nitrogen. Each made a medium, similar to that of Fig. 19-1, which is

FIG. 19-1. *A bottle of nitrogen-free medium inoculated with soil and incubated for two weeks.*

nitrogen-free. They each inoculated it with soil, and after a time obtained growth—perhaps in a bottle like that in the figure. You can do this yourself in a fruit jar at home if you like. Such a bottle typically shows some gas bubbles from the bottom, and after a while, a scum—sometimes a thick gummy layer on top. After some weeks the top layer may turn deep black. Sometimes the whole bottle becomes greenish, even in the dark. This too happened to the bottles inoculated by Winogradsky and Beijerinck.

Winogradsky was the first to succeed, and from the bottom of the bottle isolated some organisms capable of fixing nitrogen, which had the strange property, hitherto unknown, that air was toxic to them. These were the strict anaerobes. Today the types found by Winogradsky are placed in the genus *Clostridium*, the spore-forming anaerobes, and they still are

capable of fixing nitrogen, although the amount of nitrogen fixed is not spectacular. However, members of this same group are used for the production of acetone and butyl alcohol, and during this production they actually fix considerable quantities of nitrogen in the fermentation tanks. Winogradsky's organisms fixed nitrogen, indeed, but there were some reservations about how important they were in the restoration of nitrogen in nature. Most soils are aerobic, so how could these organisms grow in them? Also, the amount of nitrogen fixed was not large; could this really be enough?

In the meantime, using apparently the same general kind of media and apparatus, Beijerinck set out to isolate the nitrogen-fixing organism. But he picked the scum on the top and isolated an aerobic organism, a member of the genus *Azotobacter*. This organism fixed considerably more nitrogen than *Clostridium*. However, this too was not really enough to close the gap and recoup the loss. Compared to the amount of nitrogen fixed by the symbiotic nitrogen-fixers, even *Azotobacter* is a poor second.

The last ten years have demonstrated, especially with the use of the heavy nitrogen isotope as a tracer, that ability to fix nitrogen is not limited to members of these two genera. Table 19-1 shows typical organisms in which some degree of nitrogen fixation has been demonstrated. But among these *Azotobacter* is still the best; all of the rest fix much less nitrogen.

TABLE 19-1. *Typical Organisms which Fix Nitrogen*

	NITROGEN FIXATION IN CULTURE MEDIA		
MICROORGANISM	Conditions	Time (days)	mg N fixed/liter
Azotobacter sp.	Aerobic	4	1050
Clostridium sp.	Anaerobic	10	136
Rhodospirillum	Anaerobic, light	10	76
Blue-green algae	Aerobic, light	55	52
Aerobacter aerogenes	Anaerobic	2	60
Achromobacter sp.	Aerobic	4	17

Symbiotic Nitrogen Fixation

In practical agriculture over a century ago, it was well established that "the legumes renewed the soil." Here is a statement from 1806: "Lately the practice of sowing white clover (a legume) as a crop has become very general; only a few apathetic and indolent agriculturists or men who are firmly wedded to old opinions and customs neglect this practice." The Germans, a century ago, claimed that legumes fixed nitrogen from the air; the French claimed that all plants, even grains and grasses, fixed nitrogen. The English claimed that none did; and they argued among themselves.

Table 19-2 shows the result of a six-year experiment, published in 1837 by Boussingault in France, in which he analyzed the nitrogen in

TABLE 19-2. *Boussingault's Experiments*

		FOUND		GAIN (lb)	
Plot	Crop	Nitrogen in crop	Nitrogen added as manure	Total*	Per year*
1	Potatoes, wheat, clover, wheat, timothy	229	185	44	9
2	Mangels, wheat, clover, wheat, fallow	231	185	46	9
3	Potatoes, wheat, clover, wheat, peas, rye	323	223	100	17
4	Fallow, wheat, wheat	80	76	4	1
5	Alfalfa, alfalfa, alfalfa, alfalfa, alfalfa	980	205	775	130

* Note: years vary

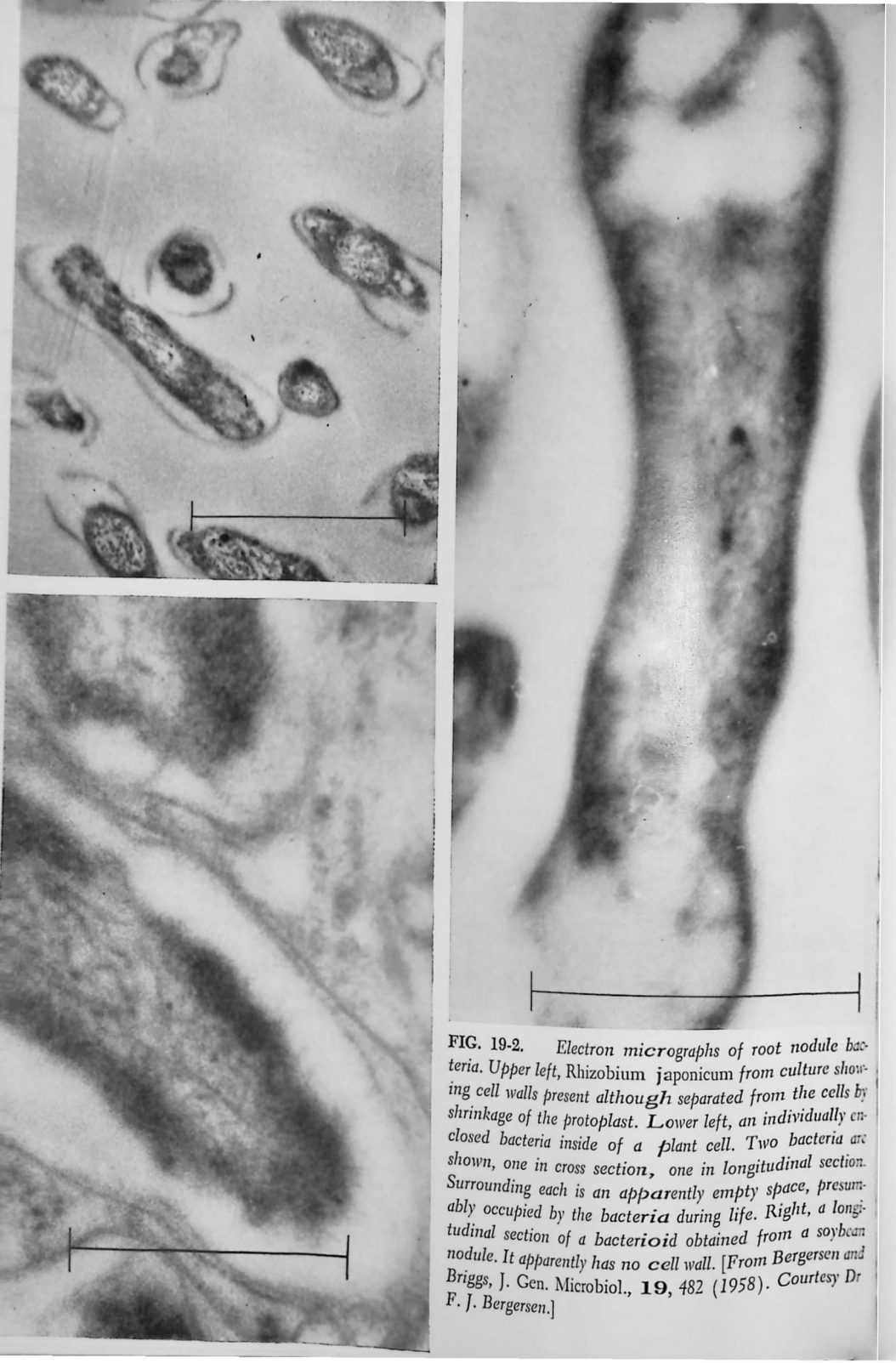

FIG. 19-2. *Electron micrographs of root nodule bacteria. Upper left,* Rhizobium japonicum *from culture showing cell walls present although separated from the cells by shrinkage of the protoplast. Lower left, an individually enclosed bacteria inside of a plant cell. Two bacteria are shown, one in cross section, one in longitudinal section. Surrounding each is an apparently empty space, presumably occupied by the bacteria during life. Right, a longitudinal section of a bacterioid obtained from a soybean nodule. It apparently has no cell wall. [From Bergersen and Briggs,* J. Gen. Microbiol., **19**, *482 (1958). Courtesy Dr F. J. Bergersen.]*

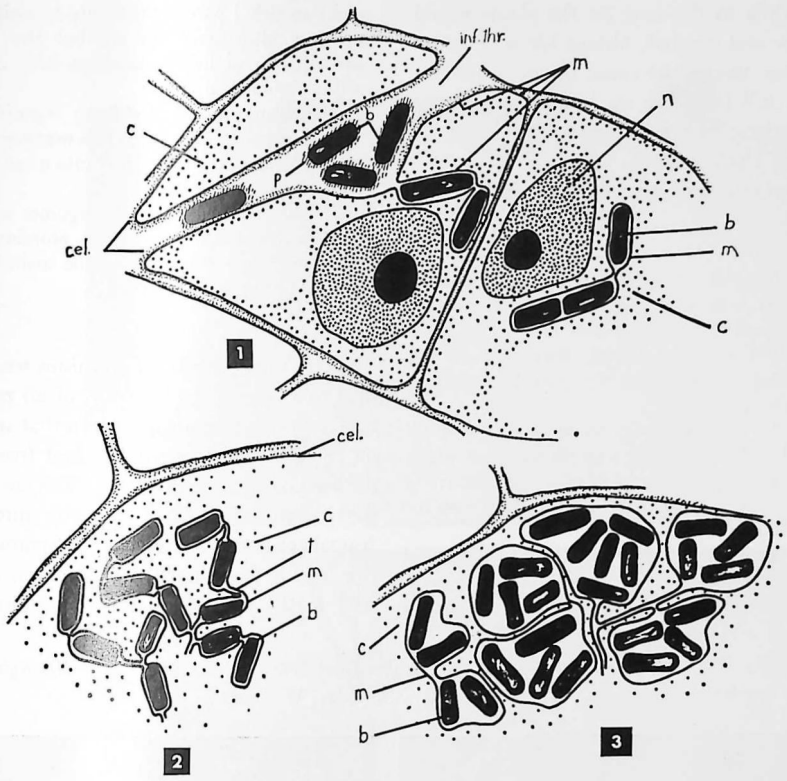

FIG. 19-3. *Diagram of infection in root nodules. Part 1 shows an infection thread with bacteria in it. These escape from the thread proper but are still enclosed by a thread membrane (m). These are actually within the cytoplasm of the cell (c), and within this protective coat the bacteria multiply (Part 2). Finally (Part 3), the cells occupy most of the cellular space. [From Bergersen and Briggs.* J. Gen. Microbiol., **19**, *482 (1958). Courtesy Dr. F. J. Bergersen.]*

the crop and the manure and struck a balance. Notice that when he had legumes (clover and peas) twice, he had twice the gain; when he had alfalfa all the time he *gained* 130 lb of nitrogen per acre per year. He concluded, "Azote (= nitrogen) may enter the living frame of plants directly . . . the observations of vegetable physiologists are not generally favorable to this view." Indeed, some years later Leibig talked him out of it by convincing Boussingault that the gain was all analytical error. But in 1883 Hellreigel and Wilfarth, two German chemists, started some experiments—rather crude ones, really—which eventually yielded the answer. What occurred was as follows. Cereal crops such as oats grew in proportion to the amount of nitrogen supplied to them, but peas, a legume, did not. What was most especially confusing was that there was a tremendous variation in the growth of pea plants within the jars to which no nitrogen at all was added. The peas in one would be tall and

green, while in the next jar the plants would be yellow and stunted. Almost like a detective story, they traced the cause of this variation: bacteria fell from the air into certain of the jars and these bacteria were responsible for the nitrogen. Their conclusions (of 1886) are still worth quoting since they describe the essential facts.

1. The behavior of legumes is fundamentally different from the cereals in regard to the absorption of their nitrogenous nutrition.
2. The cereals are solely dependent on the assimilable nitrogenous compounds present in the soil . . .
3. To the legumes a second source is available besides the nitrogen of the soil from which they are able, in a highly efficient manner, to cover their needs entirely or in part, in case the first source is insufficient.
4. This second source is the free elemental nitrogen of the air.
5. The legumes do not themselves ["haben nicht an sich"] possess the ability to assimilate the free nitrogen of the air; but the active participation of living microorganisms of the soil is absolutely necessary.
6. The mere presence of lower organisms of the soil is not sufficient, but it is necessary that certain kinds of the latter enter into a symbiotic relation with the former.
7. The root nodules of the legumes are not merely reserve storehouses for proteins, but stand in a causal relation to the assimilation of free nitrogen.

These "certain kinds" of organisms were isolated two years later (1888) by, of all people, Beijerinck. Much study has shown that neither the bacteria in culture nor the plant free from the bacteria is capable of fixing nitrogen; nitrogen is fixed only when they enter into this "causal relation." The bacteria are gram-negative rods, rather gummy, which, when examined in the nodule, are highly pleomorphic.

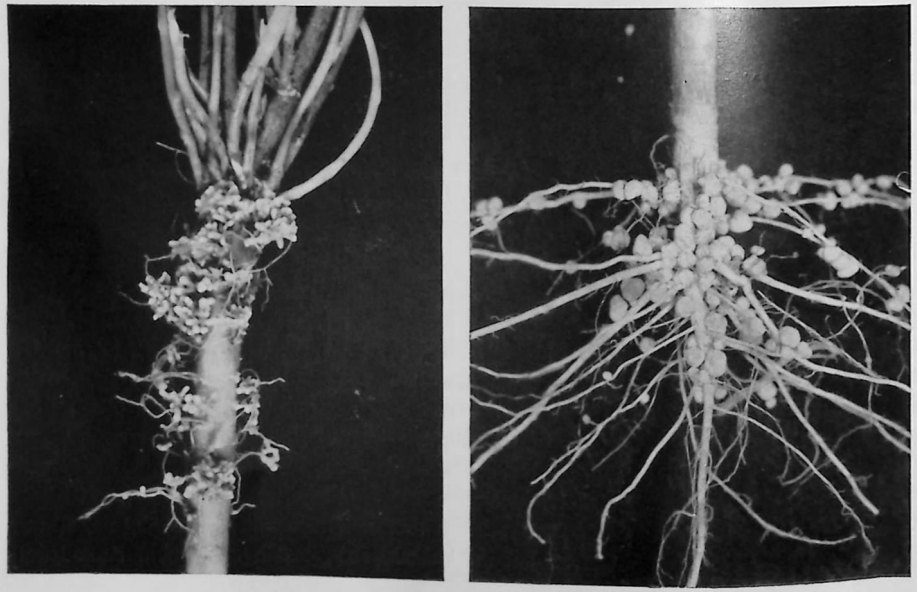

FIG. 19-4. *Nodules on leguminous plants. The plant at the left is alfalfa; that at the right, soybean.* [*Courtesy J. C. Burton, The Nitragin Co., Milwaukee, Wisconsin.*]

FIG. 19-5. *Effect of inoculation with root nodule bacteria upon the yield of plant. Upper photograph is of a field of peas. The seed planted to the left of unplanted strip has been inoculated with* Rhizobium leguminoserum; *that to the right was not so inoculated. Notice how much greener (darker color) the inoculated plants are, which is a good indication that they have adequate nutrition. The lower photograph shows crimson clover* (Trifolium incarnatum). *Note how much larger the inoculated plants are than the uninoculated. The inoculated plants are to the right and show nodules.* [*Courtesy of J. C. Burton, The Nitragin Co., Milwaukee, Wisconsin.*]

These odd forms (Fig. 19-2) are called "bacteroides" and are believed by some to be *the* nitrogen fixing form.

The process of nodule formation is as follows (Fig. 19-3). The bacteria enter the plant through the root hair. They first congregate at the tip of the root hair, and then apparently secrete a substance causing the root hair to curl. If the bacteria are of the right "cross-inoculation group" (see below) the bacteria enter the root hair and begin to penetrate the root. The plant seems to lay down a wall about the bacteria, the net result being an **infection thread.** Apparently this thread must locate a polyploid cell, one having double the normal number of chromosomes, before nodule formation takes place; only about 4% of the infected root hairs ever form nodules. Upon the approach of an infection thread the polyploid cell begins to be stimulated and soon the infection thread enters the polyploid region, bursts open, and releases the bacteria into the polyploid cell. The cells continue to divide until a nodule is formed, most of the cells in the nodule being filled with bacteria (Fig. 19-4).

However, strains of bacteria isolated from different legumes differ drastically in their ability to form nodules. For example, strains isolated from alfalfa (*Mediago sativa*, etc.) will form nodules on alfalfa and on sweet clover (*Melilotus albus*, etc.) but not on peas, soybeans, etc. These **cross-inoculation groups** are the following. The species of root nodule bacteria is usually named after the major plant in the group.

Alfalfa group: *Rhizobium meliloti*. Alfalfa, sweet clover.

Clover group: *Rhizobium trifolii*. Red, white, and alsike clovers.

Pea group: *Rhizobium leguminosarum*. Pea, sweet pea, vetch, lentil.

Soybean group: *Rhizobium japonicum*. Only soybean.

Cowpea, bean, lupine and eight other minor groups are known.

Not always is the symbiosis effective. The ability of a given symbiosis to fix nitrogen depends upon the strain of the bacteria, the variety of the plant, the presence of trace elements (especially molybdenum and calcium), and the "carbohydrate-nitrogen relationship." This last means that, in proportion to the amount of available carbohydrate, if the nitrogen is high there will be little nitrogen fixation.

Frequently the members of *Rhizobium* sp. of the proper type and nitrogen-fixing ability, are not present in the soil. Under these circumstances it is frequently profitable to inoculate the seed with the proper bacteria before planting; there are commercially available such **inoculants** which are used by most farmers before planting legumes (Fig. 19-5).

The mechanism of nitrogen fixation is not precisely known. The first product detectable by tracer studies is ammonia, but how N_2 is converted to $(NH_4)^+$ is not yet known.

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QUESTIONS

1. Define or identify:

nitrogen fixation	nodule
free-living nitrogen-fixing bacteria	cross-inoculation group
symbiotic nitrogen fixation	infection thread
legume	polyploid cell
bacteroides	carbohydrate-nitrogen relationship
	inoculant

2. There occasionally appears in the agricultural magazines an advertisement for "soil bacteria" claiming that special isolated strains of "nitrogen fixing" bacteria will improve the soil fertility, and if one adds these to the soil, for a price, one can eliminate fertilization. As a microbiologist, do you think that this is possible? Under what circumstances?
3. For many years certain eminent microbiologists claimed that cross-inoculation groups did not exist because if one put overwhelming numbers of *Rhizobium meliloti* on red clover an occasional nodule resulted. Dr. Dodder has read a paper which claims this and has recommended that the clover group be abolished. As a microbiologist engaged in the production of clover inoculants, how would you defend your position?
4. A curious phenomenon in nitrogen fixation is this: if leguminous plants, inoculated with the proper bacteria, are grown in an atmosphere with some of the nitrogen replaced by hydrogen, nitrogen fixation is inhibited. Molecular hydrogen and molecular nitrogen occupy about the same space per molecule. *Clostridium* sp. produce a great deal of hydrogen, especially in cultures given glucose. How might these facts influence your estimate as to the amount of nitrogen *Clostridium* sp. contribute to soil per acre per year? How would you go about getting a more accurate estimate?
5. *Azotobacter* has an exceedingly high respiration rate. It is maintained in a "more vigorous" form by growth on nitrogen-free media containing alcohols or organic acids; long cultivation on glucose or on media containing peptones is harmful to it. On the basis of this information, devise a simple medium which could be used in an underdeveloped but habitable country for the fixation of nitrogen.

Chapter 20

FOOD MICROBIOLOGY

"4. And the Lord said unto Moses, Behold, I will rain bread from Heaven for you; and the people shall go out and gather a certain amount every day."
"19. And Moses said, Let no man leave of it till the morning."
"20. Nothwithstanding they hearkened not to Moses; but some of them left of it until the morning, and it bred worms and stank; and Moses was wroth with them.
"22. And it came to pass that on the sixth day they gathered twice as much bread . . .
"23. [and Moses said] . . . bake that which ye will bake today and seethe [boil] that ye will seethe; and that which remaineth over, lay up for you to be kept until the morning."
"24. . . . And it did not stink, neither was there any worm therein."

Exodus 16 (probably about 1200 B.C.)

FOOD MICROBIOLOGY is concerned with three main subjects: (1) The preservation of food from the attacks of microorganisms. (2) The preparation of food by the use of microorganisms. (3) The detection and prevention of food poisoning due to microorganisms and the control of disease transmission by food.

Food Preservation

The particular problems which make food preservation necessary are both economic and hygienic. Economically, this necessity is based upon the seasonable production of foods and the necessity of preservation to tide over periods

when food is scarce. The starvation experienced by primitive man (and sometimes still by animals) because of the lack of preservation of what was harvested was probably more frequent than the starvation due to failure of harvests, although the latter was basically the result of the inability to store up enough in times of plenty. The centralization and concentration of populations in civilized countries, particularly where people live far from the food sources, demands food preservation systems, sometimes of very great complexity. This is related to the need to transport food, an ancient need indeed for armies, navies, and exploration parties, far from the sources of food.

But the hygienic problems are of equivalent importance. The spread of infections by foodstuff must be prevented and also the growth of organisms which might produce harmful substances leading to food intoxications; all of this in addition to preventing gross spoilage as such.

The problems become even a little more complex when we consider the following. We wish to preserve the energy content of the food, and its essential building blocks—essential amino acids, etc.—as well as its vitamin content. But in addition to this, we need the preservation of flavors and of other esthetic qualities of food—feel or texture, appearance, color, smell, etc. Sometimes we do not preserve all of these things—compare canned strawberries with the fresh fruit; but we preserve something which is edible and desirable, even if it is not the same thing we started with.

Not all food decomposition is due to microorganisms. Flavors are particularly fragile; they are frequently changed, particularly by metals such as copper or arsenic and sometimes from cans or sprays, so that so-called "off" flavors are found. Frequently decomposition occurs by the enzymes of the plant or animal itself. At times, in fact, this decomposition is desirable—as when plant enzymes change starch into sugar and thus add sweetening, or when proteolytic enzymes of the meat "tenderize" it. Finally, there is the natural instability of some of the compounds found in food. Even if we eliminated all the microorganisms, it would be difficult to keep certain kinds of foods forever. But that is not the point. In most cases we don't preserve food forever but only for a useful or economical time. Food can be preserved for years—thousands of years, indeed, as for example the wheat buried with Egyptian mummies; but there is some loss of flavor, texture, or palatability.

Certainly, however, a large part of the decomposition of most foods is due to microorganisms, and if these can be eliminated or checked, food can be preserved for varying periods, depending upon its nature and upon the deterioration necessary for it to become inedible. In essence, this is competition between man and microbe for the food and in many cases we wish to load the dice in our favor. The following factors determine whether an organism will grow and, with access to many kinds of organisms, will determine which organisms will grow.

Moisture: Note how frequently drying can preserve. Absolute dryness is not necessary. Increased osmotic pressure can effect a form of drying.

Oxygen: Its presence or absence.

Temperature: There are two aspects. (a) Temperatures below those of growth will slow down and eventually stop growth (note preservation by refrigerators or by freezing) but low temperatures do not kill the organisms. (b) Temperatures far above the growth range will kill, if maintained for a sufficient time.

Acidity: Curiously, very few foods are alkaline. In general, few bacteria will grow below the range of *p*H 4.5–5; streptomyces are also limited at such a *p*H, yet yeasts and molds do rather well here.

Composition of food: Its carbohydrates, fat, and proteins. The requirements of different organisms differ, so which organisms will grow will depend on the nature of the environment, i.e., on the nature of the foodstuff.

With respect to the organisms, the following table is helpful.

Organism	Distribution*	O_2	Temperature	*p*H	Special requirement
molds	wide	must have	mesophilic	acid	—
yeasts	narrow	both[1]	mesophilic	acid	need high sugar
streptomyces	wide	must have	mesophilic[2]	neutral[3]	—
bacteria	wide	both[1]	mesophilic[2]	neutral[3]	—

* The likelihood of their presence on food.
[1] Some will grow either in the presence or in the absence of air.
[2] Some thermophiles exist in these groups.
[3] Not below *p*H 4.5.

There are obviously two primary methods of preserving food. Since its spoilage is due to microorganisms, and to their growth, we may either keep out all microorganisms capable of growth or we may prevent the growth of those which may be present. Since food is taken from nature, it is a rare case that it comes packaged free from microorganisms (the egg is one such example), hence it is rare that food is free from microorganisms. Preservation consists then mainly of destroying the microorganisms present or preventing their growth. When this is done food may be preserved for years with only such loss of flavor and texture as is inherent in the instability of the compounds which compose it.

There are three basic principles in food preservation.

1. The nature of the foodstuff determines which organisms can grow in it, what changes they can cause in it, and what methods can be used to prevent it.
2. The environment in which the foodstuff is held determines what organisms can grow and what changes they can cause.
3. The organisms present and their numbers will determine which organisms will grow.

These principles lead to a variety of methods of food preservation, but before these are discussed the importance of asepsis should be pointed out. If we reduce the third factor above to zero (i.e., no microorganisms), the food will usually be preserved. Most inner tissues, as the pulp of an orange, have few if any microorganisms. But even if the complete absence of microorganisms is impossible, partial asepsis can be a great help in preservation. This cutting of the load of microorganisms has three marked benefits, as follows:

1. It cuts the chances of the kinds of organisms present and thus the chances of those which might find the environment ideal for their growth.
2. With fewer organisms, there is slower decomposition.
3. We can get rid of the organisms more easily if there are fewer of them.

Aside from the esthetic advantage of preparing and storing food under clean and sanitary conditions, there is the very practical need to do so. Methods of food preservation work best on clean food.

Types of Foods and Organisms Decomposing Them

Methods of food preservation are obviously not all applicable to different kinds of food. For our purposes it is helpful to divide foods into seven main divisions, considering human foods primarily.

1. *Cereals* are high in carbohydrate, generally starches, but low in nitrogen. The principal preservation factor is lack of water; hence, so long as they are dry, there is no trouble keeping them, if free from insects. But if wet they decompose readily. Typical foods: bread, flour.
2. *Foods high in sugar* are also low in moisture and generally low in other nutrients. They possess a high osmotic pressure generally adequate to prevent microbial growth. Some osmophilic yeasts do cause trouble, but they are rather poorly distributed in nature and hence are not generally present. Honey, syrups, etc. are relatively stable but when diluted with an equal volume of water decompose readily.
3. *Vegetables* may be considered under two types. First, the leafy vegetables—lettuce, spinach, and the like. While they are rather high in carbohydrate, most of this is starch, not sugar; unless bruised, so that the cells of the plants are damaged, they are rather stable. However, when they wilt and the cells are damaged, microorganisms, especially bacteria, readily attack them.

Second, the roots and tubers (carrots, beets, potatoes) have a relatively low moisture content. The inner portions are usually sterile and the carbohydrate is starch and not too readily available. In some cases an outer layer protects the tissue. But they do decompose.

4. *Fruits* are rather high in sugars, but usually are somewhat acid. Hence decomposition is likely to be by yeasts, as in grapes, or molds, as in citrus fruits.
5. *Milk and dairy products* are good food for man and microbe, and thus are rapidly decomposed. They will be discussed in Chapter 21.
6. *Meat* is generally rather high in nitrogen and rather low in carbohydrate. Further, it is neutral to alkaline. These factors mean that meat is likely to be attacked by bacteria, but rarely by yeasts or molds.
7. *Eggs* are, in composition, like meat, but they are protected by a natural aseptic package —the shell. When this is penetrated, they decompose rapidly.

Basic Methods of Food Preservation

The variety of methods for food preservation which can be employed depends upon the food and what can be done with it without altering its desirable character. There is no point in trying to preserve sugar by making it acid, or ice cream by boiling it.

LOW TEMPERATURE. If we lower the temperature, we can slow down—or if we lower it enough we can stop—the growth and activity of the microorganisms. This process does not generally kill microorganisms but rather simply slows down their metabolism. Low temperature suggests, of course, deep freeze. But there are really a great many preservation practices based on low temperature which are not nearly so sophisticated. Cellar storage, for example, has been practiced for generations. Root crops like potatoes, carrots, beets were simply held at a cool temperature. This was possible because the foods were high in starch and were relatively dry so that few organisms would attack them. If the temperature was too high, their own enzymes broke the starch to sugar and the vegetables rotted; if the moisture was too high they did not long withstand bacterial attack. Most of them had a tough outer skin and were relatively sterile on the inside. Mostly, too, they were kept in air; the bottom of the pile decomposed. Air was helpful since many anaerobes attack starch. Here, then, were a variety of con-

ditions: high carbohydrate, low moisture, relatively aseptic on the inside, and air—which already were favorable to preservation so that under these circumstances a very low temperature was not necessary. In a sense, it was probably because of these very properties that these tubers were selected and cultivated by man; this combination of factors prevented rapid spoilage and permitted him to survive the winter. What matter if in spring they decomposed? There was always a new crop for the next year.

Many foods, however, need a lower temperature because they do not possess these stability factors. In many cases, freezing or freezing temperatures were not absolutely necessary and under somewhat primitive conditions were difficult to maintain except during winter. Meat, for example, was chilled and held cold for long periods of time. Milk is held in the refrigerator without freezing. In most of these cases, preservation is not intended for long intervals. The housewife frequently chills leftovers or buys for a few days without intending to freeze but only to chill the food. Chilling to near-freezing temperatures without actually freezing slows down the rate of bacterial decomposition so that it essentially stops; "cold storage" generally means that the food has been held near freezing but not frozen. Many foods may be preserved for months in this fashion.

Actual freezing or "deep freeze" of food is a relatively recent process. Here, not only is the temperature lowered below that which permits microbial growth, but also the moisture is removed by converting it into ice. Many of the flavors of the original fresh food are preserved, but a great difficulty is the preservation of the texture. Before the early trials with frozen foods, few people realized how much they were conditioned to textures of food and how much this was part of the character of the food. Mushy peas, stringy peaches, woody carrots might have all the flavor of the original, but they just did not taste right. In part, much of the problem could be solved by rapid freezing, which tended to produce smaller crystals; the smaller the ice crystals, the less tearing and damage of structural parts and apparently the less damage to various colloidal dispersions upon which the texture of the food ultimately depended. In other cases, the physical conditions of freezing were important. At times a "freeze mix" was supplied; frequently this consisted of an antioxidant browning; ascorbic acid was a favorite. Large quantities of a wide variety of things were frozen commercially—baked goods, pies, fresh fruits and vegetables, orange juice, and what not. Today this method of preservation is so common as to occasion no comment, yet ten years ago it was a rarity. Originally, too, much freezing was done in the home. This is still common in areas close to sources of supply but less common in cities.

One point should be clarified. Most organisms are slowed down and stopped by freezing but are not killed. When the food warms up, they normally grow very rapidly in it. Frequently the freezing has released soluble sugars, etc. Hence food that has been frozen should, as a general rule, be cooked or consumed shortly after it is thawed. If thawed and not consumed or treated very soon, it decomposes generally more rapidly than comparable food not frozen.

HIGH TEMPERATURES. In general, **storage** at temperatures too high for microbial growth is too damaging to the food, so high temperatures are usually just used to kill organisms. This killing may be complete or it may be only partial; partial killing is used to increase the load and to eliminate certain types of microorganisms which could either grow or cause disease, particularly under conditions where greater heating would destroy the character or quality of the food.

There are two principal processes in which partial killing is used to decrease the load and One is boiling, the other is pasteurization. Boiling is commonly used in the home, generally, of course, without any particular knowledge that it is capable of killing microorganisms, but mostly as a method of preparation for reasons of flavor or even tradition. The housewife knows, however, that boiled food keeps longer. It is a very useful method of preservation where spore-formers are not likely to interfere, particularly in acid foods, such as cherries, rhubarb, or even tomatoes, or where a higher temperature will spoil the food. Most cooking in the home destroys the vegetative cells, rarely the spores. Food is held in the refrigerator, which generally prevents spore germination. Cooked food, especially if neutral, when held at room temperature for a considerable interval and then eaten cold without reheating, is a potential source of botulism (see p. 364).

Pasteurization kills part but not all of the organisms. It consists of heating the food to temperatures below boiling, which will kill off many of the organisms. It is used primarily for milk, in the sense that it is almost a standard practice in the treatment of milk. We shall discuss it in connection with milk and dairy products, only pointing out here that it is applicable to many foods—beer, cheese, and fruit juices.

Autoclaving, or heating above the temperature of boiling water, is generally applied to foods in the process of canning. Commercial canning is a widespread and familiar process. Home canning is not nearly as common as formerly, but since the processes differ slightly we shall discuss them separately. While in both the object is to kill off the living organisms with heat in some sort of a container which will keep the others from getting in—a can or jar—the facilities available in the home and those available commercially are vastly different. Commercial canning uses extensive equipment not available to the housewife; it also has degrees of control which are not known or are not available at home. In commercial canning the "tin" can is generally the standard container, and it may be manufactured in a variety of sizes and shapes. So common is this container that some cynics call our civilization the "tin can age." However, canned foods are demonstrably edible, safe, and nutritious, although it was not always so. Frequently they have as much flavor and texture as is possible under the circumstances. For the circumstance is that they must be heated. The amount of heat required varies with the following factors.

1. The acidity of food—the more acid, the less heat. Foods with a *p*H of less than 4.5—acid foods like cherries—require perhaps 3–5 minutes at 100°C (212°F); semiacid foods with *p*H 4.5–6—beans, pumpkins—may require 30 minutes at 104.4°C (220°F); nonacid foods with *p*H 6–7—peas, corn, meat—may require 90 minutes at 115.5°C (240°F).

2. The kind and number of organisms present. The more organisms, generally the more heat is required. If there are few spore-formers, there is less chance of later spoilage.

3. The nature of the substance and the nature of the container. Basically, this means the shape of the can, the consistency and heat capacity of the product, and how much heat it is capable of withstanding. The shape and size of the can determine how long it takes before the entire contents reach the necessary temperature. The consistency of the food is a similar factor. *All* of the contents of the can, even way down in the center, must be adequately heated and held there sufficiently long that all the organisms are killed. Frequently this must be done without raising the temperature of the outer layers of the can above the point where the product loses too much flavor or texture. Further, if thousands of cans are being autoclaved at a time, each can, whether at the edge or the center of the autoclave, must receive the proper treatment.

The two main objectives of canning are to preserve the food in an edible form, though not necessarily the same form as the fresh food, and to make it safe to eat, particularly to prevent the possibility of botulism (p. 364). On a commercial scale these objectives require a degree of control and an imposition of safety precautions which somewhat complicate the processing. However, these vary so widely with the nature of the food and the type of product being prepared that we shall do no more than to point out that the technology of commercial canning is a rather carefully worked out process based both on laboratory study and large-scale experience.

Home canning resembles that of commercial canning when pressure cookers are used—a common enough practice at present. But what we wish specifically to point out is that in the days before widespread commercial canning and before the common availability of pressure cooking, much food was preserved by canning: corn, beans, apple sauce, pumpkins, meat, cherries, peaches, pears—to name only a few. And this was done without temperatures much over 100°C and without elaborate temperature controls. In general, the product was frequently delicious—or at least the past generation remembers it that way. The thing was done partly by the careful choice of clean materials plus the care of the housewife in cleaning them and in "blanching" or steaming the materials before canning to cut down the load of microorganisms, plus a few odd tricks mostly developed empirically, but which tended to render the heat treatment more effective. In the case of fruits, usually a sugar solution was used—hence the acidity was combined with increased osmotic pressure. String beans and corn can stand long heating periods without becoming inedible, and the cans (generally glass jars) were immersed in boiling water, sometimes for hours.

Under other circumstances, higher temperatures were resorted to. A common device was to heat the closed jar in an oven. But imagine the hazards involved when a wood-fired oven was filled with glass jars closed tight and heated. That the housewife generally survived and that the food frequently did too is a tribute to household ingenuity. In many cases the food did not survive. All the organisms were not killed and the food spoiled. Generally this was merely economic loss, since the spoilage of the food was obvious, and no one ate the spoiled product. But on occasion, particularly in neutral foods like canned string beans, the result was tragedy; organisms then grew, but not enough to change the flavor or odor or texture —yet they produced toxins, sometimes deadly. The worst of these was botulism (p. 364).

PRESERVATION BY DEHYDRATION. This is essentially based upon removing one of the principal factors required for life—namely, water. Drying has been used for centuries as a method of food preservation, but generally the dried food was different food from the fresh, as with prunes, dried apricots, or dried apples. In some cases water could be removed to the extent that the materials left in solution produced a high enough osmotic pressure so the few organisms could grow, as maple syrup from maple sap or molasses from cane juice; but in many other cases the water was brought down to the point where there was not enough to support growth. Water is heavy and bulky, so where either of these factors were of importance in storage, drying was resorted to, largely for the purpose of decreasing bulk, and thus shipping cost, with preservation only an incidental benefit. In the cases of milk and eggs, for example, the "reconstituted" product (with added water) should resemble the original as much as possible; while some of the early products in this field left much to be desired, vast improvemnts in processing and technology have been made.

In some natural foods such as honey, high sugar content and thus high osmotic pressure was the preservative factor. To what extent is salting of meat and hides also primarily an osmotic phenomenon?

PRESERVATIVES. A further method of food preservation involves the use of additives or "preservatives." If these are supplied during the storage period and are removed before the food is consumed, they may be satisfactory. If, however, they are consumed as such, they may be poisonous to man or animal as well as to the bacteria. The older methods used mild antiseptics—sodium benzoate, boric acid, formaldehyde; in fact, the indiscriminate use of such agents and their continual and mostly unsuspected consumption by man constituted a strong force necessitating our pure food laws. Their use, except when specified on the label and under restricted conditions, has been decreased. A new development, however, has taken over; this is the use of antibiotics, especially the broad spectrum types, for the preservation of fish, meats, etc., particularly for foods which are later washed, a process removing the antibiotic, or boiled, a process destroying it.

There are more natural preservatives which have been used for generations, although we do not always recognize them as preservatives. The smoking of meat or the use of spices involves a preservative action, but of course, the nature of the foodstuff is changed. Even more drastic changes occur in the manufacture of some foods, as with sauerkraut. Here cabbage is salted, generally at about 2.5% NaCl, and allowed to ferment anaerobically. Under these circumstances, the salt tends to hold down streptococci and gram-negatives, but permits the natural plant lactobacilli, to grow. These produce lactic acid and the *p*H drops to 3.5–4.0, which kills off the lactobacilli; so long as the sauerkraut is held anaerobically, so molds cannot grow, it may be preserved for long periods. Of course, it has a different flavor than fresh cabbage, but many regard this an improvement, and its nutritive value is not greatly impaired.

OTHER METHODS OF PRESERVING FOOD. Methods of killing the organisms in food by ultraviolet light, supersonic sound, excessive pressure, or radiations of various sorts may have some possibilities but are not at present widely used. Fundamentally any preservative depends upon slowing or stopping growth, as with low temperature; killing off organisms, as with high temperature; holding the food, or altering it, so that organisms which may be present cannot grow, as in drying.

Special Spoilage Problems

The spoilage problems of food are obvious from the method of preservation; whenever these are unsuccessful, spoilage occurs. But there are special problems of particular foods that merit some discussion.

Bread is especially susceptible to mold growth, particularly when the outside humidity is high. Most bread is sprayed on the surface with propionic acid or a similar material which tends to inhibit the mold.

Pickles are made from cucumbers by a fermentation process much like that of sauerkraut. Sometimes a capsulated organism grows, especially if there is access to air, and we obtain a slime, causing "slippery" pickles.

Canned foods are subject to three special kinds of spoilage.

1. In "flat sours" organisms grow, producing acid but no gas; the can does not swell. Frequently there are facultative or anaerobic sporeformers which have not been killed in the heating processes. Special difficulty is caused by thermophiles. The spores of these may have a greater heat resistance but may cause no trouble so long as the storage temperature of the can

is normal. But warehouses, piled high with boxes of cans, exposed to the midsummer sun can get quite warm, which permits the thermophiles to grow and spoils the product.

2. "Bulges" or "swells" are caused similarly by anaerobes (usually thermophiles) which produce a great deal of gas. The can first bulges, and frequently explodes. Generally this is accompanied by a particularly disagreeable odor.

3. "Sulfide stinkers" result from protein decomposition, liberating hydrogen sulfide and other ill-smelling substances inside the can. These are frequently undetected since the can does not smell and shows no outward evidence of decomposition until opened—but then!

The Preparation of Foods by the Use of Microorganisms

The preparation of sauerkraut, pickles, olives, fermented milks, and silage for animal fodder is the result of microbial action on the original material, generally to produce a usually acid product that keeps much better than the original material; hence the action is primarily one of preservation.

There are, however, many instances in which the character of the food itself is dependent on the microorganism. An important instance of this is the preparation of fermented beverages —beer, wines, and whiskey. Since bacteriologists are widely employed in these industries and are expected to know something about them, we shall discuss these materials in more detail than would otherwise be warranted. However, so many of the details of the production of these foods resemble industrial processes, that it is convenient to consider this production as part of industrial microbiology. Hence the information on beer, wines, and distilled liquors will be found in Chapter 22.

Vinegar is traditionally the product of oxidation of alcohol and contains 4% acetic acid. Acetic acid is almost entirely responsible for the sour taste of vinegar; indeed, a slightly sweetened 4% solution of chemically pure acetic acid make a passable substitute for vinegar. The general method of production consists of taking a solution of hard cider or any other nutrient liquid containing alcohol, and allowing it to be oxidized in the presence of *Acetobacter* species, which oxidize with alcohol to acetic acid. Frequently, the solution is trickled through a tank which contains shavings or similar material and air is blown through this column in the opposite direction, the vinegar being collected at the bottom. Vinegar may, of course, be manufactured in the home, but is done on a commercial scale and provided so widely that it is no longer necessary to prepare it domestically.

Sauerkraut. There are many processes in which the action of the microorganisms is desired but a product is not isolated; yet the action is predominantly that of a preservative. Perhaps the most widely used process of this nature is the preparation of sauerkraut. The sauerkraut consists of a fermentation of cabbage by bacteria which are normally present on the cabbage plant. These are generally *Lactobacillus* species, which grow on the cabbage to produce primarily lactic acid. Under commercial conditions pure cultures of lactobacilli are sometimes used; usually, however, the process is allowed to proceed naturally, depending on a heavy inoculum of lactobacilli on the cabbage. Salt is mixed with layers of shredded cabbage; this prevents the growth of many undesirable bacteria and also tends to draw out the juices of the cabbage. Sometimes sauerkraut is prepared merely by cooking the cabbage in the vinegar but this lacks the true rich flavor of fermented sauerkraut. The acid produced is such that eventually it stops the growth of bacteria and other organisms, and the sauerkraut may then be preserved for a long period, so long as it is kept away from air. The acidity of finished sauerkraut is such that most bacteria will not grow, but it is not below that where mold growth is possible; hence, sauerkraut is packed under unaerobic conditions or at least kept away from air so that mold growth will be discouraged.

Silage or ensilage. A similar type of process occurs in the formation of ensilage. Here the product is used for animal fodder. The material —wheat, corn plants, or other material having high sugar or starch—is packed in cylindrical tanks, silos, or other suitable containers and fermentation proceeds, primarily due to the lactic bacteria. Lactic acid is formed and this will preserve the material so long as it is not exposed to the air. It is necessary that the material being fermented have a relatively high carbohydrate content so that acid will be produced relatively rapidly; this will prevent the growth and development of organisms which will attack the proteins and produce undesirable odors and flavor in the ensilage. Alfalfa, for example, and other legumes are generally too high in nitrogenous material so that they cannot be placed in a silo and fermented; instead of an acid fermentation, there would be a proteolytic and putrefactive fermentation. However, it is possible to produce a desirable fodder which is relatively high in nitrogen from alfalfa and other legumes by one of two processes. One is to mix them with suitable quantities of a high carbohydrate for fermentation, such as corn; thus the acid produced from the corn will preserve the alfalfa. The other is to add sufficient molasses to provide carbohydrate or acid produced in the legume silage or to add sulfuric acid itself to adjust the *p*H down to about 4.5–5 so the more valuable and more highly nitrogenous legumes may be preserved in a similar fashion.

Pickles and olives are prepared by a similar kind of process. Pickles are also the result of a lactic acid fermentation. Cucumbers are packed down in 10 to 20% salt solution, although somewhat less (8%) salt for dill pickles, with or without various kinds of flavoring condiments. The organisms are not definitely known but they are probably primarily lactobacilli that are highly salt-tolerant. A high salt concentration keeps out undesirable organisms. The difficulties of decomposition are that there are many salt-tolerant molds and unless the conditions are held anaerobic after storage, these molds tend to grow on the preparations and decompose them. There are also moldlike yeasts which are able to grow under these conditions somewhat anaerobically and if the cucumbers are heavily contaminated with these organisms, one may obtain a slimy pickle due to their growth. In a similar fashion, olives are prepared by an alkaline fermentation.

Fermented milks, which are certainly partially preservative in character, are discussed elsewhere (Chapter 21).

Bread. A further product of action of microorganisms is involved in the production of bread. Unleavened bread consists of wafers, crackers, tortillas, etc., but leavened bread was probably discovered by accident quite early. Primitive leaven was probably a mixture of bacteria and yeast. The purpose of the leaven was to furnish CO_2 or other gases which make the dough light, and a more palatable product results. It is usually best to use pure cultures of yeast but in some kinds of bread, a "starter" is saved from each batch to inoculate the next batch. There are three basic types of rising breads. There is the white or common bread in which yeast is added to a paste of flour and water and salt, with a little sugar often added as the food for the yeast. Otherwise, the yeast must depend on the diastatic action of the flour for soluble sugar. Yeast converts soluble sugar into alcohol and CO_2, which is retained in the dough, causing it to swell or rise; the alcohol is volatilized in the baking process.

Saccharomyces cerevisiae is the common type of bread yeast. When much of the bread was baked in the home, it was very important to

have some starter cultures of yeast available and, under somewhat primitive conditions, one of the things that the pioneers carried with them was yeast cultures for bread making. There are many reports of cases where the yeast cultures died out and the bread was unpalatable; pioneer families might have gone hundreds of miles in order to obtain another yeast for bread starter.

The second type of bread is a sour dough, from which generally a starter is saved to inoculate the next batch. The organisms appear to be *E. coli* and *Aerobacter* species which produce a mixed lactic fermentation, that is, accompanying the gas there is always some lactic acid which tends to make the bread taste sour. The third is salt-rising bread dependent upon the spontaneous fermentation of (probably) wild yeasts and common contaminating bacteria, *E. coli* and *Aerobacter* types. Commercial starters sold at present are clostridia. In this case salt is added to the bread, which cuts down some of the extraneous contamination and simply lets it ferment by itself.

Food Poisoning and Disease Transmission

The third aspect of food bacteriology is the attempt to control and prevent the development of food poisoning and to prevent the spread of disease by foods. The problem of food poisoning is the most important, and so is discussed here. The aspect of transmission of disease—largely tuberculosis, brucella infections, *Salmonella*, and some animal parasites—is merely that food can serve as an agent of transmission. This aspect can be controlled by preventing contact between the diseased individual and food consumed by another; it is discussed later under disease transmission (Chapter 23).

Food Poisoning

While toxicity of foods may be due to the presence of metals (mercury salts, for example) or the accidental incorporation of toxic plants, what is generally meant by "food poisoning" is the toxicity introduced into food by the growth of certain bacteria. The dangerous ones are those which grow in the food, but do not decompose it enough to be especially noticeable. No one will eat spoiled food, although what is spoiled to one person is not necessarily so to another; for example, certain peoples like "spoiled" eggs, such as the quail eggs eaten in China. But if the food is not especially off-flavor or visibly disintegrated, a person may eat it—sometimes merely to be polite. It was earlier supposed that the products of putrefaction were the cause of food poisoning; these were called **ptomaines**, which supposedly gave rise to ptomaine poisoning. It could be demonstrated that extracts of rotted meat injected into animals were toxic, but no one would eat such materials. However, when taken experimentally by mouth, they were not especially toxic, so the food poisoning could not be due to ptomaines. Rather, these effects are due to the growth of bacteria. There are three types of food poisoning:

1. *Staphylococcus* poisoning, in which the victim has nausea, vomiting, sometimes chills, sometimes prostration due to shock. These symptoms appear from 45 minutes to 5 hours after the toxic food has been consumed; the mortality is **nil** and virtually everyone recovers. Some individuals are completely resistant, although resistance to the poison of one area of the country, original or acquired, does not necessarily make one resistant on travel. It is caused by the growth of staphylococci in the food, usually pastry of some sort. Usually there is no change in the appearance and frequently not in the taste of the food. The staphylococci produce an enterotoxin, which means simply that consumption of the bacteria results in toxicity. Large amounts of toxin are required to cause poisoning in most persons, and outbreaks may be single cases or a few cases, even when many people have eaten the food. When a victim develops the symptoms, not much can be done other than supportive therapy. One should generally suspect custard and cream-filled cakes and pastries, sauces, milk, meat to which salt has been added, as in salads or sandwiches, particularly if these have been allowed to stand at room temperature or above for several hours. The toxin is heat-resistant and can be boiled for 10–20 minutes without detoxication, so recooking the food helps little. Bacteriological examination of suspected foods for staphylococci is diagnostic when these are found in large numbers. However, not all staphylococci produce enterotoxin and experimental methods for detecting such toxins are not good. Relatively few of the pathogenic staphylococci possess toxin; see p. 136 for distinction between pathogenic and other forms. Pigmented, coagulase-positive staphylococci are generally involved.

2. *Salmonella* poisoning, the second type of food poisoning, is more serious. It is caused by the actual infection by *Salmonella* species. It generally occurs 4–12 hours after consuming the food, rarely 24 hours later. The general symptoms are the same as in staphylococcus poisoning but with a longer incubation period, but when and if infection becomes generalized the disease resembles typhoid fever, sometimes with a very high temperature. Unless there is a generalized infection, which can be very dangerous, the mortality is low. Complications are appendicitis, cholecystitis, and persistent fever. The susceptibility rate is high when there are adequate numbers of organisms, but people tend to develop a certain immunity to the *Salmonella* species in their own region; they are more likely to succumb while traveling. Cooked or warmed-over foods are suspect; preserved foods are rarely at fault. In most cases there is little overt indication of food spoilage. For diagnosis both the excreta of the patient and the suspected foods are examined for the *Salmonella* organisms. The organisms themselves are gram-negative rods not fermenting lactose. The available facts suggest that *Salmonella* strains may be divided into three large groups: those found only in man, where they are invasive and cause typhoid or paratyphoid fever; those found in both animals and man and external environments, which are not as invasive and cause only food poisoning in man; and those which seem to be associated with specific animals and have little effect in man. The various species (there are over 350) are capable of causing some disease in man and may be separated by **biochemical tests**—cultural tests such as acid and gas from sugars; but

TABLE 20-1. *A Few Salmonella Pathogens*

Species	Pathogenic to Man	Cause Food Poisoning	Pathogenic to Animal	Remarks
S. typhosa *S. paratyphi* *S. schottmulleri*	+	−	−*	Enteric fever, typhoid fever, or paratyphoid fever
S. typhimurium *S. enteritidis* *S. suis*	−	+	+	Natural pathogens to rodents, hogs, etc.
S. pullorum	−	−	+	Disease restricted to chicks

* While they may experimentally cause disease, they are not a natural pathogen to animals.

they are usually identified serologically. The details of the various species are not our concern here, but a few are so common as to require a little attention; they are outlined in Table 20-1.

3. *Botulism.* The third type of food poisoning is caused by a gram-positive, anaerobic, spore-forming rod which belongs to the genus *Clostridium* (p. 445) and one species in particular, *Cl. botulinum.* This organism can grow in foods and there produce a toxin, one of the most, if not *the* most, poisonous material known. It is estimated that one ten-thousandth of a milligram (0.1 μg) will kill a man. The organism may grow in the food without altering its flavor or appearance. The result of consuming such food is generally not noticed for 24 to 48 hours, in which time the toxin penetrates to the nerves. The onset of the disease is accompanied by marked muscular weakness; rarely is there stomach upset, and rarely pain. There is usually loss or disturbance of those functions of the body which are greatly dependent on nerve action: vision, speech, pulse, and temperature. Different strains produce slightly different toxins, Types A, B, and C, and antibodies may be prepared for each; that for A does not work on B or C, etc. Most human outbreaks are type A; B and C are mostly animal diseases. When food containing the toxin is consumed, virtually everyone gets the symptoms and about two-thirds die.

When the disease strikes, the toxin is already in the tissues, although the organism does not grow in tissues. As there are ways of determining the type rapidly, one should supply the specific antitoxin and keep the patient in absolute quiet. One should always suspect preserved foods, meat products, or home canned foods consumed 24 hours earlier, and examine the suspected food for toxin by animal inoculation into mice or other animals, and culture the food for the presence of spores. (How would you do this?) Fortunately, the toxin itself is destroyed by boiling for 12–20 minutes; most food is not actually boiled this long. The organism is fairly widely distributed in nature and is found in many soils. It grows well in slightly alkaline medium with not too much sugar, from which it would produce acid and gas, and is rather proteolytic, so it grows well in materials high in nitrogen. What better place to find anaerobic conditions than in the center of a can of string beans or canned meat? For commercial canning the heat treatment is such as to eliminate the spores of the organism, and a close watch is kept at canneries for any suggestion of botulinic-type decomposition. There have been few if any cases attributable to commercially canned food for the past decade. The main trouble is in home canned food or in food that has become recontaminated and incubated after removal from the can. The organism does not grow in the intestinal tract, nor does it invade the body, so it is only its toxin which must be feared. Other pathogenic members of the genus *Clostridium* cause tetanus (lockjaw) and gas gangrene; these are discussed in Chapter 26.

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QUESTIONS

1. Define or identify:

deep freeze	vinegar
cold storage	sauerkraut
browning	silage
canning	bread starter
blanching	food poisoning
dehydration	ptomaine
preservative	*Staphylococcus* poisoning
slippery pickles	*Salmonella* poisoning
flat sour	botulism
bulger	
sulfide stinker	

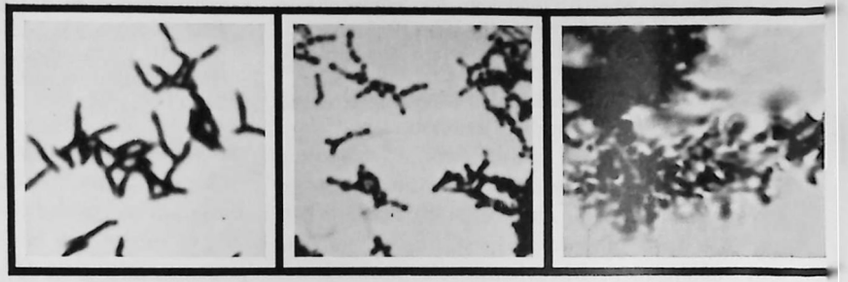
2. In many processes of canning, the food material is subject to blanching before canning, especially if the canning is done commercially. This usually consists of a short steaming or immersion in hot water. From the microbiological point of view, what benefits might be expected from the blanching process?

3. A food product of increasing importance, although it has been a staple for centuries, is fish or other seafoods. What special spoilage problems would you expect to find? What preservation methods would you recommend?

4. How would you demonstrate that the browning of fruit is an enzymatic and not a microbial process?

5. You are the vice-president of the International Wooden Nutmeg Company, and a chemist in your research laboratories finds that by adding 0.2% streptomycin to your "Mother Murphy's Mangrove Pies" their shelf life can be increased for weeks and there is no danger of *Staphylococcus* or *Salmonella* poisoning. Would you recommend that this product be so modified? Why?

Chapter 21



MICROBIOLOGY OF MILK AND ITS PRODUCTS

"I often wish that his phrase, 'applied science,' had never been invented. For it suggests that there is a sort of scientific knowledge of direct practical use, which can be studied apart from another sort of scientific knowledge, which is of no practical utility, and which is termed 'pure science.' But there is no more complete fallacy than this. What people call applied science is nothing but the application of pure science to particular classes of problems. It consists of deductions from those general principles, established by reasoning and observation, which constitute pure science. No one can safely make these deductions until he has a firm grasp of the principles."

HUXLEY, *Science and Culture*

MILK IS AN EXAMPLE of the food which we will examine in detail since it has most of the problems which are connected with other food plus some more peculiar unto itself. Dairying is an important industry, and it is an area in which microbiological control is reasonably far advanced. There is a certain degree of competition between varied forms of life for food. Any food is fair game. The object, then, of dairy microbiology is to prevent the spoilage of milk, letting man get it first, and to produce desirable products from the milk. Another object of dairy microbiology is to prevent the spread of disease.

TABLE 21-1. *Composition of Milk*

Water	87.3%
Butterfat	3.8%
Casein	2.5%
Lactose	5.0%
Albumin and globulin	0.7%
Ash	0.7%

*p*H, 6.7; specific gravity, 1.035

Contamination of Milk

The composition of milk is shown in Table 21-1. It is roughly 87% water, 5% sugar, 4% fat, and 3% protein. It is a good medium for the growth of most microorganisms. Furthermore, it is opaque; we can obtain considerable growth of bacteria in milk without visible change. In addition, the conditions for milk production are such that microorganisms are bound to get into it. Initial aseptic conditions can not be used, but naturally the fewer organisms can get into the milk, the fewer have a chance to grow. There are several principal sources of contamination of milk (Table 21-2): the utensils in which the milk is handled, the coat of the cow, the udder of the cow itself, the air, and the milker. But utensils are by far the largest source of contamination. They may account for as much as 100,000 to a billion organisms per milliliter. The organisms involved are the coli-aerogenes group and the lactic acid type. By utensils are meant the pail, the strainer, and cans, coolers, pipes, bottles, vats, pumps, and all those things which are used for the handling of milk. It is in the cracks and crevices of these, rather than on smooth surfaces, that organisms survive. The utensils must be rigorously cleaned with hot water—steam, if possible—with hypochlorite, lye, or trisodium phosphate, to dissolve the small quantities of protein which may lodge in such cracks and crevices and on which bacteria may grow. The cow's coat contributes relatively less: 10,000 to 100,000 organisms per milliliter—spores, coli-aerogenes bacteria from the manure, and sometimes lactic acid types. These may be held down to a minimum by keeping the cow clean and wiping down the outside of the udder before milking.

The third source of contamination, the udder, may contribute as many as 500 to 1,000 organisms per milliliter. These are generally micrococci. The udder itself has organisms in it. These are, of course, higher when the milk is first drawn than later; discarding the first milk that is drawn from the animal generally will eliminate most of these organisms. Mastitis, which is a disease causing inflammation of the udder, will of course contribute considerable numbers of organisms, sometimes even blood cells, into the milk. If all of the milk is not drawn out of the udder but remains between milkings, it may spoil within the udder itself and thus increase the count tremendously. The air of the barn or the milking shed may contribute as many as 40–50 organisms per milliliter, mostly spores and the coli-aerogenes group. Of course, if this air is dusty or dirty, it

TABLE 21-2. *Sources of Milk Contamination*

Source	Numbers Contributed per ml	*M. tuberculosis*	*Br. abortus*	Pathogenic streptococci	Typhoid	Diphtheria
Utensils	100,000–1,000,000	–	–	+	+	+
Cows coat	10,000–100,000	–	–	–	+	–
Udder	500–1,000	+	+	+	–	–
Air	40–50	–	–	–	–	–
Milker	1–10	–	–	+	+	+

may contribute a bit more. It is very questionable whether the sprays which are sometimes used to cut down air contamination actually do very much good. Probably their principal effect is to reduce the number of flies, but the main point of keeping the conditions clean and sanitary is to not raise dust. In general, unless it is very dirty, the air contributes relatively little.

The milker contributes very few organisms, but these are of considerable importance since they may well be human pathogens. If the milker has a cold or typhoid fever or diphtheria or something of this sort, such pathogenic organisms may enter the milk in this fashion, and the importance of the contamination contributed by the milker is simply that it is the most dangerous.

Preservation of Milk

Milk may be preserved for longer periods of time by the application of four basic processes. The first of these is to cut down the number of organisms as much as possible. The value of any sample of market milk is inversely proportional to the number of organisms that it contains. If in the process of its preparation, these numbers can be held to the lowest possible level, milk will maintain its keeping qualities for a longer period of time. The second factor in the preservation of milk is the time since the milking and the temperature at which the milk is held. The dairyman must get the milk rapidly to as low a temperature as is economically possible and transport the milk to the consumer, either to the individual household or to the manufacturer of a particular product, as soon as possible. After all, the milk when it leaves the animal's body is at body temperature, so it is the initial cooling—both the rapidity and the degree of cooling—which is a very important factor in the quality of milk. Further, on the individual farm cooling facilities are not always adequate.

The third thing that can be done is to remove the organisms from the milk. They can not be filtered out but they can be centrifuged out. There are machines called **clarifiers** through which the milk can be run, in which the organisms are sedimented out.

Pasteurization

The fourth process is to pasteurize the milk. Pasteurization is the process of heating to a temperature considerably below the boiling point with the object of destroying a large percentage of the organisms present, including all pathogenic types. Its main object is to reduce the total numbers by 90 to 95% and thus improve the keeping quality of the milk. Although pasteurization kills all the pathogens, it does not injure the food value, the flavor, or the creaming ability.

When we look at the time temperature curves, such as that shown in Fig. 21-1, we notice that the most resistant pathogenic organism likely to be found in milk is *Mycobacterium tuberculosis*, and that for a heating period of ten minutes, it would require a temperature of 62°C to kill a reasonable population of the tubercle organism. But this degree of heating would also have killed off *S. typhosa*, *C. diphtheriae*, and virtually all of the other pathogens. Heat at slightly more than this temperature for a period of 10 minutes would also destroy the cream line and the enzymes in the milk; still higher heat would destroy the taste. However, by heating precisely at 62°C for 10 minutes, it would be possible to kill the tubercle organism without influencing the creaming properties of the milk, the enzymes which might be present, or its taste.

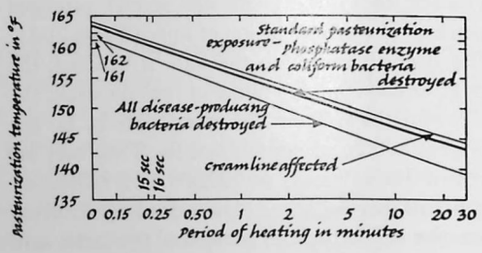


FIG. 21-1. *The relationship between time and temperature and the retention of desirable properties of milk.* [*Redrawn from P. R. Elliker,* Practical Dairy Bacteriology, *McGraw-Hill, New York, 1949.*]

There are two fundamental methods of milk pasteurization. The first of these, **flash pasteurization,** uses heat at a relatively short time at a relatively high temperature. This temperature is considerably far from boiling and amounts to 71.1° to 85°C (160–185°F) for 1–0.2 minutes; that is, heat at 71.1°C for 1 minute or at 85°C for 0.2 minute. In the past, the difficulty has been the rate of heat exchange. All of a large volume of milk must be heated to precisely this temperature for precisely this time and then cooled rapidly. But these difficulties, which were primarily engineering difficulties, have now been solved; the advantage of the flash pasteurization is that a large volume of milk can be handled in a relatively small time and space.

The other type of pasteurization is the **holding process,** which employs heat from 60 to 64.4°C (140–148°F) for 30 to 20 minutes; 142 to 145°F for 30 minutes is the most common compromise. The typical organism requires 142°F for 30 minutes to kill, and 145°F for 6 minutes to kill, so that holding the milk for 30 minutes at these temperatures gives a large margin of safety, without influencing the cream line, the enzymes, or the taste of the milk. However, while such a process is somewhat easier to control than is flash pasteurization, it requires large vats which can be held at these temperatures. This frequently is difficult.

The bacteria which survive pasteurization are of course spore-formers: some of the lactobacilli, the micrococci and the streptococci, and thermophiles. Most of the lactics[1] have been killed off, but some must survive. This is particularly important because milk customarily spoils by turning sour. If it spoils in any other fashion, it is not considered a proper way of spoilage. If the milk spoils because spore-formers grow in it—which do not produce lactic acid but digest the proteins—then the housewife is suspicious that something has gone wrong with the milk supply.

In all of the pasteurization processes, a rapid cooling is needed. Otherwise the thermophiles, which are mostly lactics, will grow too rapidly. It is further necessary to prevent the recontamination of milk by any possible pathogen. And of course some lactics must be present so the milk will decompose by souring. The older objections to pasteurization are no longer pertinent and were never real. The advantages of pasteurization are that it prevents the spread of pathogens, reduces infant mortality, and improves the keeping quality of the milk.

What kinds of organisms grow in milk? There are first the lactics, whose nature we shall describe in a subsequent section (pp. 433). Another type of organism is the coli-aerogenes group, which get into the milk from manure and hay. These organisms form acid and gas from lactose, the principal sugar from milk. When *Escherichia coli* is found in water, it has quite a considerable sanitary significance. But the very conditions under which milk is produced imply that certain quantities of *E. coli* will be present; hence the presence of *E. coli* in milk does not have the sanitary significance that it has when in water. There is an easy trick which may be employed for determining manure contamination. While a certain amount of *E. coli* is bound to be present in the milk, there should not be any great numbers of anaerobic spore-forming bacteria,

[1] By "lactics" we mean organisms producing predominantly lactic acid—especially *Lactobacillus* and *Streptococcus.*

which are present in considerable quantities in the intestinal tract of man and animals. If, therefore, the number of *Clostridium* species is particularly high, it is an index of manure contamination.

Due to the growth of microorganisms present in the milk, a variety of changes may occur on storage. The typical souring due to the lactics with the formation of a solid "hard" curd may be replaced by other changes. Most of the organisms of the coli-aerogenes group form a softer curd and not too much acid; they form gas in milk, but generally the lactic acid formers grow so fast that they form enough acid to prevent the coli-aerogenes group from producing any appreciable quantities of gas in milk. There are in addition both aerobic and anaerobic spore-formers. In the case of the aerobic spore-formers, they grow rather well on lactose but are generally quite proteolytic and if they gain the upper hand they may well cause foul-smelling decomposition and off-flavors. The anaerobic organisms generally produce a **stormy fermentation;** the curd which is formed shrinks and gas bubbles or gas foam is produced above the milk. They generally produce butyric acid and butyl alcohol. However, again, the lactics generally grow so fast and produce so much lactic acid that these organisms are crowded out.

The kinds of organisms obtained are greatly influenced by temperature. At very low temperatures, less than 10°C (less than 50°F), there are mostly the spore-forming proteolytic organisms growing on long storage; under such circumstances clear serum may be obtained, rather than a curd. Storage at slightly higher temperatures, 10–15°C (50–60°F), which are the storage temperatures in most refrigerators, permits primarily the growth of the lactic acid bacteria. Storage at outside room temperatures, 15–35°C (60–95°F), permits rapid growth of the lactics, especially *Streptococcus lactis*. But the treatment between the production and cooling of the milk is very critical for determining how many lactic organisms there may be in the milk with a given degree of contamination. Higher temperatures, body temperatures or above, permit the growth of thermophiles in milk, quite a few of which produce lactic acid. Note in all of these conditions that—except at very low temperatures—the coli-aerogenes groups of organisms or the spore-formers are generally outgrown by the types producing lactic acid. It is only when the lactics are not there that there is an appreciable growth of the others. Milk can be frozen, but it will not yield milk when it has thawed; the physical properties have been changed so that the material is not "reconstituted."

Milk and Disease

Milk can serve as a carrier of a disease. Generally the danger is due to the inoculum, not to the growth in the milk itself. Usually, under modern conditions, the temperature of the milk is held normally at such a low point that the pathogen rarely grows in the milk. There are two kinds of passage which may occur; one is from the animal to man. There are diseases of the animal which also affect man. The origin of these, then, is in the infected animal and not in the milk. There are various examples. One is bovine tuberculosis, a disease of cattle which can also infect man. When man becomes infected with the bovine strains of tuberculosis, he generally has an intestinal or a bone tuberculosis which is not visible on chest X-ray and frequently causes a considerable degree of debilitation. Tuberculosis of cattle can be eliminated as an animal and as a human disease by eliminating tuberculosis in the herds. The animals are tested with tuberculin (see discussion of tuberculosis in Chapter 26); in "TB tested" herds all the animals which have shown a positive tuberculin reaction have been eliminated.

Another disease is undulant fever or Bang's disease, which in cattle is known as contagious abortion. It is caused by an organism of the brucella group (*Brucella abortus*) and is passed from animal to man through the milk (see discussion of undulant fever in Chapter 27).

Sometimes streptococci pathogenic for both animal and man lodge within the udder and contaminate the milk. But all these examples of potential danger to man may be eliminated by pasteurization of milk.

Milk may also serve as the vehicle for disease transmission from man to man. In this case the pathogenic organism comes from the milker. He contaminates the milk and thus infects the individual who may drink the contaminated milk. There are three diseases which may be transmitted in this fashion. These are scarlet fever, diphtheria, and typhoid fever; the last may also come from the wash water and the utensils. These diseases too may be eliminated by milk pasteurization.

Decomposition

One of the curious things about milk is that we expect it to spoil in a particular way; that is, it must turn sour. During pasteurization enough of the lactic organisms are knowingly left so the milk will spoil by lactic acid production. But there are other kinds of milk spoilage that occur on occasion, and when they do they generally cause difficulty considerably out of proportion to their seriousness.

The first of these is **proteolytic decomposition,** which results when aerobic spore-formers grow in milk and the lactics do not grow fast enough to inhibit them. Proteolytic enzymes are formed and these enzymes break down the protein of the milk, resulting in a **serum.** The milk becomes clear and sometimes develops bad odors.

Another kind of decomposition of milk results in ropy milk. In this case, organisms, generally of the *Alcaligenes*[1] genus, grow in the milk and produce heavy capsules, sometimes so heavy that the milk becomes sticky; if poured from a bottle, the entire bottle of milk may come out. If a loop or utensil is dipped into the milk and pulled out, the milk pulls into long strands or ropes. *Alcaligenes viscosum* grows at somewhat colder temperatures than room temperature and it frequently grows during the period between milk delivery and the time the housewife puts it into the refrigerator.

A third kind of milk decomposition is **blue milk,** and results from the growth generally of *Pseudomonas*[1] organisms. They produce a bluish green pigment which tends to give the milk a watery color; the housewife thinks the milk has been diluted with water and is not good milk.

Another kind of decomposition results in **"bloody" milk.** *Serratia marcescens,* a red-pigmented organism, grows frequently in the form of discrete colonies within the milk, causing small lumps which appear very red and bloody. This itself would not be especially serious but it happens that *S. marcescens* is a lipolytic organism; that is, it decomposes fat very readily and causes off-flavors in the milk—to say the least.

There are certain standards by which milk is judged. Certified milk is milk which is collected under special conditions in sterilized containers in a clean dairy; the animals providing the milk are given a health examination at frequent and specified intervals and the milkers and other personnel engaged in preparation of the milk are examined medically at specified and frequent intervals. It may be provided raw or it may be pasteurized. If raw, it must have less than 10,000 organisms per milliliter. If pasteurized, it does not necessarily have any specified count but it must have been prepared from certified

[1] What kinds of organisms are these? See Chapter 7.

raw milk. Obviously preparation of certified milk is a very expensive process and rarely are all of these precautions either necessary or even particularly desirable.

Most of the milk produced in this country is produced under much more economical conditions. There are three principal grades of milk: grades A, B, and C. Grade A raw milk should have less than 50,000 organisms per milliliter, should require longer than eight hours to reduce methylene blue,[1] and should have been produced under certain specified conditions, although these specifications vary somewhat from state to state. When used for the preparation of Grade A pasteurized milk, raw milk should have less than 200,000 organisms per milliliter; after pasteurization the milk should have less than 30,000 organisms per milliliter, and must have been produced under grade A conditions and processed in a plant which conforms to certain sanitation ordinances.

Grade B milk should have less than 1,000,000 organisms per milliliter, require more than 3½ hours to reduce methylene blue, and after pasteurization have less than 50,000 organisms per milliliter.

Grade C milk may have more than 1,000,000 organisms per milliliter, reduce methylene blue in less than 3½ hours, and should not be used for pasteurization.

Most of the milk for human consumption in this country is Grade A milk; Grades B and C are used generally in commercial production for other purposes. A high count does not necessarily mean that the milk is unsafe; that is, that it contains pathogenic organisms; but a high count does mean that decomposition of the milk has progressed considerably.

[1] The methylene blue test is a rough index of the number of organisms in the milk. In the absence of oxygen, methylene blue may be reduced to a colorless material. The more active the organisms present, the sooner this reduction takes place.

Milk Products

Milk is converted into a variety of different products, some of which will be of interest for us to examine. **Evaporated milk** is prepared by evaporating milk under a vacuum to one-half or one-fourth of its original volume, placing this evaporated milk in cans, and sterilizing them in an autoclave. Such milk, of course, may be kept for a long time and may be reconstituted to its original concentration by adding the water that has been removed by evaporation. The milk has a cooked flavor and will never pass for untreated milk, but it nevertheless is a suitable food. So long as it remains in the can there are no problems of bacteriological spoilage.

Condensed milk is milk which has been evaporated under vacuum to one-fourth of its volume and then 40% sugar added. It is canned, but the cans are not sterilized. Yeasts and molds are killed during the process of evaporation. The high sugar concentration prevents bacteria from growing and the can keeps out the air and further contamination. Under these circumstances, the material is preserved for long periods. Milk may also be dried. There are several types of processes for drying milk and the dried powder can be reconstituted to a food material which under many circumstances is very similar to milk. Under dry conditions, so long as there is no moisture, there is little chance of bacterial decomposition.

Ice cream is made by taking milk, adding various ingredients (as shown by the formula in the chart below), and freezing. Here there are two bacteriological problems. First of all, the freezing does not kill too many organisms and if there were pathogenic organisms in the milk, there is a reasonable chance that they will survive in the ice cream. Hence the manufacture of ice cream always starts with pasteurized milk from which any possible pathogens have been eliminated. Second, the freezing process is somewhat hard on the physical structure of the milk and the ice cream itself has a "feel" on the tongue which is quite characteristic. If we start to produce ice cream from milk in which there are already too many bacteria growing, the physical structure of the resulting ice cream may be undesirable.

Ice Cream

MIX COMPOSITION

(Varies according to state regulations, local preference, and cost of ingredients. In New Jersey plain ice cream must contain at least 10% fat, while fruit or nut ice cream must contain at least 8% fat; a gallon of finished ice cream must weigh at least 4.5 pounds.):

Fat	8–14%
Nonfat milk solids	10–22%
Sugar	14–16%
Stabilizer	0.3–0.5%
Emulsifier	0.0–0.1%

SOURCES

Fat: Cream (fresh or frozen), butter, butter oil.

Nonfat milk solids: Condensed whole or skim milk, skim milk powder.

Sugar: Sucrose, other sweeteners such as corn sugar (dextrose), corn syrup, corn syrup solids (dried corn syrup) or invert sugar may substitute for 25–30% of the sucrose.

Stabilizers: Gelatin, sodium alginate, Irish moss, Irish moss extract, sodium carboxymethyl cellulose.

Emulsifier: Mono and diglycerides of C_{14}–C_{18} fatty acids.

A mix of the following composition:

Fat	12.0%
Nonfat milk solids	11.5%
Sugar	15.0%
Gelatin	0.4%

Using 40% fat cream, condensed skim milk (30% nonfat milk solids), 3.5% fat milk, cane sugar, and gelatin, would require:

Cream	27.03 lb.
Milk	33.92 lb.
Condensed skim milk	23.65 lb.
Sugar	15.00 lb.
Gelatin	0.40 lb.
	100.00 lb.

The mix is pasteurized, homogenized, and then cooled. Flavoring is added shortly before freezing. In the freezer, air is incorporated into the ice cream so that an increase in volume of 60–100% results.

Butter is made from churning cream. In the past, sour cream was used. Butter making was something of a home industry; each farm had a pantry or buttery, in which cream was allowed to sour. The souring caused the fat to coalesce and come to the top, and the sour cream was skimmed off and churned. Today, virtually all butter starts from pasteurized cream. But it is still possible to make a sour-cream butter; in this case, the cream is inoculated with lactic organisms. In the dairy industry such an inoculation is called a "starter"; the cream is allowed to sour and the butter fat is processed in a way very similar to that of the past. Sweet-cream butter may also be prepared if the fat is removed without souring the cream. It has considerably less flavor; in fact, if it has been prepared from pasteurized milk which itself has had little decomposition, the sweet-cream butter has virtually no flavor at all. Under such circumstances the butter may be flavored in either an artificial or a natural manner, depending upon how you look at it. That is, you may grow the flavor-forming organisms in milk or cream; if the churned butter remains in contact with the flavored milk and cream, the butter will absorb the flavor and taste like natural butter. The flavor of butter is due to a large extent to the presence of diacetyl (see p. 174). Butter may be kept for long periods by low storage temperature and by salting the butter—unsalted butter decomposes much more rapidly; since there is little carbohydrate in the butter, it keeps for long periods. **Buttermilk** used to be the liquid that was left after the cream had curdled and was churned. The fat was taken off as butter and the residue became the buttermilk. This contained the ash, the lactic, etc., from the cream. With sweet-cream butter, a sweet buttermilk was obtained. Today we use largely **cultured buttermilks**, which generally have little to do with butter

TABLE 21-3. *Types of Fermented Milk*

Name	Raw Material	Conditions of Production	Microorganism Involved
Cultured buttermilk	Pasteurized Skim milk	Held at 20°C (U.S.A.)	*S. lactis, S. cremoris,* or butter starter cultures
Bulgarian buttermilk	Pasteurized Skim milk	Held at 37°C (Balkans, U.S.A.)	*L. bulgaricus*
Acidophilus milk	Pasteurized Skim milk	Held at 37°C (U.S.A.)	*L. acidophilus*
Kefir	Whole milk; cow, goat, sheep	Originally fermented in leather bags, temperatures probably about 20°C (Balkans)	Kefir "grains," small curds containing lactobacilli, streptococci, lactose fermenting yeast. May have 1% alcohol
Yoghurt (leben, dadhi)	Whole milk; cow, goat, sheep	Milk heated to 42–45°C and fermented hot (Russia, Turkey, India)	Dried yoghurt. Organism *S. Thermophilus* and *L. bulgaricus*. Some claim special flora
Kumiss	Mare's milk	Simply inoculate with previous batch. Incubate from 12 hours to several days (Russian Nomads)	Mixed flora: *S. lactis, L. bulgaricus,* and yeast. Old Kumiss may have 2% alcohol. Sometimes distilled to form an intoxicating drink
Taette	Fresh milk	Inoculate from previous batch (Scandinavia)	Variety of *S. lactis;* somewhat stringy and highly acid

itself. Frequently, they are even prepared from skimmed milk. There are two principal types. There is the so-called **Bulgarian buttermilk** or **Acidophilus buttermilk.** The origin of these milks goes back to the microbiologist Metchnikoff, who dreaded the idea of dying. In the course of his vast readings he had run across the reports of two old ladies who had become so old that they felt no more desire to live. This is the way one should die, thought Metchnikoff. The thing to do is to find a way to live long enough so that you are ready to die. He interviewed many old people in old people's homes and traveled all over France, interviewing people of advanced years. Finally he decided that the oldest people in the world were the peasants in Bulgaria and that they lived to a ripe old age because they drank a particular kind of buttermilk.[1] So he set out to culture the organisms which were present in this buttermilk and raised such a fuss, indeed, that many people began to drink Bulgarian buttermilk. For nearly 20 years, he lived austerely. He did not drink alcoholic drinks, he did not smoke. He drank essentially nothing but Bulgarian buttermilk. He died at the age of 71.

The basis of Metchnikoff's theory was that when we eat protein foods, we become intoxicated because of the protein breakdown products, alleged to be poisonous. Therefore the object would be to prevent the breakdown of proteins in a particular fashion in the intestinal tract. By drinking vast quantities of buttermilk, he felt that one could introduce the *Lactobacillus bulgaricus,* which he had isolated from the Bulgarian buttermilk, as a permanent inhabitant of the intestine; this would prevent decomposition of proteins, and thus one might live for long periods. It was found later that *L. bulgaricus* does not survive for very long in the intestine, but *L. acidophilus* does, and it too makes an excellent tasting buttermilk. As a matter of fact, the use of buttermilks of one

[1] It never occurred to him that this was perhaps all that they had to drink.

sort or another is partly based on custom, but does have undoubted beneficial effects in cases of certain diseases. In many parts of the world there have developed particular kinds of fermented milk drinks such as kefir, kumiss, yoghurt, etc., each of which has particular kinds of properties, as shown in Table 21-3.

Cheese. A large part of the milk produced is used in the manufacture of cheese. Cheese is the partially degraded concentrate of milk fat and casein, with a small amount of milk fluid. The concentration of the fat and casein is effected by the curdling of the milk, which may be done in various fashions: by acid; by treatment with **rennet,** an enzyme from the stomach of calves which causes a curdling of milk; by acid and rennet, which is actually the most common method of curdling of milk; and by the use of acid, rennet, and heat. Having been prepared, the curd must shrink and expel moisture. Sometimes this process of moisture removal is aided by pressing the curd. By a combination of these processes, we may make cheeses of varying density, of varying texture, and of varying flavor. Starting with a pail of milk and rennet and salt, we can obtain over 400 varieties of cheeses without adding anything further, simply by altering the conditions under which the cheese-making process is carried out.

We may divide cheeses into two large groups, the acid-curd cheeses and the rennet-curd cheeses. The acid-curd is represented best by cottage cheese. The rennet-curd cheeses may be divided into two further groups: the soft rennet-curds, and the hard rennet-curds. The soft curds have generally poor keeping qualities and a short ripening period. Those which are prepared by bacterial action we know as Limburger and Liederkranz and their varieties; those prepared by the action of molds we know as Camembert and Brie.

The hard rennet-curd cheeses have a long ripening period, generally with good keeping qualities; in fact, they may be preserved for years. Bacteria are primarily involved in the curing processes of cheddar cheese. If the curdling and the treatment with rennet is carried out under high temperature, we get Swiss cheese. If the ripening process is carried out primarily by molds, we get cheeses like Roquefort, Iowa blue, or Danish cheeses.

It is of interest to provide brief descriptions of the main characteristic cheeses. First, an acid-curd cheese—cottage cheese. To prepare cottage cheese sour the milk, allowing *Streptococcus lactis* or lactobacilli to grow in it until they produce sufficient acid to allow the milk to curdle; drain off the whey, add salt to the curd, and the cheese is ready for consumption. The differing flavors of cottage cheese depend to a considerable extent on the organism that did the souring. In practice, you can control the flavor by pasteurizing the milk and then inoculating it with the organism you desire. There is no curing process, of course, since there is rapid spoilage. Molds particularly can grow on cottage cheese very rapidly; hence, it is packed in closed containers, the absence of air preventing mold growth, and is stored in the refrigerator.

Cream cheese is a combination of rennet and acid curd. Instead of relying solely upon acid to precipitate the casein, you help it along a little by the addition of rennet. This cheese has the advantage of being smoother and more easily spread upon bread, crackers, etc.; thus it is preferred over the more lumpy acid-curd cheeses. Otherwise it has essentially the same properties as cottage cheese.

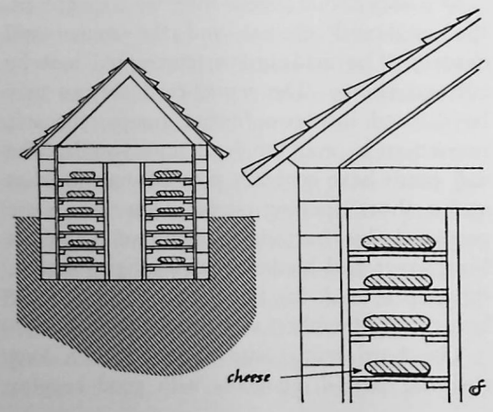
The soft rennet-curd cheeses are prepared basically in the following fashion. Add rennet to fresh milk to coagulate the milk. The whey is left in; there is relatively little pressing of the cheese. You make small cheeses—perhaps six inches square and two to three inches deep —and under these circumstances you obtain aerobic growth on the surface of the cheese.

The acid which was left in the curd is destroyed by the growth and putrefaction sets in; that is, the proteins are broken down to amino acids, and the amino acids to other degradation products. There are two main types of such soft curd cheeses: the bacterial types such as Limburger and Liederkranz, and the mold-ripened types such as Camembert and Brie. In Limburger relatively little salt is added to the curd and the cheese is handled frequently so that bacterial growth will occur on the surface. These bacteria destroy the acid and permit the partial putrefaction and decomposition of casein, giving the characteristic flavor of Limburger. Liederkranz is very similar, except that considerable salt is added to the curd. This promotes a different flora of organisms on the surface of the cheese, and thus a different flavor is obtained—in Camembert, particularly *Oidium* and a white *Penicillium* growth on the surface. In Brie a similar mold type is responsible. In all of these cases some fat is decomposed as well, particularly in the mold-ripened cheeses, and this too contributes to the flavor.

The hard rennet-curd cheeses are probably more familiar to most people. American cheese (cheddar cheese) can be made in any size you wish—as large as a house or as small as a baseball; the usual size about 150 pounds. The best type of cheddar cheese should be free of holes, although in the market you usually find some holes in it. For the preparation of cheddar cheese, you precipitate the curd, after acidification, with rennet. The whey is drained off the curd, and some of the acid is retained in the cheese and prevents putrefaction. The curd is salted and then the excess whey is pressed out of it by pressing the curd in a cheese press. The cheese is stored and gradually develops a flavor due to slow bacterial action. It is stable, lasts a long time; it can be melted and blended. When it has been so melted and blended and reconstituted, it is called **processed cheese.** Frequently it will be melted and poured into molds. Its flavor depends somewhat on its age. The older the cheese, the greater the degree of decomposition of proteins to amino acids, and the sharper the flavor. The amino acids themselves tend to have a sharp and not unpleasant flavor and, as the cheese ages, proteins gradually break down to form these flavor-contributing elements.

For the preparation of Swiss cheese, you take fresh sweet milk and heat it in a large kettle to 130°F (56°C). This is, you will recall, almost pasteurizing it. At this temperature you add rennet and a "starter" culture containing thermophilic organisms (*Streptococcus thermophilus*), some thermophilic lactobacilli, and some propionic acid bacteria. It is the latter bacteria which gives Swiss cheese its characteristic flavor and the gas produced causes holes. At this higher temperature you allow the starter culture to act for a period at this comparatively high temperature. After a suitable time rennet is added; a very fine curd is formed, which is cut while it is still hot with a cheese rake into very fine particles. These particles are then fished out of the hot tank in a cheesecloth net, which is then put into a mold or a press. About

FIG. 21-2. *A typical cheese house for the ripening of Swiss cheese.*



200 to 300 pounds of curd are used to make a single cheese. The pressed curd, after the whey has drained off it, is placed in a brine bath to harden the surface, and then it is placed on shelves in a cheese house (Fig. 21-2). The shelves in this house which contain the cheeses are at different temperatures, those near the top of the building being warm, and those near the bottom of the building being considerably cooler. The cheese maker decides whether the cheese is ripening fast enough or not by thumping with his thumb on the outside of the cheese and listening to the sound. If it is not ripening fast enough, he will raise it a shelf higher so that the process will go faster. Each time the cheese is handled, and it is handled essentially daily, the outside of the cheese is salted. The cheese maker must judge the rate of penetration of the salt into the cheese and the rate at which the bacteria are working inside the cheese so he will obtain the proper distribution of holes within the cheese for good quality. This quality is reflected in the final flavor of cheese, but actually Swiss cheese may be graded on the number of holes, their size, and whether or not the inside is shiny.

Roquefort cheese is a mold-ripened, hard-curd rennet cheese; the mold involved is *Penicillium roquefortii*. Theoretically it is made only from goat's milk, but actually other milk is frequently used in the preparation. Its flavor is due largely to the decomposition of fat in the cheese.

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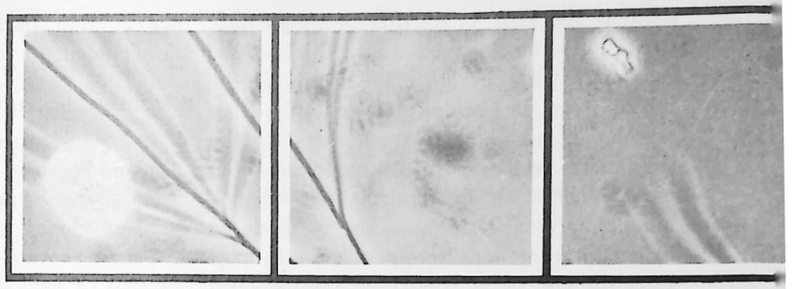
QUESTIONS

1. Define or identify:

market milk
clarifiers
pasteurization
creaming properties
flash pasteurization
hold pasteurization
curd
stormy fermentation
milk serum
ropy milk
blue milk
butter
buttermilk
cultured buttermilk
acidophilus milk
kefir
koumiss
yogurt
cheese
rennet
cottage cheese
cream cheese
bloody milk
certified milk
grades A, B, C
evaporated milk
condensed milk
dried milk
ice cream
cheddar
Limburger
Liederkranz
Camembert
Brie
Swiss cheese
Roquefort

2. You are the manager of a dairy plant producing a pasteurized certified grade A milk. You are having difficulty in meeting the minimum standards for the raw milk entering your plant. How would you correct this difficulty?

3. A new public health officer, perhaps inadequately trained, condemns the milk from your pasteurization plant because he has found *E. coli* present. You, on the other hand, are convinced that your milk is safe and properly pasteurized. How would you find out which of you is correct? How would the situation differ if the sample containing *E. coli* were taken before pasteurization rather than after pasteurization?
4. The local pasteurization plant has 20,000 gallons of milk which tests out as grade C and has too much bacterial growth to risk putting it through their flash pasteurization process. (Why can't they?). You operate a spray-drying plant but your drying equipment is under contract use for the next two days. You are offered the milk at a very attractive price. What could you do to preserve it until it can be dried?
5. The cheese industry has operated for centuries without scientific microbiological control. You have been trained as a microbiologist. What advantages might such a training be in improving cheese production and quality?



Chapter 22

INDUSTRIAL MICROBIOLOGY

"Myself when young did eagerly frequent
Doctor and Saint, and heard great argument
About it and about: but even more
Came out by the same door where in I went,"

"And much as wine has played the infidel
And robbed me of my Robe of Honor—well,
I often wonder what the vintners buy
One half so precious as the stuff they sell."

OMAR KHAYYAM, *The Rubaiyat*

DIRECTING THE ACTIVITIES of certain microorganisms so that they may produce products useful to man is as old as antiquity. Beer, wine, the fermented milk, the leavened bread go back to antiquity. But modern microbiology has been able to control microbial activity to such an extent that microorganisms may be used for the direct production of pure chemical substances. It is here that real "industrial" application, in the sense of factory production of a substance, is most evident. But there is a wide range of other applications which have commercial application, and we shall undertake to discuss some of these as well.

Product of Microbial Action Isolated

There are two kinds of substances which are produced by microbial action, isolated, and known to commerce as pure chemicals. One of

these is the bulk production of simple substances, ethanol, for example. In this case the economics are the controlling factor. If the ethanol can be made more cheaply from petroleum, it will be so made. Most of the substances which were once produced as organic chemicals by fermentation—ethanol, butanol, acetone—are now largely produced by chemical methods from petroleum. In most cases the cost of isolation from the microbial processes overbalances the relative economy of raw material involved in the microbial process. Those cases where the gross chemical production is still by fermentation are either those in which the product is not isolated, such as ethanol in beer, where the product is much more difficult to produce chemically or where the product is exceedingly simple and economical to isolate.

The other phase of the production of pure chemicals of commerce by microbial action is that in which the substance cannot be produced otherwise, and is so valuable that the cost of isolation can be borne. Examples of this type are the antibiotics and vitamin B_{12}, whose sources today are primarily microbial fermentation. These obviously are mostly "fine" chemicals, those sold in quantities of grams or milligrams, compared to "bulk chemicals," those sold in terms of pounds, tons, or carloads.

Bulk Chemicals Produced by Microbial Action

Alcohol, Ethanol

A typical process is shown in Fig. 22-1, the proportions and sizes being approximately those required to produce 10,000 gallons of alcohol. The kind of yeast employed is of considerable importance, and mutant strains able to produce higher yields of alcohol are employed. The fermentation system is not aseptic but the low *p*H, lack of oxygen, relatively high sugar, and the time essentially restrict microbial development to yeasts.

Acetone, Butanol

In the early part of World War I, when the airplane began to be used, large quantities of acetone were employed in the formation of airplane "dope," the varnish used to strengthen the canvas covers of the wings, and in the production of explosives. Hence during the war there was a tremendous demand for acetone, far beyond what could be produced by the previous processes of distillation of wood. About this time it was discovered by several people, including a man by the name of Weizmann, that it was possible to prepare acetone by the fermentation of grain, using a special organism, *Clostridium acetobutylicum*. On setting up this kind of a process, the needs for acetone were solved; as part reward for its development, Weizmann, a Zionist, obtained a grant from the British Government which enabled him to set up the state of Israel, and he was its first president. Of course, there were many other complicated factors in the development of this state, but certainly the discovery of the acetone-butanol fermentation played an important role in the development of Israel.

During World War I what was wanted was the acetone; the butyl alcohol was an undesirable by-product. After the war, much effort was spent in trying to develop a use for the large quantities of butyl alcohol which had accumulated. But just about this time the automobile industry began to expand. Up until this point automobiles had been painted with slow-drying oil paint, but it took so long for the paint to dry that it was seriously limiting the production of autos. Acres and acres of dust-free area were required for simply allowing the paint to dry. Hence it was imperative to develop quick-drying paints and lacquers, and when this was done it was found that the primary ingredient was butyl alcohol; hence there was a further

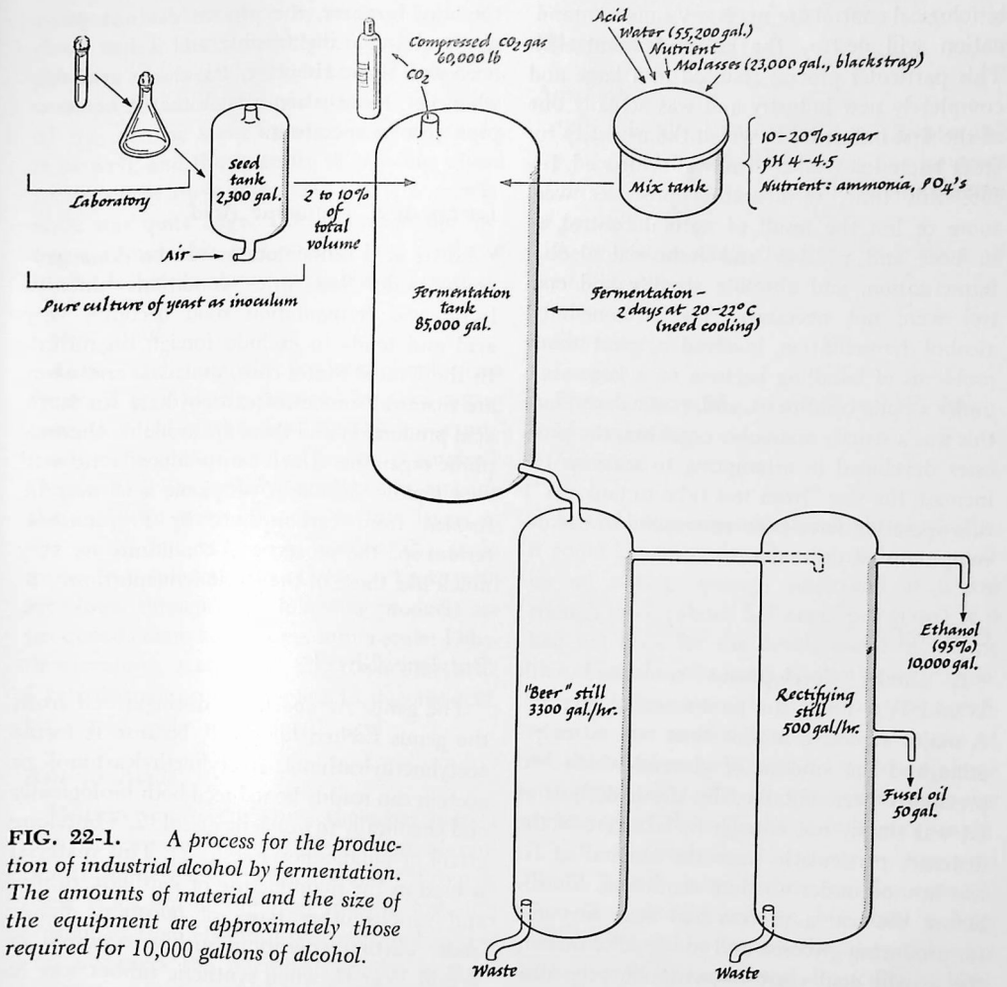


FIG. 22-1. *A process for the production of industrial alcohol by fermentation. The amounts of material and the size of the equipment are approximately those required for 10,000 gallons of alcohol.*

demand for large quantities of butyl alcohol and this time the acetone was the undesirable by-product.

Both corn and molasses are used in commercial production, the starch of the corn being hydrolyzed to sugar by diastatic enzymes in the acetone-butyl alcohol organism (originally called B.Y.), *Clostridium acetobutylicum*. The process is completely anaerobic, takes from 48 to 72 hours, and the average yield from 100 pounds of corn is 8 pounds of acetone, 16 pounds of butanol, 3 pounds of ethyl alcohol, 20 pounds of hydrogen, and 20 pounds of carbon dioxide.

The fermented mash thus contains about 2% solvent and is concentrated to about 50% solvent in a continuous still. It is then separated into its components by further fractional distillation and shipped in drums and tank cars. Throughout the process, mechanical and bac-

teriological control are necessary since contamination will destroy the entire fermentation. This particular process resulted in a large and completely new industry and was actually one of the first industries in which the necessity for strict bacteriological control was evidenced. Before this time, fermentation processes were more or less the result of natural control, as in beer and whiskey and industrial alcohol fermentation, and absolute sterility and control were not necessary. The acetone-butyl alcohol fermentation involved a great many problems of handling bacteria on a large scale under aseptic conditions, and, particularly since this was a strictly anaerobic organism, the processes developed in attempting to scale-up (= increase the size "from test tube to tank car") this operation have been very useful in the development of other fermentations.

Glycerol

A similar development connected with World War I was the production of glycerol. A major explosive at this time was nitroglycerine and the amount of glycerol which had previously been obtained by the hydrolysis of fat was simply not enough to take care of the demand, particularly since the amount of fat was limited under wartime conditions. Shortly before the war a process had been invented for producing glycerol, and consisted of making the usually acid yeast fermentation very alkaline. A similar result is obtained by adding sodium sulfite. Under these circumstances the acetaldehyde which would normally be an intermediate in the formation of ethyl alcohol is tied up. One of the intermediates in the fermentation (dihydroxy acetone phosphate) is reduced to glycerol phosphate, from which glycerol is formed. The yields of this process were sufficient to make it commercially feasible and during the war Germany made a considerable amount of glycerol in this fashion. After the war, however, the process did not prove to be economically feasible and it has rarely been used since. However, it is always available whenever the situation is such that it becomes profitable to operate it.

Lactic Acid, Propionic Acid

Lactic acid fermentations can be done generally under less strict conditions since a lactic acid fermentation soon becomes very acid and tends to exclude foreign organisms. In the United States corn, molasses, and whey are practical sources of carbohydrate for lactic acid production and there are available thermophilic organisms which can produce lactic acid very rapidly. Similarly, propionic acid may be formed from carbohydrate by *Propionibacterium* sp.; the process and conditions are very much like those of the lactic fermentation.

Butylene Glycol

The genus *Aerobacter* is distinguished from the genus *Escherichia* partly because it forms acetylmethylcarbinol. Acetylmethylcarbinol or acetoin can readily be reduced both biologically and chemically to butylene glycol (2, 3-butylene glycol or butane-diol, Fig. 22-2). This material is used in the manufacture of synthetic rubber and various other types of polymers; hence under wartime conditions such as occurred in World War II, when synthetic rubber was in great demand, there was a tremendous effort

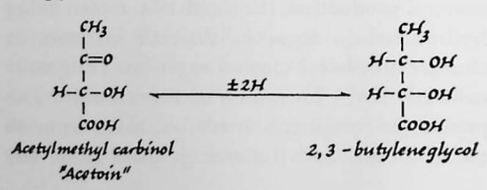


FIG. 22-2. *Acetylmethylcarbinol and butylene glycol.*

to obtain butylene glycol by bacterial fermentation. It was discovered that not only is the property of acetylmethylcarbinol formation widespread among the *Aerobacter*, but many of the aerobic spore-forming bacteria possess it as well, and the quantity of butylene glycol produced by these organisms on a commercial scale was quite large. The process at the moment is not able to compete with synthetic methods but is always available if necessary.

Citric Acid

Citric acid, originally produced from citrus fruits, is now largely prepared by microorganisms. A mold of the *Asp. niger* group is normally used and an aerobic process is required. Originally it was probably produced by growing the mold on sugar solutions in thin layers in flat pans, but in today's process the mold grows in a submerged condition in tanks with air blown through. The following products are produced commercially on a minor scale: Dihydroxiacetone, acetic acid, 2-ketogluconic acid, 5-ketogluconic acid, gluconic acid, itaconic acid, kojic acid, gallic acid, fumaric acid.

Amino Acids

Within very recent years, there has been a new development of the production of particular amino acids by fermentations. Of course, these acids can be obtained from hydrolyzing protein or from chemical synthesis, but in several instances the microbial process is more economical and yields a better product. Especially are glutamic acid, lysine, ornithine, valine, tryptophene, and alanine produced in this fashion. These processes are based upon finding bacteria which form relatively large quantities of the specific amino acid in its culture fluid. For example, an organism called *Micrococcus glutamicus*, growing in a glucose-mineral salts medium with a little peptone and yeast extract, produced 15 g of lysine HCl per liter in 72 hours at 28°C.

Fine Chemicals Produced by Microbial Action

Antibiotics

Virtually all the antibiotics (see Chapter 14) are produced by microorganisms. For purposes of brevity we shall describe only the production of penicillin.

A typical medium for the production of penicillin is

Corn steep liquor	3%
Lactose	5%
Phenylacetic acid	0.05 to 0.1% daily
Mineral salts	
Antifoam	

As we have mentioned, it is doubtful whether it could have been possible to produce penicillin on a large enough scale and at a low enough price (about 6–7 cents per gram) if it had not been for the development of aseptic "know how" in the butyl alcohol fermentation. But penicillin not only needed aseptic conditions, it also needed tremendous amounts of air; there had to be provision for blowing large quantities of *sterile* air (and provisions for sterilizing it) through the fermentation tanks. Initially, the organism was grown in large petri dishes, then in large bottles (on the surface), then in shaking machines, but until a large fermenter could be devised, the labor costs were so high as to be impractical. Penicillin in 1943, when it first came on the open market, was fifteen dollars per 100,000 units packaged in vials; in 1960 the same material was 7.5 cents; in bulk it was only 3 cents.

It was early found that penicillin was best produced from lactose and that corn steep liquor, originally a waste product in starch manufacture but later made specifically for penicillin production, contained something which stimulated penicillin formation. These materials are still used in commercial produc-

tion. Phenylacetic acid (comprising the R group of penicillin G; see Chapter 14, Fig. 14-2) is added. Since there is vigorous aeration there is a need to cut down foaming. The course of an industrial fermentation is shown in Fig. 22-3.

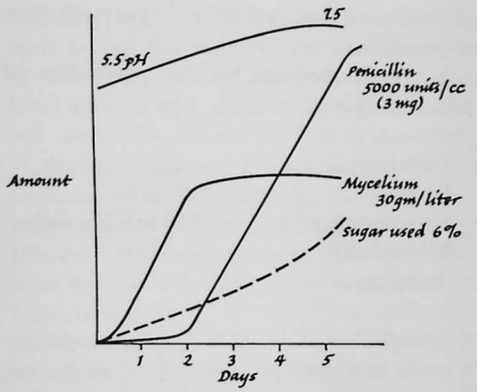


FIG. 22-3. *The course of an industrial penicillin fermentation.*

Notice that the penicillin begins to form just after the growth of the mycelium ceases, and thereafter continues to rather high levels. These amounts, 3 mg/ml penicillin produced after 6 days, may seem fantastic when compared with the early yields, but they are the result of careful strain selection, media selection, and control of operating conditions.

The mold, under the conditions of a large fermentation tank, is not the large green mass you see on a culture but rather exists on small pellets of mycelium. It is filtered off and the filtrate is treated with charcoal or other absorbents, to which the penicillin is absorbed. The washed charcoal is treated with acetone to elute the penicillin and several further chemical steps of purification are involved. An alternative and more common process is to extract the penicillin from the fermentation broth with amylacetate or butyl acetate, purify with countercurrent extraction, and crystallize the penicillin from the solvent.

Vitamins

Two vitamins are at present produced by microbial action under industrially controlled conditions, namely **riboflavin** and **vitamin B_{12}**. In the case of riboflavin (vitamin B_2, vitamin G), which can also be produced synthetically, the microbial production depends on yeastlike organisms producing quite remarkable quantities of riboflavin from raw materials of relatively low cost, i.e., whey. In the case of vitamin B_{12} or its relatives, the cobamides, fermentations are the principal source of it.

Giberellins

These are substances with a closely related and complex structure that cause the growth stimulation of certain plants. They are produced by certain fungi and are used in gardening and horticulture.

Other Chemical Uses of Microbial Action

In all of the foregoing, the microorganisms produced the bulk chemical or the fine chemical by starting with its food material, generally carbohydrate. But there is another use of microorganisms in the production of the chemicals of commerce, in which the organism accomplishes just one step. Generally, a specific substance is added to a growing or to a grown culture, the microorganism acts upon it in a restricted and characteristic manner, and the transformed substance is isolated. We cite two examples.

Ephedrine Manufacture

In this process, shown in Fig. 22-4, the step using benzaldehyde and adding a two carbon unit to it is difficult to do chemically; hence the yeast is employed to carry out this step and the product is then isolated and converted by chemical synthesis to ephedrine.

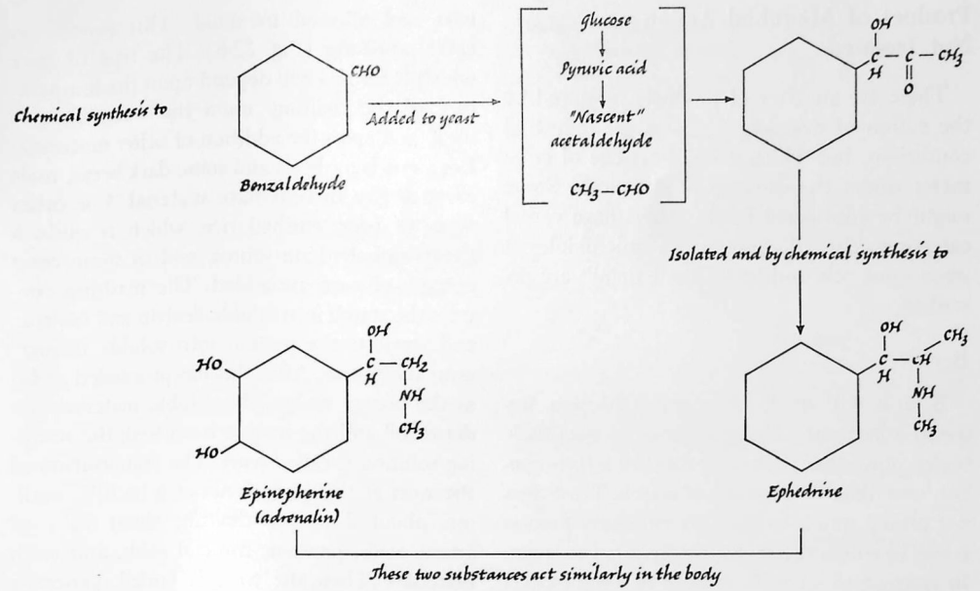
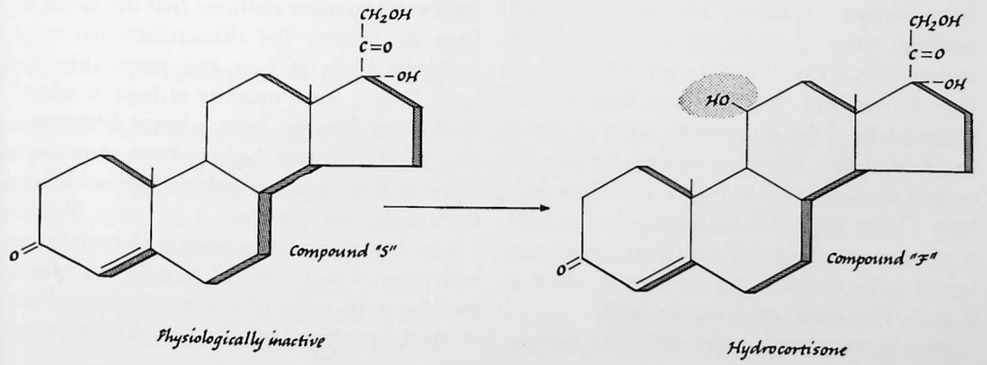


FIG. 22-4. *The use of a fermentation step in the manufacture of ephedrine.*

Steroid Transformations

This process is illustrated in Fig. 22-5. Here a steroid found widely in nature but with no hormone activity, called "compound S," can be converted by microorganisms—for example, *Cunninghanella blakesleeana*, *Streptomyces fradiae*, or some *Pseudomonas*—to hydrocortisone, an active steroid hormone of the body. It is most difficult chemically to introduce a hydroxyl group at the position shown in Fig. 22-5, and an oxygen or hydroxyl is needed here for hormone activity. Thus this is a commercial process whereby a less valuable steroid is converted to one of great value.

FIG. 22-5. *The transformation of steroids into active hormones by microbial action.*



Product of Microbial Action Not Isolated

There are an array of products produced by the action of microorganisms under industrial conditions, but which are not articles of commerce under the category of chemicals. Some might be considered foods, others have varied categories. In all, however, a microbiologist plays some role and hence some details are described.

Beer

Beer is the result of a grain infusion fermented by yeast. The grain used is essentially barley plus other materials, most of which contain considerable amounts of starch. Yeast does not attack starch so the first necessary process is one in which the starch is converted to sugar. In contrast to scientific control in some aspects of the fermentation industry, the producer of beverages still depends to a great extent upon those "skilled in the art." The traditional Braumeister contributes practical knowledge to the process and under present circumstances we do not yet know how to replace his services in a scientific fashion. The main problem at the start is to convert the starch to glucose, and this is done from barley. Barley itself does not have any great amylase activity, but when it is sprouted the barley seed starts to form digesting enzymes which contain amylases, proteolytic enzymes, and others. This process is called **malting.** After sprouting the barley is heated and dried in a kiln to kill the plant but still retain the enzymes that had been formed. The temperature of the kiln, usually 120°F, is raised to 145°F if the malt is to be used for pale ale or light beer, or raised as high as 220°F for dark beer. Under the latter circumstances the malt is partly carmelized and contributes a characteristic color and flavor to the beer in which it is used. The dried powder is the **malt.**

Malt is mixed with water and other ingredients and allowed to stand. This process is called **mashing** (Fig. 22-6). The type of beer which is formed will depend upon the temperature of the malting, upon the water that is used, and upon the addition of other materials. For some lager beers and some dark beers, malt alone is the carbohydrate material. For other types of beer, crushed rice, which is quite a cheap carbohydrate source, and in some cases extracts of corn are added. The mashing converts the starch into soluble dextrin and maltose and converts the protein into soluble nitrogenous substances. After this has proceeded as far as the brewer wishes, the soluble materials are drawn off and the residue is washed; the resulting solution is called **wort.** The composition of the wort at this stage is about 6 to 10% maltose, about 2 to 5% dextrin, about ½% of amino acids, peptone, mineral acids, and color materials. Then the wort is boiled, generally with hops, in a steam kettle. The boiling operation sterilizes the wort, stops any further diastatic action, extracts the hops, and precipitates easily precipitated protein. It finally concentrates the wort to any desired strength.

Hops are a kind of a plant which is grown widely in Europe. From hops an extract can be prepared which contains oil, alkaloid, resins, and a bitter principle. The hop extract is what gives the bitter flavor to many beers. Originally it was used as a preservative in beer; this is no longer necessary but beer has been made in this fashion for so many centuries that the flavor of hops has become the characteristic flavor of particular kinds of beer and people like its taste. Only a small quantity of hops is added to stout or Bavarian lager, whereas Bohemian ale (pilsner) is very high in hops. American lagers tend to have less and less hops added to them as the time goes on.

The boiled wort is cooled and inoculated with yeast. The process of inoculation, called **pitching** with yeast, uses about 10–30 pounds of yeast to a hundred-barrel vat. The type of yeast used depends upon the beer desired. Stout or porter use a "top" yeast that ferments best at between 50 and 75°F and the fermentation generally goes for 3 to 5 days. German and most American lagers use "bottom" yeast that has an optimum temperature of between 40 and 50°F (4–10°C or refrigerator temperatures), and the fermentation goes between 8 to 16 days. During this principal fermentation, most of the soluble sugars are converted into alcohol and CO_2. After the 3 to 5 days for the high-temperature beers and the 8 to 15 days for the low-temperature beers, the beer is run into storage vats and held at almost freezing temperature. Here a slow fermentation occurs which may take 6 weeks to 6 months. The

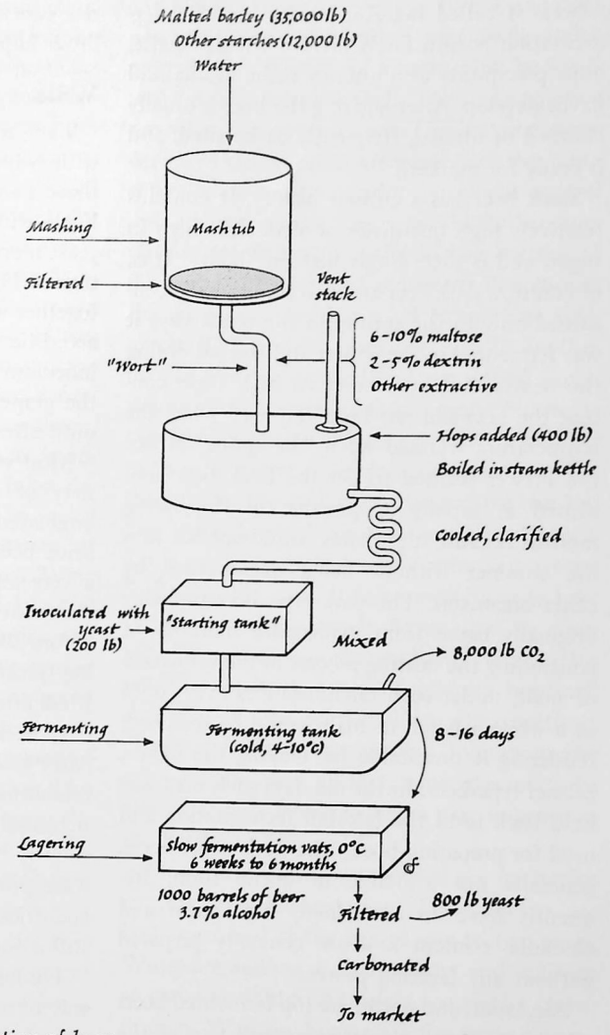


FIG. 22-6. *A process for the production of beer.*

process is called **lagering.** A further slow fermentation occurs, finely divided colloids settle, hops precipitate as a gummy resin, aroma and flavor develop. After lagering the beer is usually clarified or filtered, frequently carbonated, and is ready for market.

Bock beer has a curious history. It contains relatively high quantities of alcohol, is high in sugar, and is exceedingly high in "body." It is, of course, a dark beer and it is traditionally consumed only in the spring. In the olden days it was fermented in the winter and lagered during the winter, but because of its high sugar content the beer did not keep very well when the temperature warmed up in the spring. Hence the brewer wanted to get the bock beer consumed as rapidly as possible in the spring months because it probably would not last into the summer without being decomposed by other organisms. The dark type beer probably originally came from inadequate methods of controlling the heating process in the formation of malt; under such circumstances every once in a while a batch of malt would be scorched, rendering it unsuitable for making the lighter pilsner type beer. In the old days such malt was held back until the late fall fermentation and used for preparing bock. The dark sweet beers, generally not available in bottle form, frequently have an exceedingly high sugar and alcoholic content and are generally prepared without any lagering process.

Ale, stout, and porter are top-fermented beers which now form a relatively small part of the total American beer production, but they have retained much of their popularity in England to the virtual exclusion of lager beer. **Ale** has a pale bright color, a rather bitter pronounced hop flavor, and a more tart taste, with an alcoholic content of 5–6%. **Porter** is a dark brown beverage with a malty flavor and less hops flavor than ale, and an alcoholic content of about 5%. **Stout** possesses a very dark color and a rich sweet malty flavor combined with a strong bitter hops taste.

Wine

The microbiological problems connected with wine making are much simpler than those encountered in the manufacture of beer. Each wine district has a certain species of yeast more or less indigenous to that particular region. These yeasts tend to grow on the grapes together with the grape, give the wine its characteristic flavor, odor, aroma, and taste. The inoculum is usually natural; the yeasts are on the grape and no pasteurization is attempted until after the wine has been made.

Most of the world's wines come from a variety of single-grape species which probably originated around the Caspian Sea and have since been carried to all continents. Wine is a very ancient product and there is evidence that wine was produced in Egypt and in China before 2000 B.C. From Asia Minor wine growing spread with civilization to the shores of the Mediterranean. The Romans are believed to have borrowed wine growing from the Greeks. After Caesar's conquest of Gaul, the Romans established wine production as an important agricultural pursuit in Western Europe. Every country along the Mediterranean produced wine principally for table use. In most Latin countries wine became centuries ago what it still is today, a universal daily beverage.

Throughout the Middle Ages the Church was actually the greatest single initiator in the spread of vineyards. This has resulted today in some of Europe's most famous vineyards, which were established by the religious orders centuries ago.

Fermentation of the grape begins essentially as soon as the skin of the grape is broken. This may be done either by mechanical crushers or by treading in tubs or vats as is done in some of the Old World wine regions. Wine grapes when ripe contain about 12–30% of natural

grape sugar, glucose, 70–85% water, and many kinds of acids, minerals, and other constituents, whose presence in varying quantities produces the variations in body, color, taste, and bouquet of wine. The fermentation is carried out primarily by *Saccharomyces ellipsoidies* or "wine yeasts." These differ from the yeasts that are used in the production of beer and distilled beverages, and particular kinds of yeasts tend to develop in particular regions and on particular grapes, which gives to the finished product a particular and desirable flavor. If beer yeasts, for example, are introduced into a grape mash, the resulting product is much less desirable and frequently has a somewhat beery flavor. If wine yeasts isolated from particular wines are used to ferment pasteurized grape juice, the nature of the product obtained may be varied considerably, depending upon the strain of yeasts employed in the fermentations. Wine makers, therefore, consider it beneficial—and for some operations essential—to employ cultivated strains of wine yeasts that have proved their efficiency. It is today almost a universal practice to inhibit the spoilage bacteria and the wild yeast by adding a small amount of sulfur dioxide to the pressed grape juice. This pressed material, frequently containing the seed of the grapes, is called **must.** The alcholic content resulting from the fermentation is governed by the sugar content of the juice but as a rule normal fermentation produces an alcoholic content of about 14%. The duration of the fermentation varies considerably, from a few days to several months.

After fermentation the "new" wine is drawn into storage containers to begin aging, which may take years, depending on the type and quality of wine. The aging usually starts in large casks or tanks holding as much as 50,000 gallons, generally made of wood, sometimes of concrete lined with a protective coating. The temperature for aging must remain quite uniform, ideally around 55°F for most white wines and 65°F for most red wines. To provide coolness, many old wine cellars were built underground. Newer ones, of course, may be artificially air-conditioned. During the aging period the wine uses some oxygen, undergoing some degree of oxidation as air penetrates the pores of the wood cask or is admitted under control into concrete tanks. The wine slowly becomes clarified by depositing solid in the bottom of the cask in the form of sediment. Periodically the wine must be drawn off from these sediments. The transfer from one cask to another for this purpose is called **racking,** during which some air is incorporated into it again. Normally the wine is drawn off to successively smaller casks as it grows older and more mellow, but the smaller the container, the greater the surface through which air can penetrate. Eventually a point is reached where wine can age no further in the cask. If left longer, it may take on an excessively woody flavor, lose its character, and become overoxidized. When this point is obtained, wine is "right for bottling" and so it is then bottled. Even after bottling, extensive further improvement follows in the case of some wine. In fact, wine appears to be about the only beverage which may improve in the bottle.

Most table wines are bottled six to twelve months after fermentation. The majority are best consumed at the age of one to two years. Wines are endless in their variety and differ in so many attributes as to defy too rigid a classification. We would recognize five main classes: red table wine, white table wine, appetizer wine, dessert wine, and sparkling wine.

The **red table wines** are produced for consumption at meals. They constitute the vast bulk of the world's wines and are usually dry, or low in sugar. They are generally tart and sometimes even astringent in flavor. Their alcoholic content is generally around 10 to 14%. They are roughly grouped under two names,

claret and burgundy, the latter having greater body and flavor and a deeper color.

The **white tables wines** are also designed for consumption at meals. They are generally extremely dry and tart, although some special types may be sweet and mellow with a delicate flavor which blends best at meals. Traditionally, they are served cold. In color they range from pale straw to deep gold and have about the same alcoholic content as the red wines. Most white wines are produced by the fermentation of the grape juice alone, without the grape skin, and have little of the astringency of the red wines which are produced by the fermentation of the juice plus the grapes skins and the seeds. However, some white wines are fermented from grapes which do not possess a red or purple skin and are produced by fermenting white grapes; hence they may also have some of this astringency. The two principal types of wines under this classification are the sauterne and the Rhine wine. Rhine wine is frequently also called hock.

Appetizer wines are usually used before meals. They range from fully dry to semisweet. The main types are sherry and vermouth. Sherry is characterized by a nutty flavor that is usually obtained by the addition of pure grape brandy to the fermented or partly fermented juice in the initial period of aging. Most sherries have an alcoholic content of 17 to 20%. Vermouth is an aromatic flavored wine. Brandy has been added during production, followed by further aging. The principal types are the dry and pale amber, French vermouth, and the sweet and dark amber, Italian type. Vermouth has from 15 to 20% alcohol.

Dessert wines are used chiefly after meals. The name has a legal significance in many countries, for wines having more than 14% alcohol are grouped together for taxation purposes as dessert wines. More typical of the class of dessert wines, however, are port, which may be either white or red, and muscatel. They contain around 20% alcohol. In the production of dessert wines much of the grape sugar is retained in the wine by adding brandy to arrest the fermentation of the juice, and generally very specialized types of grapes are used in the preparation.

The **sparkling wines** are table wines that have been made effervescent by a second fermentation conducted in closed containers, generally in the bottle itself. (Noncarbonated wines are called **still wines.**) They may be white or pink or red. Champagne is the most widely known of its class, but the others usually take the title "sparkling," followed by the name of the wine that is used for the base —e.g., sparkling burgundy. For the production of sparkling wine most wine makers require a blend of table wines. To these are added a little sugar and a yeast capable of acting in the presence of the already developed alcohol. The second fermentation is conducted in bottles, with strong corks clamped on to prevent the escape of CO_2, or in large bulk containers under pressure. When the production of the CO_2 is due to natural causes, the wine may be considered a sparkling wine. In a group apart from the naturally sparkling wines are many wines which are made effervescent by artificial carbonation at the time of bottling. These are less expensive and are marketed as carbonated wine.

Distilled Liquor

The manufacture of distilled liquors, primarily whiskey, has many aspects in common with the manufacture of alcohol for industrial purposes. Nevertheless, there are several aspects which merit particular attention. The fermented liquid is distilled to a high alcoholic content and no particular difficulty is encountered in its preservation. The source of carbohydrate is usually a grain—rye, corn, or barley —and this, basically, is malted and mashed by a process similar to that for beer manufacture. The resulting infusion of soluble carbohydrate is inoculated with a pure culture of yeast of a high alcohol-yielding capacity, the fermentation is allowed to go to completion, and the alcohol is distilled off. For beverage purposes the distillate is stored in charred casks for aging. During the period of aging, higher alcohols produced during the fermentation are converted into compounds which give characteristic aroma and taste.

Bourbon whiskey is distilled from a mash containing not less than 51% corn; **rye** from a mash containing not less than 51% rye grain, etc. In the United States whiskey must be stored in charred, white oak containers for at least two years, after which it may be designated as "straight whiskey." If it is a combination of several whiskeys or has been diluted and its alcohol content restored with industrial alcohol ("neutral grain spirits"), it is called "blended whiskey."

Scotch and Irish whiskeys differ in a minor extent; the process of manufacture is quite the same. However, they are prepared by mixing various grains with malt, the nature and proportion being subject to considerable variation. The malt used has been dried over peat fires and is the origin of the typical smoky flavor of these whiskeys. They must also be distilled in a pot still rather than a continuous still.

Gin is simply alcohol to which an extract of juniper berries has been added. **Vodka** is also alcohol, originally from potatoes, but now from any source (including petroleum), suitably diluted and treated with charcoal to remove any traces of flavor.

All of these are labeled "proof," which is the alcoholic content times 2; i.e., 50% alcohol = 100 proof; 85 proof = 42.5% alcohol.

There are further cases of industrial action of microorganisms in which the product is not isolated as a chemical of commerce. Some of these of interest to us are the following.

Production of Linen and Hemp: Retting

The long fibers of flax, hemp, jute, and sisal are embedded in the plant in heavy layers of hemicellulose, a material which resembles cellulose in being a polysaccharide. But it has a structure more like that of starch or pectin, rather than the long regular chain structure of the cellulose. The object of the **retting** is to remove the hemicellulose, thus freeing the long cellulose fibers; they can then be collected and woven into linen or used in the form of ropes or packaging. There are two basic processes for retting, and in both cases, it is a matter of encouraging the growth of organisms which will attack the hemicellulose without permitting the growth of organisms which will attack the cellulose fibers themselves. The first process is one in which the flax or hemp is spread out on the ground, particularly under somewhat acid conditions such as would occur in peat moss, and is exposed to the air and rain for some period of time. Under these peculiar circumstances the molds and any aerobic bacteria can grow, but many of those which attack cellulose itself are inhibited. Retting is achieved but the process is somewhat uncertain; it depends on the weather during the time the retting is progressing and there are frequently organisms that produce pigment or some growth on the cellulose fiber and thus color or weaken it. However, this process is practiced, particularly in Ireland and Scotland, where peat bogs abound. The production has established a reputation for quality that is difficult to match; "Irish" linen is known throughout the world for its softness, pliability, and absorbency.

The basic plant involved in the production of linen is flax. But a very similar plant, hemp, is involved in the formation of somewhat cruder fibers. This, too, may be retted by an aerobic process as for flax, but generally an anaerobic process is employed. In Italy, under natural con-

ditions, the hemp is placed in a flowing stream and is allowed to remain there for several days; during this time the anaerobic organisms attacking the hemicellulose are able to grow and the natural fiber can be removed from the resulting fermentation. In other places, where flowing water is not employed, artificial retting vats are set up, but rather crude control is exercised over the microorganisms involved. The aim is to build up a heavy population of hemicellulose-decomposing organisms and to keep the vat sufficiently acid so many of the cellulose-decomposing organisms will not grow. The process, when it is properly operated—not necessarily on exactly scientific principles—yields a very nice fiber which can be made into "linen of quite good quality." Jute and sisal are tropic plants and are subjected to retting generally under higher temperature conditions and generally under water in bays and lagoons or sometimes flowing streams. The product from these operations is relatively crude but also relatively cheap. Much of the rope fiber and packaging cloth used throughout the world originates from these plants.

Yeast

In this case the organism itself is wanted. The process is much like that for industrial alcohol but, instead of having anaerobic conditions, air is blown through the tanks to obtain as many cells as possible. Similarly, root nodule bacteria, dairy starter cultures, vaccines, and others are commercial products where the microbial cells themselves are what is wanted.

Others

We only mention the production of fats, steroids, vinegar, enzymes, etc. as articles of commerce produced by microbial growth. Mushrooms are grown both in the old conventional fashion and in submerged fermentation tanks as food flavoring agents. Garbage disposal, the generation of burnable gases, composting, and the like are all semi-industrial activities of microorganisms.

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For background information, see p. 495.

QUESTIONS

1. Define or identify:

bulk chemical	acetoin
fine chemical	acetone
dope	cornsteep liquor
B.Y.	phenyl-acetic acid
mash	giberellin
cobamide	hormone
ephedrine	mashing
compound	lagering
hydrocortisone	wort
steroid	ale
malting	hops
Pilsner	bock
pitching	must
bottom yeast	racking
top yeast	retting
wine yeast	dry wine
sparkling wine	straight whiskey
proof	blended whiskey
butylene glycol	neutral spirits

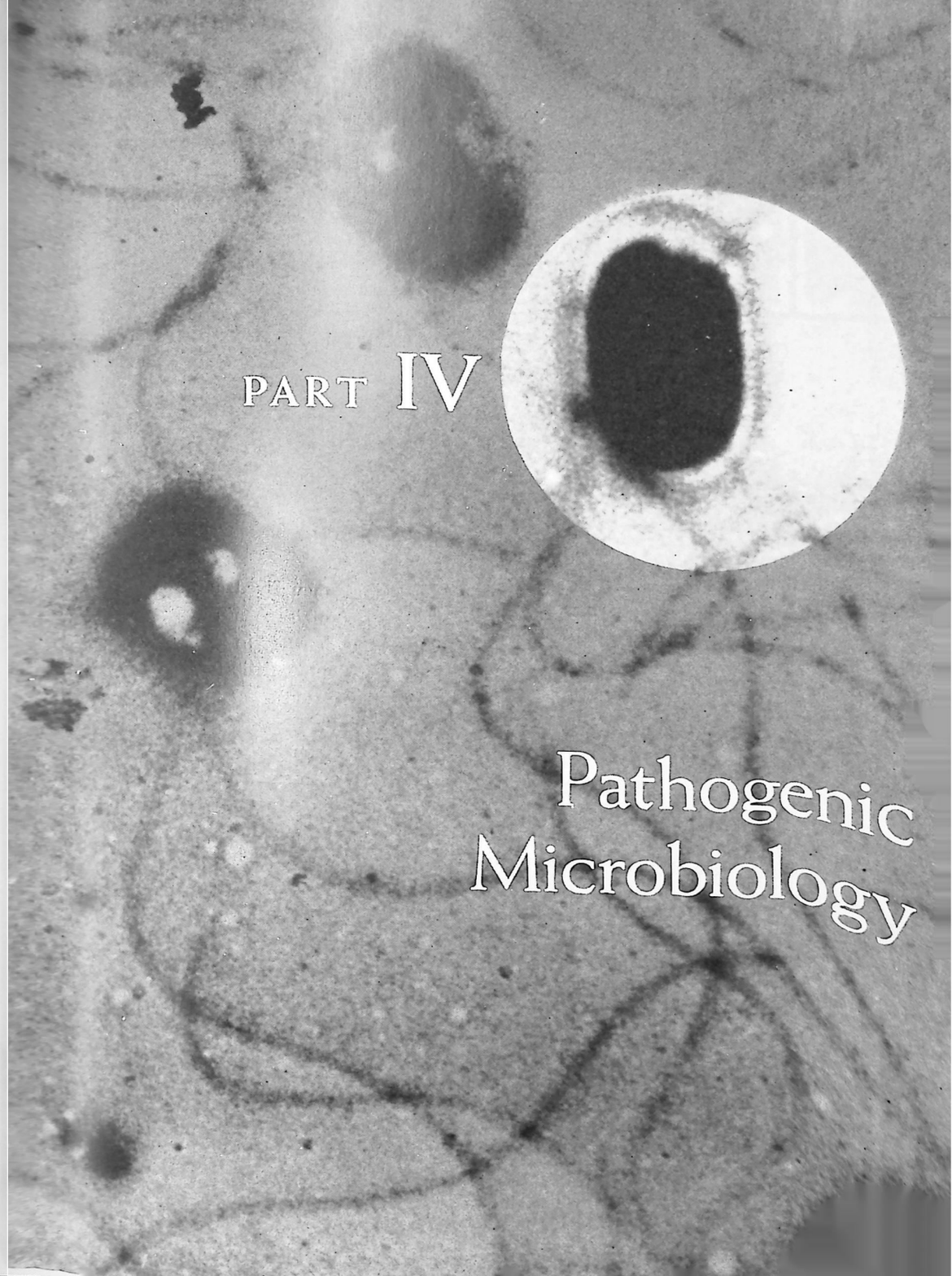
2. You are asked to follow the course of growth of an organism producing propionic acid in an industrial plant where you must sample ten closed tanks aseptically every thirty minutes. What method would you use? Why?

3. Old Hockenshlock Beer has been giving its customers a real pain. Instead of the fine mellow flavor the boys at the tavern are used to, the flavor is tart, the odor musty, and the foam hardly rises. What would you suspect and how could you go about correcting the situation?

4. The Industrial Disposals, Inc., a firm engaged in garbage disposal, is thinking of buying the patents on a new process whereby all the garbage is placed in a large tank, inoculated with thermophile, and allowed to heat up. It is claimed that in 24 hours a tank 3 feet deep and 20 feet in diameter will dispose of a ton of garbage and generate enough heat to distill out the fats and oils. What problems might you encounter in operating such a unit?

5. A year ago the price of vitamin B_6 was 17¢/g, riboflavin 3.6¢/g, thiamin 3.6¢/g, and vitamin B_{12} \$138/g. You have organisms capable of producing each of these, which will grow on the same base medium producing (depending on the precursors supplied) 2 mg B_6/ml, 17 mg riboflavin/ml, 5 mg thiamin/ml, or 2 mg vitamin B_{12}/ml. Your cost of isolation is 9¢/g for vitamin B_6, 1¢/g for thiamin and riboflavin, and \$30/g for vitamin B_{12}. Which substance will it be most profitable to produce?

Facing page, *S. typhosa* after 30 seconds' contact with anti-typhoid sera. [From Mudd and Anderson, *J. Am. Med. Assoc.*, **126**, 570 (1944).]



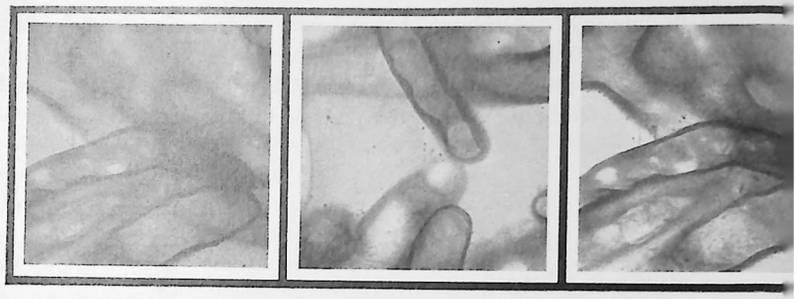
PART IV

Pathogenic Microbiology

THROUGH long and darkened centuries, man lived and died and knew sorrow and disease, but understood little of their origin. Again and again diseases struck him and his fellows, swiftly, terribly, and then left in their wake, not only death, but disfigurement, disability, and devastation. A little less than a hundred years ago the study of microbiology began to reveal that some large portion of disease was caused by microorganisms. Further, microbiology developed the methods and the means to hunt out and hunt down these scourges of the past. Rarely was a science capable of so much good and launched with such spectacular success. In two decades men had solved what had been ancient plagues and pestilences, visitations of the hands of watchful gods, against whom all defense was useless. Not only were their secrets known, but most were trapped, controlled, subdued. No wonder men flocked to bacteriology, as it was called in those days; no wonder they gave their lives to it.

Today, a scarce hundred years later, those miracles are commonplace. But there is still much to do, much to learn and much to discover. For infectious diseases have been wily antagonists and man has never quite wiped them out, once and for all. They are there to spring at us again—and do.

Chapter 23



TRANSMISSION OF INFECTIOUS DISEASES

"After the aforesaid pestilence, many buildings, both large and small, in all cities, towns and villages had collapsed and had completely fallen to the ground in the absence of inhabitants. Likewise, many small villages and hamlets were completely deserted; there was not one house left in them, but all those who had lived in them were dead. It is likely that many such hamlets will never again be inhabited. For all the beasts and cattle that a man possessed wandered about without a shepherd and everything a man had was without a caretaker."

The chronical of Henry Knighton (1350); the impact of the Black Death

PATHOGENIC MICROBIOLOGY is the study of microorganisms which cause disease. It is not, usually, a study of the disease itself—its symptoms, its occurrence, and its treatment. While pathogenic microbiology touches upon some of these, its center core is knowledge of the microorganisms causing disease, their nature and properties, their recognition, and whatever distinguishing characteristics they may have for differentiating them from similar organisms not causing disease. Since we are here concerned with microorganisms and not with disease per se, it is convenient to discuss the pathogens in groups related to their biological character as microorganisms, but they are so arranged that groups of similar diseases, or groups with similar modes of transmission, tend to occur together. The subsequent chapters

dealing with the subject of pathogenic microorganisms are arranged in this fashion. However, this chapter and the one following are devoted to the more general aspects of disease as such.

The Role of Microbiology in the Study of Disease

Disease is a disturbance in function. Literally it means "lack of ease." But it may be defined in a more sophisticated manner as "the failure of adequate function of a tissue or an organ of the body." The disease process itself may be called "a reaction of the body to injury."

Disease may be caused by a variety of alterations: physical difficulties, such as burns or sunstroke; mechanical alterations caused by the enviroment, such as a wound, an obstruction of the nasal passages, or a chemical injury. Bacteria and most disease-producing organisms produce disease in a chemical fashion.

There are, of course, as you might suspect, a variety of different diseases. There are eight basic disease types quite generally accepted by most people: (1) wounds, (2) intoxications, (3) metabolic diseases, (4) endocrine disorders, (5) nutritional deficiency, (6) nervous diseases, (7) cancer, and (8) communicable or infectious diseases. Pathogenic microbiology is concerned fundamentally only with the eighth type of disease, that is, the communicable diseases. These are due to infectious agents and are characterized by being transmissible from one individual to another. For our purpose the terms **transmissible** and **communicable** mean the same thing: they mean that it is possible to pass the disease from one person to another, from one plant to another plant, from one animal to another. A broken arm, for example, is not transmissible, but the common cold is. There are other terms in general use which have rather precise meanings—although used somewhat loosely by many people. It is desirable that we clearly define and distinguish them.

An **infectious disease** is one caused by a microbial agent, whether or not it is transmitted easily or directly. There is always the implication that the microbial agent is growing in or, more rarely, on the body. For example, lockjaw, caused by the growth of *Clostridium tetani* in wounds, is infectious but not easily communicable since it has no natural means of reaching a similar wound in another individual.

A **contagious** disease is a communicable disease which spreads readily. The term used to have the connotation that it spread by touch or contact, but today it means only that it is easily transmitted. Malaria is a transmissible disease, an infectious disease, but not a contagious disease; it will not spread readily from mere contact with the malarial patient but needs an insect bite to start a new case.

An **endemic** disease is one that occurs constantly among a population.

An **epidemic** disease is one in which a large number of cases develop in a given population in a short time, above the normal prevalence.

In addition to these terms distinguishing diseases, there are some others having to do with the disease-producing properties of the microorganism.

A **pathogenic** microorganism is one capable of producing disease. This implies nothing about how easy or how hard it is to produce the disease or how severe or mild the disease may be, but only that the microorganism is able to produce the disease.

A **virulent** organism is a pathogen that is readily capable of causing disease in a specific host, with the general implication that the disease is more severe. This is generally thought of as the degree of damage done by a pathogenic organism and its ability to overcome the host defences. The fewer microorganisms required to overcome a given host resistance, the more virulent is the microorganism. An organism may be virulent for rabbits but avirulent for mice.

A **saprophytic** microorganism is one that lives on dead organic matter.

A **parasitic** microorganism is one that lives on living cells, i.e., growth conditions are more favorable for it within the living body than elsewhere.

With these terms we are able, for the most part, to characterize the nature of the disease and the organisms in any particular case. All communicable diseases have a body reaction which is reasonably characteristic. The invading microorganism is foreign to the body and the body begins to defend itself. The first reaction is inflammation and the movement of fluid into the invaded tissues, thus causing swelling. Next there is usually fever. When the "battle" between the body and the pathogen has been won by the body there is repair of the damage, if this is possible; many times an immunity has been established. We shall examine the nature of this immunity in the next chapter, but these processes—inflammation, fever, repair, and immunity, especially the last—are always associated with communicable disease. Each of these processes vary both in the specific disease and the specific host.

It was only about a century ago that the germ theory of communicable disease was established. The man who established it was Koch, on studies with anthrax. Before this time, indeed for many years before, there had been suspicion that microorganisms caused disease. The Middle Ages, with their plagues and pestilences, contained the beginning of the idea, which swelled into almost certainty by the 1860's. But not quite. The problem came down to this: how can you *prove* that a given microorganism produces a disease? How do you know that it causes the disease rather than only happening to be there? This problem was solved by Koch in his studies on anthrax, and a set of rules for such proof, called "Koch's postulates," were set down in 1883; see pp. 30–31.

Historically, this was the original role of bacteriology: first, proof that the rod-shaped organism, which we call *Bacillus anthracis*, was the cause, and the only cause, of the disease anthrax.

The next role was to demonstrate the organisms associated with other diseases. In these studies, not only were bacteria found to be involved, but also yeasts, molds, protozoa, rickettsiae, and viruses. This expanded the role of the bacteriologist—he became a microbiologist. To some extent, especially in the field of virus study, the modern microbiologist still is concerned with the discovery of the etiologic agents of disease. But the great majority of medical microbiologists are involved in diagnosis, prevention of infectious disease, or assistance with therapy.

The diagnosis of infectious disease is usually begun by the physician through physical examination. From this he may have some diagnostic suspicions, and the microbiologist makes appropriate tests or attempted isolations to confirm the diagnosis or help in the decision. This may involve search for organisms or for characteristic immune reactions. If, on the basis of these, the physicians can still not diagnose the case, further microbiological research is necessary.

The prevention of infectious disease is made possible by studies on the nature of the pathogenic organisms, their modes of transmission, and the development of proper steps to be taken to prevent the spread of pathogenic organisms. In this area it is the microbiologist who has the predominant role.

The cure of disease is eminently in the domain of the physician. But here he is assisted by the microbiologist in one of three ways. First, in the employment or development of immune reactions—as with diphtheria antitoxin. Second, in the development and production of therapeutic substances for the physician's use, such as penicillin. Third, in determinations of

drug or antibiotic sensitivity of particular organisms from a particular patient—as, for example, determining that an organism isolated from a patient is sensitive to penicillin and that this, therefore, would be a suitable treatment.

Control of Communicable Diseases

Before medicine can cope with a communicable disease, knowledge of certain factors concerned with its cause, its spread, and its treatment must be obtained. These factors are the following.

1. Cause
   (a) The infectious agent
2. Transmission
   (b) Source of infection
   (c) Mode of transmission
   (d) Incubation period
   (e) Period of communicability
   (f) Methods of control
3. Treatment
   (g) Methods of treatment

Much of the earlier discussion and much of the material in subsequent chapters deals with the infectious agent. The problem of treatment is a part of medicine and we touch upon it only briefly in microbiology. The remainder of this chapter is therefore devoted to the transmission of communicable disease.

A disease starts from somewhere, and that somewhere is where the pathogenic organism is. It can be, and frequently is, a sick individual. But it can be elsewhere; *B. anthracis*, for example, may survive in soil for years.[1] But, from this somewhere—this source—the infectious agent must leave, it must travel to a susceptible individual, and it must enter into this individual. There is then an "exit," a "route of transmission," and a "portal of entry." We chose these words for a definite purpose. If we can, at the "exit," at the "route," and at the "portal," do something to prevent the pathogen from accomplishing its natural purpose, we may prevent the spread of the disease. In a communicable disease, this is to prevent the disease.

Means of Exit

We may first begin with an individual who has the disease. He is thus our source of infection. If there is another source of infection, the soil for example, we shall come upon it in discussion of the route of transmission. So we shall start with the infected body. As shown in Fig. 23-1, there are six exits: saliva, feces, urine, skin, milk, and insect bites.

The exit through **saliva** may occur through coughs, sneezes, and through what one might call "droplet infections." When a person sneezes, droplets of saliva are ejected into the air and these may contain the organisms. The diseases which are commonly transmitted through the saliva by way of such droplet infections may be divided into bacterial and viral diseases. Relatively few fungal diseases are transmitted in this fashion, except by dust. Typical bacterial diseases are scarlet fever, septic sore throat (caused by streptococci), lobar pneumonia (caused by diplococci), meningitis (caused by *Neisseria* and other organisms), diphtheria, tuberculosis, and whooping cough. The viruses spread by droplet infection are the common cold, influenza, measles, German measles, mumps, smallpox, chicken pox, and psittacosis.

[1] Since anthrax is a spore-former, the spores may survive; indeed, the organism may grow outside the body. In other cases, especially in those diseases in which the disease is transmitted from animal to man, we speak of a **reservoir** of infection, i.e., the species which more or less continually has the disease from which it may be transmitted to man. For example, from the rat which carries the bacterium causing plague, it is transmitted to man by the flea. Such a living transmission agent is called a **vector.** Man himself may be a reservoir, as in malaria or typhus fever.

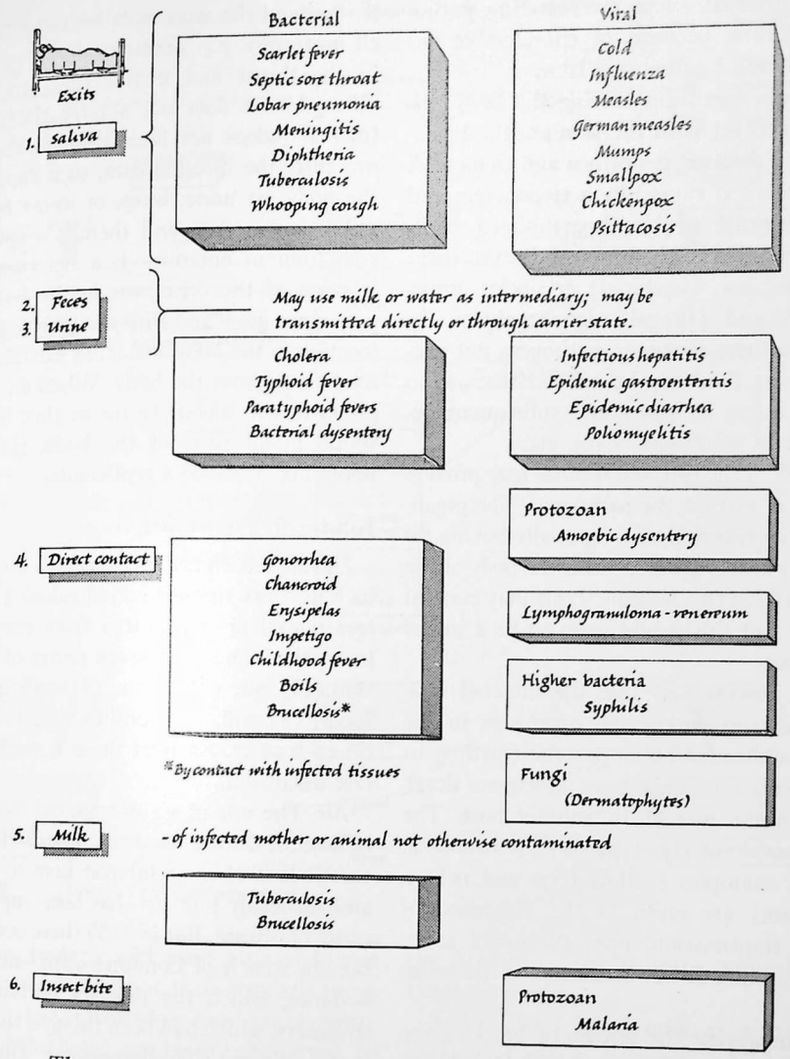


FIG. 23-1. *The means of exit.*

Organisms may also leave an infected individual by way of the **feces** and the **urine**, and under circumstances where either feces or urine, get into water or milk, such diseases may be milk- or water-borne. The bacterial diseases are Asiatic cholera, typhoid fever, the paratyphoid fevers, bacterial dysentery, and brucellosis. The one important protozoan disease in which the organisms leave the body in the feces is amoebic dysentery. The viruses are infectious hepatitis, poliomyelitis, epidemic gastroenteritis, and epidemic diarrhea. It is in organisms leaving the body by feces and urine that the problem of the carrier state, in which an apparently

healthy individual keeps on excreting pathogenic organisms, becomes of considerable importance; it will be discussed later.

Pathogenic organisms leaving the body primarily by contact with the **skin** are the bacterial venereal diseases, gonorrhea and chancroid; syphilis, which is caused by a treponema; and the viral disease of lymphogranuloma venereum. In addition to the venereal diseases there are skin diseases: (bacterial) erysipelas, impetigo, boils, and (fungal) dermatophytes. In addition to these, however, pathogens not normally leaving the body by contact may do so through trauma or injury and subsequent development of secretions, scabs, etc.

The **milk** of the infected mother may provide a channel of exit for the pathogens. The organisms causing tuberculosis (especially bovine tuberculosis) and brucellosis leave the body of the cow typically in this fashion. Tears may contain pathogens but this would seem to be a minor route of exit.

Finally, **insects** may bite the infected individual, suck up pathogenic organisms in the blood consumed, and then pass these pathogens (which may or may not have undergone development in the insect) to another host. The prime example of this type of exit is malaria, and other examples (yellow fever and rickettsial diseases) are given in the discussion of routes of transmission, since the insect serves not only as an exit but as a route of transmission.

We can prevent the disease by blocking the exits. We can prevent insect bites. Direct contact may be avoided. For body secretions, mainly milk, we may not be able to prevent the secretion, but we can prevent its use. For saliva, feces and urine, we cannot prevent their exit, of course, but we can so handle them as infectious agents that they never get into the routes of transmission.

One thing further should be pointed out about the means of exit. These means are restricted and the same exits are not available to all organisms. For example, diphtheria thrives in the throat and it therefore can exit via saliva, but it does not survive the intestinal tract and does not filter through the kidney nor enter the blood stream, so it cannot leave the body via urine, feces, or insect bite. This restriction in exit—and there is a comparable restriction in entering—is a reflection of the location of the organisms in the body. Most pathogens grow and thrive in only a particular location in the body and rarely are they distributed throughout the body. When an organism grows in the blood stream so that it may be found in all parts of the body, this "blood poisoning" is called a **septicemia.**

Routes of Transmission

Having left an exit, an organism must "make its way" to an uninfected individual. It must be transported or transmitted from one location to another. There are seven routes of transmission: (1) air, (2) water, (3) milk and other foods, (4) soil, (5) fomites, (6) insects, (7) direct contact. Each of these is worth a separate discussion.

Air. The role of air as an agent in the transmission of disease has been discussed in Chapter 17. It is of some interest here to note that air—especially foul air—has been supposed the cause of disease. But in 1859 there occurred the famous stench of London—apparently a smog—during which the diseases of typhoid and dysentery, which had been thought to be caused by foul air, actually disappeared. Thus foul air as such was eliminated as a cause of disease. By far the vast majority of diseases transmitted by air are, of course, respiratory diseases; in fact there is a distinct and altogether natural link between an exit via saliva, an air-borne route of transmission, and a portal of entry through the respiratory tract (Fig. 23-2). Air is involved as a transmitting agent since it may carry dust in which organisms have survived. While most

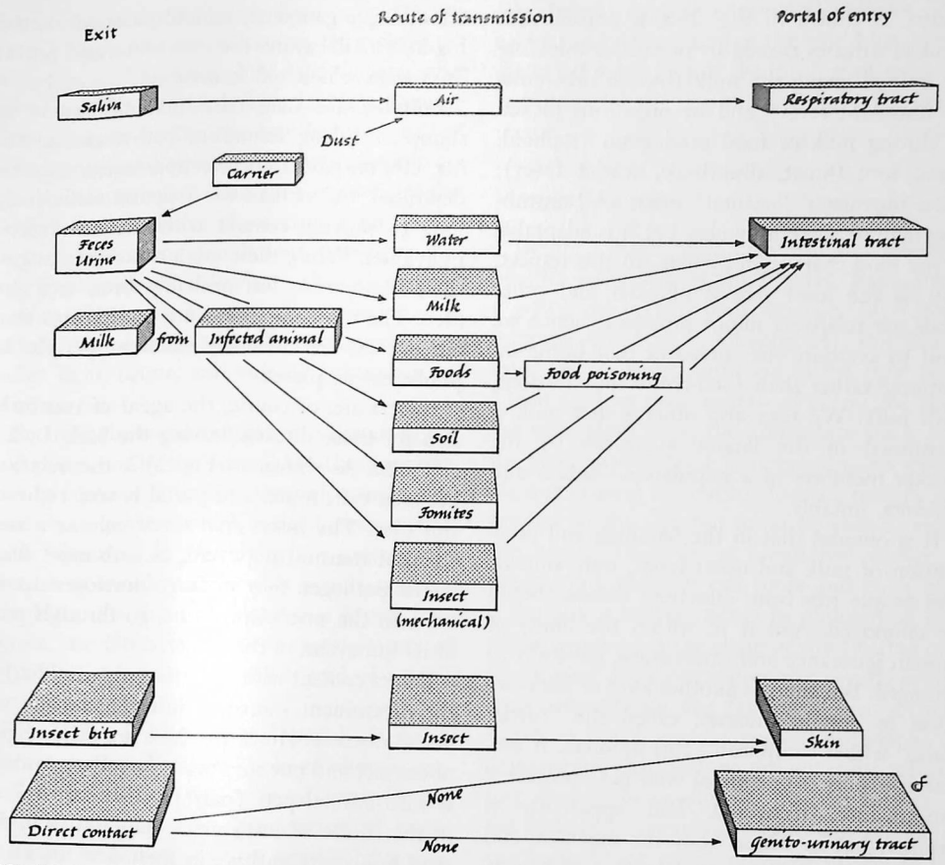


FIG. 23-2. *Routes of transmission.*

pathogenic bacteria and most viruses do not survive drying and are not, then, regularly transmitted by dust, tuberculois (and on occasion, anthrax of the lung) can be so transmitted. In addition, many of the mycoses and actinomycoses are dust-borne and thus can be air-borne.

Water. The role of water as a disease transmitter has been discussed in Chapter 17. Obviously, these are diseases of the intestinal tract—typhoid, dysentery, cholera. They are especially prevalent during war or disaster, when fecal material can enter the water supply. There is, as shown in Fig. 23-2, a connection between the fecal-urinary exits, water transmission, and the entry portal.

Milk. The role of milk as a transmission agent of disease is discussed in Chapter 21 and that of other foods in Chapter 22. There are two kinds of disease types transmitted by foods: first, the infectious diseases in which the organisms invade the tissue; second, the food poisonings, i.e., toxemias, discussed on p. 362. We are here considering only the first type—the transmission of infectious disease. Of course, those which can be transmitted by water can also be transmitted by milk and other foods. But milk

is also, as shown in Fig. 23-2, a transmission agent of diseases caused by organisms infecting the animal giving the milk (bovine tuberculosis, undulant fever) and by organisms picked up during milk or food production (typhoid, septic sore throat, diphtheria, scarlet fever); these organisms' "natural" mode of transmission may be something else, but it is adaptable to the food chain used by man. In this respect, milk is the most serious offender and other foods are relatively minor problems; hence we tend to associate the spread of food-borne infections, rather than food-borne intoxications, with milk. We may also observe that milk is consumed in the largest quantities by the weaker members of a population—babies and children, notably.

It is obvious that in the handling and preparation of milk and other foods, only animals and people free from infectious disease should be employed. And it is, within the limits of human ignorance and carelessness, generally so arranged. But there is another kind of phenomenon in certain diseases, called the "carrier state," which complicates this problem. A **carrier** is a person or animal who has contracted the disease, recovered, and apparently is healthy, but in whose body the organism still persists and is still excreted by way of its normal or sometimes altered exit. Such persons serve as a focus of infection and are difficult to detect. The famous case of "Typhoid Mary" is briefly told on p. 453, but the carrier problem occurs in other diseases as well. Such carriers are especially dangerous in the milk and food handling industries.

Soil and dirt as a source of infection and as a route of transmission are not so important as once believed; many quite healthy people have lived and do live in filth. It is only when infected fecal material is a part of the filth that the soil becomes an agent of transmission. However, there are a few diseases caused by organisms that can live for years in soil—anthrax, tetanus, gas gangrene, and blackleg of cattle. Figure 23-2 illustrates the exit, route, and portal links with which soil is involved.

Fomites are inanimate materials such as stamps, drinking fountains, bathrooms, clothing, etc. on which an infectious agent may be deposited and remain for a period sufficiently long to provide contact with the uninfected individual. While these are a source of danger, their importance has probably been exaggerated. The most important are the fomites that are successively used by different people in public eating places.

Insects are, of course, the agent of transmission for those diseases leaving the body by insect bite. As shown in Fig. 23-2, the relation between exit, route, and portal is very tight in this case. The insect may serve only as a mechanical transmitting agent, as with most flies, or the pathogen may undergo development or even, in the case of protozoa, go through part of its life cycles in the insect.

Direct contact with the infected individual is the preeminent source of infection in the venereal diseases. However, there are other types of contact and one somewhat forgotten contact is with animal pets (parrots, for example) or in the course of one's work; handlers of hides tend to acquire anthrax in their skin. In all of these cases it is perhaps more sensible to think that there is no special agent of transmission, that the proper exit merely has contact with the proper portal of entry.

It seems obvious that we can prevent the spread of an infectious disease and thus prevent the disease by cutting the lines of transmission. For the air-borne diseases we might, as discussed in Chapter 17, attempt to kill off microorganisms in the air; but there are two factors which tend to limit the spread of disease by air, and it is by enhancing these that control can be achieved. First, with the exception of diphtheria and tuberculosis—and here there are other factors which limit their spread—the organisms causing respiratory diseases do not survive long in the air. If we can have them exposed to air long enough to kill, the spread of disease can be stopped. This factor is the one that accounts for the decrease in the spread of infection when hospital beds are placed an adequate distance apart. This time of travel or distance from the source of infection is an evident factor in the recommendations, especially during epidemics of influenza, that one avoid crowds. Of course, if we can make the air more lethal, then distances can be shortened; ultraviolet light, ozone, and volatile antiseptics are of some use.

Second, the organisms get into the air in the first place, not by the normal breathing process, but by droplet infection through coughs and sneezes, a process which indeed characterizes these diseases. If, therefore, we prevent the coughs or avoid contact with people who are coughing, the line of transmission is cut. Face masks, air filters, air particle precipitators all contribute to preventing the spread of respiratory disease.

The role of water in the spread of enteric disease is the classic example of disease controlled by cutting the lines of transmission. Purification of sewage, purification of water, and sanitary testing of water supplies have eliminated most of the diseases borne by this route. Their ready return when disaster strikes shows that cutting the transmission lines holds these diseases at bay.

To milk can be added a further safeguard—that of pasteurization, a process applied to many other foods as well. Pure water, pure milk, pure foods; these cut the transmission lines.

Soil and dirt, though lesser in importance than others, can be eliminated as disease sources by cleanliness. And for fomites, especially in public places, dishes, glassware, etc. should be clean and sanitary. Modern dishwashing methods on a large scale, employing alkaline detergents and steam, do an effective job. Insects can be eliminated and screened against and whole areas of the world have been made habitable by just this process of reducing or eliminating the transmitting agent, the insect vector.

Portals of Entry

Before a pathogenic organism can attack a healthy individual, it must enter. Even then, it will meet certain body defenses (and body response, taken up in Chapter 24). But first it must get in, and it must get in the right way. There are four main portals of entry: the skin, the respiratory tract, the alimentary tract, and the genitourinary tract. An organism, for example one causing respiratory diseases, may be deposited on the skin but never be able to enter this route. There is, as mentioned earlier, a natural link between exit route and entry and if the link is broken, either by eliminating one of the members of the chain or substituting one for another, the disease does not usually occur. This accounts for the failure of early experiments in which certain persons attempted to demonstrate that bacteria *did not* cause respiratory diseases by swallowing large quantities of the pathogenic organisms—especially *Diplococcus pneumoniae*, which does not survive in the stomach or intestinal tract; the organism can very well cause the disease, but the portal of entry was changed.

SKIN. As a general rule, the unbroken skin is an impassable barrier to microorganisms. By skin, we mean that external to the body; the conjunctiva of the eye and membranes in the respiratory tract, mouth, stomach, and intestinal tract are not here regarded as skin. The skin, being in contact with the outside world, does have organisms on it—indeed, there appears to be a "normal flora" of the skin, mostly white staphylococci, *Sarcina* sp., diphtheroids, and sometimes acid-fast organisms, all of which

have a low or negligible degree of pathogenicity. The body, especially the hands, may come in contact with pathogens and they may therefore be transitorily present. Direct penetration of the skin is very rare; the bacteria causing tularemia (*Pasteurella tularensis*) and a few of the viral and fungus diseases are all that penetrate unbroken skin without some help. This help is usually an insect—mosquito, louse, tick, etc.—which bites through or into the skin, thus destroying its value as a barrier. Important insect vector diseases are Rocky Mountain spotted fever (rickettsiae), plague (bacteria), typhus (rickettsiae), malaria (protozoa), and yellow fever (virus).

Of course, if there is a wound or burn, the value of skin as an absolute barrier is also damaged. Tetanus, gas gangrene, anthrax, rabies (from a dog bite) normally enter in this fashion.

But in addition to this, the skin itself is a source or substrate of disease. Many of the fungi causing the dermatophytic ("skin-loving") diseases such as athlete's foot actually live right in the skin itself. Further, invasion of the sweat glands and hair follicles causes skin diseases. Such invasion is frequently caused by staphylococci and streptococci; acne, impetigo, and boils have their origin in such invasions.

The conjunctiva of the eye, being a delicate membrane and differing obviously from skin itself, is yet in contact with the outer world. It is mechanically protected by the eyelid and by the fluids of the eye; there is always a mechanical flushing out of foreign objects. The secretions of the eye contain an enzyme, lysozyme, which we have described earlier (p. 64), that is able to dissolve certain constituents in the cell walls of many bacteria. The tissues of the eye, then, are specially equipped to repel invasion.

RESPIRATORY TRACT. This area we will define as including the nasal passages, the pharynx, trachea, bronchi, and lungs. While it serves as an entry for respiratory diseases, as can the mouth if air is breathed through the mouth, it has its own defense against invasion.

Bacteria and other particles are rapidly removed from the inspired air by passage through the winding nasal passages lined with mucus to whose moist surface they cling. So effective is this process that few particles reach either lung and few leave the body. Expired air contains few bacteria, which is doubtless why we may pipette cultures with sterile pipettes with a minimum of contamination; the cotton plugs in pipettes are to prevent bacteria from being sucked in and are not necessary if nonpathogens are used. Only when droplets are violently expelled, as in a cough or sneeze, do bacteria leave the mouth.

The bacteria trapped in the mucus are moved by means of cilia which slowly sweep the mucus and its attached particles to the oropharynx, where it is swallowed. In short, the body switches portal of entry from respiratory to gastrointestinal.[1] This movement of the thin layer of mucus is indeed remarkable—the material in the nasal passages is changed under normal circumstances once every hour, on the average. When colds or other infections intervene, the ability of the nasal passages to manufacture mucus results in runny noses, etc.; that is, the amount of mucus produced is so far above that capable of being handled by the cilia that it must find some other exit. Lysozyme is present in mucus and there may well be other factors tending to kill off bacteria or viruses, but the antimicrobial activity is not especially remarkable.

The respiratory tract serves as a portal of entry for the respiratory diseases—influenza, the common cold, the various pneumonias, diphtheria, scarlet fever, plague, whooping cough, and pulmonary tuberculosis.

[1] This accounts for some intestinal diseases occasionally being transported via air, especially typhoid.

THE GASTROINTESTINAL TRACT (THE ALIMENTARY CANAL) consists of the mouth, stomach, and intestinal tract, including both the upper and lower bowel. The pathogens enter by way of the mouth in essentially all cases from food, water, or fomites. The mouth contains a predominantly normal flora and a minor transient one. The predominant normal flora are organisms living on and between the teeth on dental plaques, etc.; remember that Leeuwenhoek found bacteria on teeth! With food, there is a tremendous upsurge of numbers but generally the food does not remain in the mouth long enough, except for that trapped there to influence the flora. The flora of the mouth may well be related to the degree of tooth decay and may indeed be the cause of it, but we are here concerned with the mouth as a portal of entry of pathogens.

Microorganisms from teeth, from food, or from anywhere are swallowed and enter the stomach. Here they are subject to the strongly acid gastric juice. Gastric juice has an effect on bacteria greater than just its acidity and causes most of the swallowed bacteria to be killed. Some few do reach the intestinal tract—possibly because they were protected by being imbedded in small food particles—or because the time of exposure was not long enough to kill all of them. Thus, of course, the greater the number of pathogens consumed, the greater the chance that a few may escape. But we should certainly regard the gastric juice as an important barrier to invasion.

Both the small and large intestine contain large numbers of bacteria, but these normal intestinal inhabitants have come mostly from growth in the intestine rather than from passage of any great numbers through the stomach. Once having escaped the stomach barrier, the pathogen in the intestine must now compete with the normal flora there. If this is successful, the disease may then be caused in one of two ways. First, the pathogens may grow in the intestinal tract and there produce a toxin, as happens with paratyphoid fevers and cholera. This, of course, is the invasive counterpart of the externally produced toxins which survive passage through the stomach, as in staphylococcus food poisoning and botulism. Second, the pathogen may invade the intestinal wall and thus the body, as happens in typhoid fever. Ordinarily the intestinal wall is quite a good barrier to bacteria; billions live in the intestine and rarely under normal circumstances, are they capable of penetrating it. But under conditions of severe stress, especially in surgical or radiation damage, the intestinal barrier may be weakened, and under these circumstances even normal intestinal bacteria may invade and be found in the blood stream.

THE GENITOURINARY TRACT in both male and female is generally remarkably free from bacteria, probably because of the flushing action of the slightly acid urine and the relative lack of available food. But certain organisms, primarily those causing venereal disease can live under the circumstances. (We shall regard "childbed fever" as due to trauma.) Infections of the urinary tract by *Proteus* and *Pseudomonas* are found.

Control of Infectious Diseases

It seems obvious that the body, while it has portals of entry, has also guardians at these portals and that these are not open doors to pathogens. It is always a matter of conjecture whether the entry is to be successful. The factors favorable to entry are outlined here.

1. The numbers of pathogens; the more there are, the more chance there is of getting through.

2. Easier entry by the usual or advantageous route; typhoid will usually not invade if rubbed into a skin abrasion.

3. The virulence of the organism, i.e., the ability to overcome host defense.

4. The resistant state of the host or, to put it otherwise, the susceptibility of the host and the state of defense.

There is not too much that we can do to close the portals of entry since, in two cases, the respiratory and the gastrointestinal tract, the doors must be kept open for the intake of vital materials. Here we must cut the lines of transmission, with pure food, pure water, pure air. The skin, while it is not always possible to keep intact, can be aided by antiseptics on wounds, etc. The venereal diseases can be avoided by avoiding venereal contact with infected persons.

Although we can not close all the portals of entry, we must remember not to neglect the guardians that serve to prevent disease entry. If they are weakened through fatigue or neglect, the chance of infectious disease is thereby increased.

REFERENCES

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For background information, see p. 495.

QUESTIONS

1. Define or identify:

disease	Koch's postulates
contagious	etiologic agent
infectious	exit
endemic	route of transmission
epidemic	portal of entry
pathogenic	reservoir
virulent	droplet infection
saprophytic	fomite
parasitic	carrier state
inflammation	insect vector
fever	dermatophytic
immunity	mucus

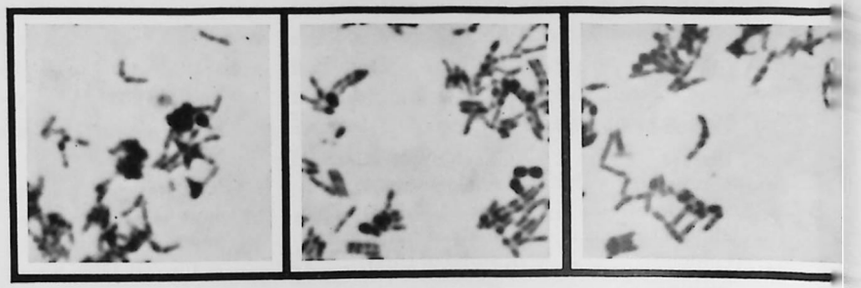
2. A bacterium originally capable of causing disease may lose its virulence by long-continued cultivation on laboratory media. Frequently the virulence can be restored by "animal passage." What do you deduce that "animal passage" means? How would you conduct such a passage?

3. A popular book on microbiology says, "Good health is synonymous with a high degree of resistance to bacterial attack." If true, why is it true? If false, why false?

4. The Institute for the Protection of the Tit-Wit consults you about a problem. It seems that Tit-Wits are dying rapidly and from the dead Tit-Wits a bacterium has been isolated. How could you prove that this either was or was not the cause of the disease?

5. You are a Public Health Officer and you find that in the northern half of your town there is an increasing amount of an illness characterized mostly by high fever. What would you suspect? What could you do about it?

Chapter 24



THE INTERNAL STRUGGLE IN INFECTIOUS DISEASE—IMMUNITY

"Some of our cells, although they are part and parcel of us, have not even fixed coherence with our 'rest.' Such cells are called 'free.' . . . The cells of our blood are as free as fish in a stream. Some of them resemble in structure and ways so closely the little free swimming amoeba of the pond as to be called amoeboid. The pond amoeba crawls about, catches and digests particles picked up in the pond. So the amoeboid cells inhabiting my blood and lymph crawl about over and through the membranes limiting the fluid channels in the body. They catch and digest particles. Should I get a wound, they contribute to its healing. They give it a chance to mend, by eating and digesting bacteria which poison it and by feeding on the dead cells which the wound injury has killed. They are themselves unit lives and yet in respect to my life as a whole, they are components in that corporate life."

SHERRINGTON, *Man on His Nature*

IF, IN SPITE OF all efforts, a pathogen has penetrated through the portals of entry and is within the internal milieu of the body, a further "struggle" goes on. While we should not think of a conscious battle between two foes, we should consider that a pathogenic organism produces disease both by tissue destruction (altered structure) and by toxins

(altered tissue biochemistry). These we label—for want of a better term—the offensive weapons of the organism. The body has further "defenses," i.e., it reacts to the presence of the pathogen. Its defenses are phagocytosis, walling off, and antibody formation, an aspect of immunity. It is all these matters that we shall take up in this chapter.

The "Weapons" of the Pathogen

Once a pathogen has entered, it may cause disease in several ways. One of these is by producing or possessing a material toxic to the host. These materials are called "toxins" and are divided into (1) **exotoxin,** a poison diffusing away from the cell, and (2) **endotoxin,** a poison on the cell surface or associated with the cell body. Both of these are best illustrated by examples.

Exotoxin—diphtheria toxin. We have earlier described an exotoxin, *Clostridium botulinum* toxin (p. 364) and we shall describe another here. The two will suffice as typical examples of exotoxin formation and action. Diphtheria is a disease caused by a "higher" bacterium, *Corynebacterium diphtheriae* (p. 439), in which the organism does not invade the body but grows most frequently in the throat and on the tonsils; while there it produces a toxin which diffuses into the body and can be, if unchecked, rapidly fatal.[1] Many strains of *C. diphtheriae* can also produce this same toxin in artificial media when grown in pure culture. The ability to produce toxin in vitro and the ability to cause a fatal toxigenic disease are not directly related. There is a further complication that pathogenic and toxin-producing strains are all infected with a bacterial virus. But these matters do not concern us here and will be taken up in part later (pp. 439 and 466). What is here important is that in artificial media, growth of the organism results in the presence in the medium of a protein which can be separated, purified, and which is exceedingly toxic, 1 μg (1 millionth of a gram) being able to kill a guinea pig; a little over 10 times this will kill a child.

This organism causes the disease by the production of a poison, a toxin. The formation of exceedingly potent exotoxins by pathogenic bacteria is not too common; they are diphtheria, botulism, tetanus, and a few species of dysentery bacteria. Some are very potent—7 ounces of botulinum toxin, it is estimated, would kill the entire human population of the world. All these organisms have very little invasive power but the toxin penetrates. Exotoxins are also found in snakes, spiders, scorpions.

Endotoxins are poisons which occur either inside the cell or on the cell surface and are not released into the surrounding environment unless the cell disintegrates. Injection of the cells themselves, even if killed, produces the toxicity. Not much is known about these toxins but it is known that some organisms produce disease because they possess such endotoxins. They are usually found in gram-negative bacteria.

Other "Weapons" of the Pathogen

In addition to toxins, certain organisms have other substances which can cause harm. Some bacteria have **hemolysins,** substances which dissolve red blood cells. Others have **leucocidins,** substances toxic to white blood cells. Some, notably among the staphylococci, possess **coagulase,** an enzyme promoting blood clotting. Others (especially many streptococci) contain enzymes which tend to lyse clots and to prevent the blood from clotting. There is also a certain amount of tissue damage and

[1] There is another form of diphtheria, cutaneous diphtheria, in which the organism grows on ulcers in the skin (desert sore, tropical ulcer), there producing its fatal toxin. The disease is especially dangerous since it is not always recognized as diphtheria.

destruction, called **necrosis**, whose origin is not clearly known.

The Defenses of the Host

While the pathogen, once past the barriers at the portal of entry, can begin the disease process, the host does not stand idly by. The pathogen must overcome the host defenses before overt disease occurs. The ability to overcome the host defense on the part of the pathogen is called its virulence. The ability of the host to withstand the pathogen is called **host resistance.** Without trying to define the amount of resistance, or the "degree" of resistance, we can point out that resistance as such is composed of three things.

First, there is a group of resistance factors, which we might call constitutive, in the same sense that a constitutive enzyme is so named. These are somewhat nonspecific factors due to species, race, and individual inheritance, conditioned by sex, age, and general physiological well-being—especially nutrition, fatigue, and exposure.

A few examples will suffice to illustrate, but not to define and delineate, this constitutive type of resistance. Man is essentially immune to cattle plague. This is resistance due to species. Another example: cold-blooded animals are not normally infected by warm-blooded organisms or vice versa. A chicken, whose body temperature is higher than that of animals, will not contract anthrax. But if the chicken is cooled, then it can be made susceptible to anthrax; this was an experiment first done by Pasteur. There seems to be an immunity due to race. Certain races seem to be particularly susceptible to particular diseases and other races relatively immune to them. The reasons for this are really not clear and the actual fact of its existence is no more than suspected. Still another type of resistance resides within the individual. It is known that individuals vary tremendously in their susceptibility to a disease; one individual may come down with a disease when another having essentially the same exposure and meeting the disease under the same conditions does not contract the disease. These constitutive factors—species, race, and individual—are conditioned and controlled by yet another group of secondary variables: age, sex, climate, nutrition, and fatigue. Again, examples must suffice. In a general way resistance to infection *increases* with age, possibly due to gradual development of adaptive immunities, with the exception of susceptibility to pneumonia. A number of diseases show a marked sex incidence, and males tend to be more susceptible. Many of the relationships supposed, and which are demonstrable in the statistics obtained, are not completely clear, and may result from a related social habit. Thus, the fact that in the past more males than females contracted typhoid may only reflect the greater mobility of the male—he traveled more and thus had a greater chance to come in contact with contaminated water.

Second, there is a group of body responses whose action is dependent upon the fact that the body is an organized community of cells. There are the **phagocytes**, certain cells of the body which rush to the scene of infection and attempt to engulf and digest the pathogen. They will attack essentially any foreign body, whether pathogenic or not. There are several types of phagocytic cells; leucocytes (white blood cells) and macrophages found in the tissues are good examples.

Digression on Blood

While the nature and properties of blood are widely known, a microbiologist needs to know a few more technical details. The gross separation of blood (Fig. 24-1) shows that cells make up just under half the blood. The remainder (the plasma) is over 90% water and the rest is mostly protein, a large part of which

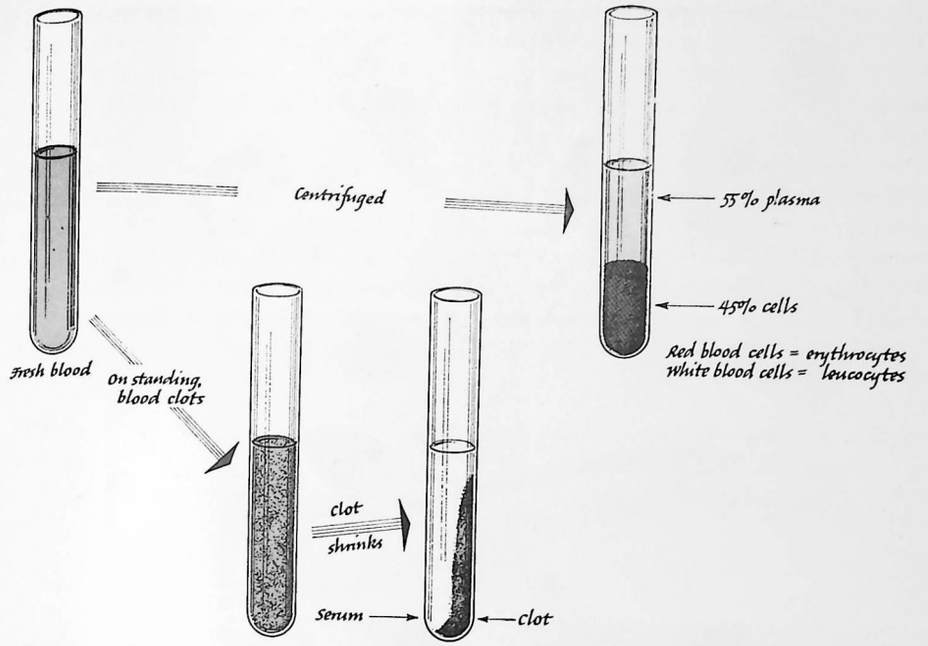


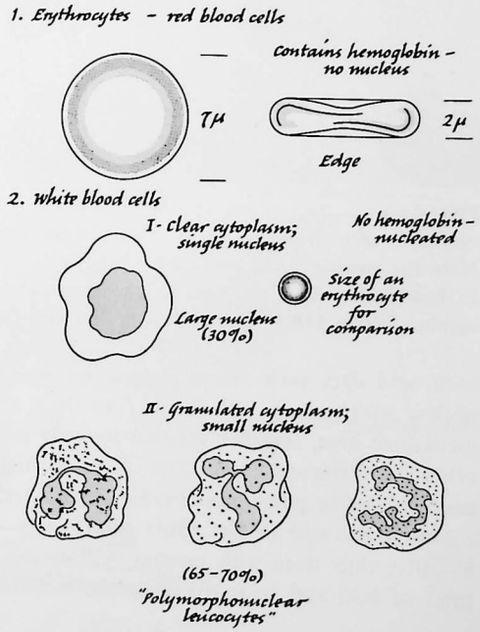
FIG. 24-1. *The composition of blood.*

is involved in the clotting mechanism. But it is the cells which principally interest us here. These are as follows.

The red cells, the erythrocytes, occur in by far the largest numbers; 4 to 6 millions per cubic millimeter, or 10^9 per milliliter; these are the carriers of the hemoglobin.

The white cells, the leucocytes, while larger than the red cells, are much less numerous (5–10 thousand per cubic millimeter or 10^7 per milliliter—roughly 1 white cell to every 500–600 red cells). There are two main groups of white cells—those with a single nucleus and a clear cytoplasm and those with a lobed nucleus and granules in the cytoplasm (Fig. 24-2). Those with a clear cytoplasm are of several types, and altogether they constitute about 30% of the white cells; their origin, nature, and function are too far from our subject to warrant discussion here. The granulated cells are of three types: the **eosinophiles**, which stain

FIG. 24-2. *The leucocytes.*



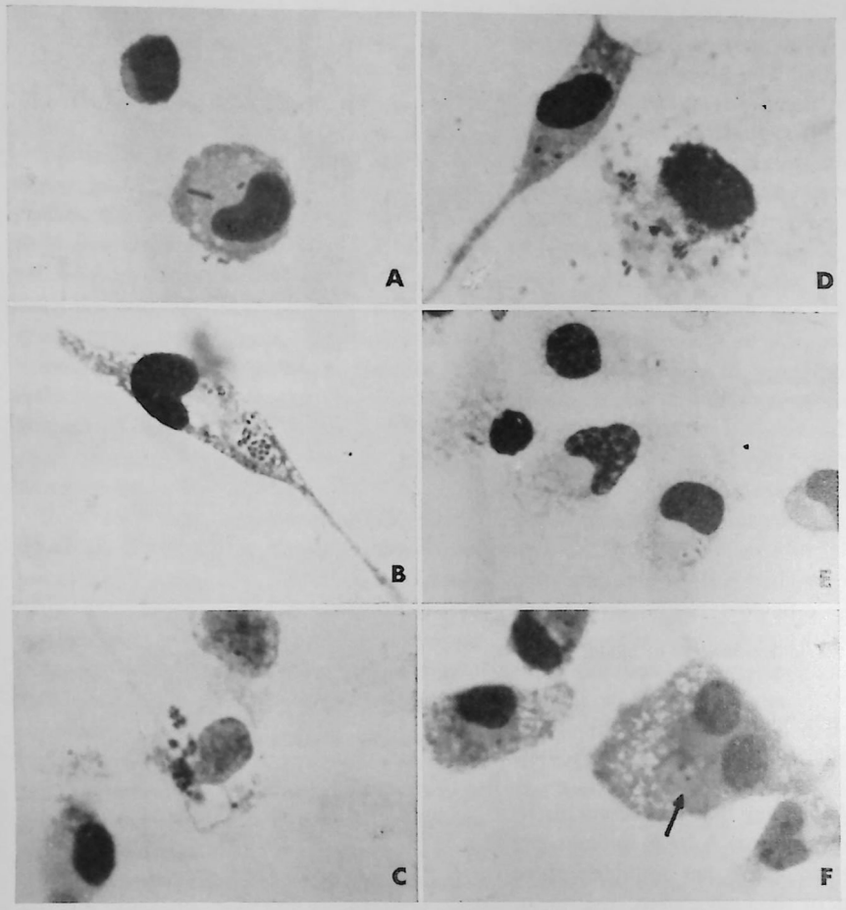


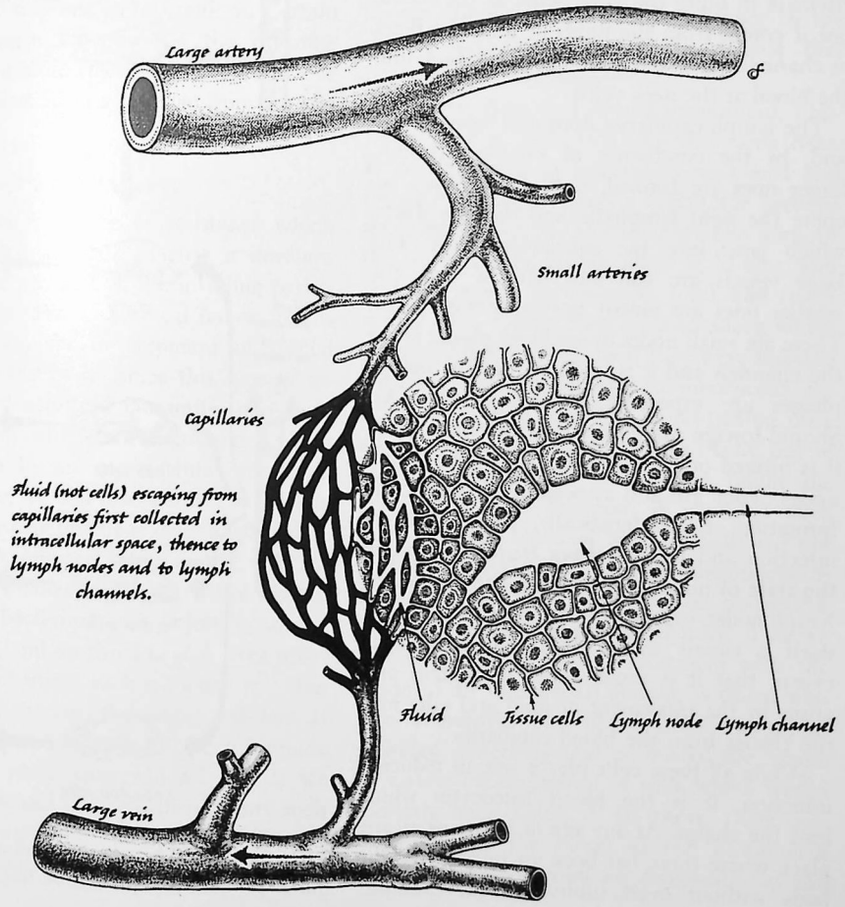
FIG. 24-3. *The course of phagocytosis.* A *is a photograph of a virulent* Salmonella enteritidis *immediately after ingestion by a mononuclear phagocyte (mouse);* B *is 3 days later;* C *is 5 days later. Note the growth of the bacterial cells after ingestion and the destruction of the phagocyte.* D, E, *and* F *show inhibition of the same strain when exposed to phagocytes from mice immunized with killed vaccine. [From Mitsuhashi, Sato and Tanaka,* J. Bacteriol., **81,** *863 (1961). Courtesy Dr. Mitsuhashi.]

with acid dyes such as eosin, and the **basophiles,** which stain with basic dyes such as methylene blue, together account for only another 5% of the white cells. The remainder, the **neutrophiles** or polymorphonuclear leucocytes, comprise the rest of the white blood cells—65–70%; they stain with neutral dyes or mixtures of acid and basic dyes and the nucleus has many forms, there being five "stages"; see Fig. 24-2. The granules in the cytoplasm are quite small but the nucleus is unmistakable. It is these cells in which we are most interested. They are actively ameboid in character, are capable of locomotion, and are able to ingest and in most cases destroy foreign particulate matter (Fig. 24-3). The cells are constantly

changing in shape and indeed resemble amoebae in their activity.

These blood phagocytes are not the only phagocytes of the body, although they are the most mobile; they can leave the blood vessels and rush to any part of the body where they are needed. But outside of the blood stream there are other cells capable of phagocytosis, considerably larger than the blood phagocytes, and for this reason are called **macrophages** or sometimes **histiocytes,** which means only "tissue cells." They can be detected by this very property of phagocytosis, since otherwise it is hard to tell them from the tissues in which they are imbedded. The macrophages all stain with weak solutions of colloidal dyes—pyrrole blue, tryptan blue, lithium carmine, etc. Such dyes are not in solution but are suspended as dispersed particles, which are phagocytized by the macrophages; the dyes are concentrated enough to give color to these cells. Such macrophages occur all over the body and serve, with the polymorphonuclear leucocytes, as a defense against invasion. They are especially found in

FIG. 24-4. *Blood and lymph.*



liver, spleen, and lymph nodes. Such phagocytic cells are considered to be related to each other and are called the reticuloendothelial system (the RES).

Lymphatic Defense System

The **lymph nodes,** or lymph glands, have a curious defense function, by virtue of their being loaded with phagocytes (especially macrophages) and it is worth some space to describe briefly the lymphatic system. We often concentrate so much upon the blood circulatory system that we tend to forget that the tissues too are bathed in a fluid. True, it does not circulate in quite the same sense as the blood, but it comes from the fluids of blood, it flows in channels and vessels, and it pours back into the blood at the neck veins.

The lymph capillaries drain the tissue spaces and, by the confluence of smaller channels, larger ones are formed, until finally they become the right lymphatic and thoracic ducts, which pour into the subclavian veins. The large vessels are real and have a wall; the smaller ones are mostly spaces between cells. There are small nodes or swellings placed along the channels and it is in these that the macrophages are especially located (Fig. 24-4). Should foreign matter get into the tissue space, it is filtered out and disposed of in the lymph nodes. They are also associated with antibody formation. Characteristically, they swell on infection and the physician frequently judges the state of infection by feeling the prominent lymph nodes, especially in the neck. The lymph itself is closely similar to the blood plasma except that it is much lower in protein and arises by the movement of fluid and salts into the tissues from the blood capillaries.

While all these cells play a role in reducing infection, it is the blood leucocytes which lead the charge. At any site of injury or at any place where there has been an invasion of the body without overt injury—as, for example,

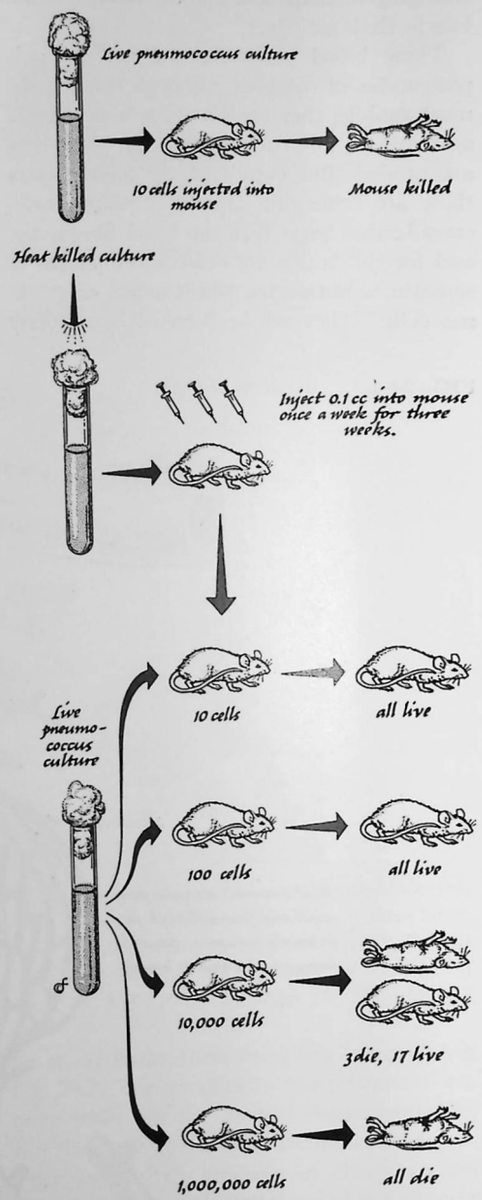


FIG. 24-5. *The experimental approach to acquired immunity. A protection test.*

the intestinal wall—the first reaction of the body is inflammation. Fluid begins to flow into the area and there is swelling and tenderness. The leucocytes, normally mostly in the blood, squeeze through the walls of the blood capillaries and make for the site of inflammation. Here they surround and attempt to engulf the foreign bodies. When there are many leucocytes and foreign bodies, the resulting exudate is known as pus.

Structural Defense Systems

There is, however, a second type of resistance to invading organisms due to cellular organization. This type of tissue response is especially evident in the response to tuberculosis. Certain body cells begin to surround the invading organisms, and more and more pile up until the invader is walled off in a tubercle from the rest of the tissue.

Adaptive Defense Immunity

Third, there is a type of resistance which might be called adaptive in that it develops because of the presence of the invading pathogen. It includes also a chemical factor, and is dependent upon the development of special substances in the body. Since this type of resistance, called **acquired immunity,** may be a little confusing when first encountered, it is best to begin by an experimental approach. As an example, we will take a strain of a pneumococcus; the experiment is diagramed in Fig. 24-5. Ten cells injected into a mouse will kill the mouse. If we now take a heat-killed suspension of this bacterium and inject it into a mouse—say 0.1 ml on three days, a week apart—and then we inject such a mouse with ten cells of pneumococcus, the mouse will live. If we inject one hundred cells into such immune or vaccinated mice, they will all live. If we inject ten thousand live cells into twenty such mice, three might die and seventeen live. If we inject a million live cells of pneumococcus into such immune mice, they will all die. The injection of a heat-killed suspension into the mouse has done something so the mouse is now able to survive ten times, a hundred times, even a thousand times as many organisms as would originally kill it. But it could not survive a million organisms.

In this experiment the mice have been protected by the previous contact with dead organisms; they were vaccinated against the disease. A **vaccine** is a suspension of organisms (living, dead, attenuated) or the soluble products therefrom, which, when introduced into the animal body, tends to stimulate active immunity. By **active immunity** we mean that the agents causing the increased resistance to the disease were developed in the animal that possesses the immunity.

We may then do a second experiment, illustrated in Fig. 24-6. We take this same heat-killed suspension of pneumococcus type I and inject it repeatedly into a rabbit for perhaps six or eight months. We then remove some of the blood of the rabbit, allow it to clot, and take the serum, for example, 1 ml, and inject it into mice. Under these circumstances, where untreated mice would die if they were injected with ten cells of the live pneumococcus, we inject a hundred cells; the serum-treated mice all live. If we inject ten thousand cells, out of twenty serum-treated mice, eight might die and twelve live. However, if we treat such mice with the blood of a rabbit that had not been vaccinated with heat-killed pneumococcus cells, then the mice would be as susceptible to pneumococcus as untreated mice. This increased resistance, due to treatment with the blood of an immunized animal, is called **passive immunity.** The blood from the rabbit provided something that permitted the mouse to survive concentration of cells which would formerly have killed it. In short, there was something in the blood of the immunized rabbit which conferred an immunity on the

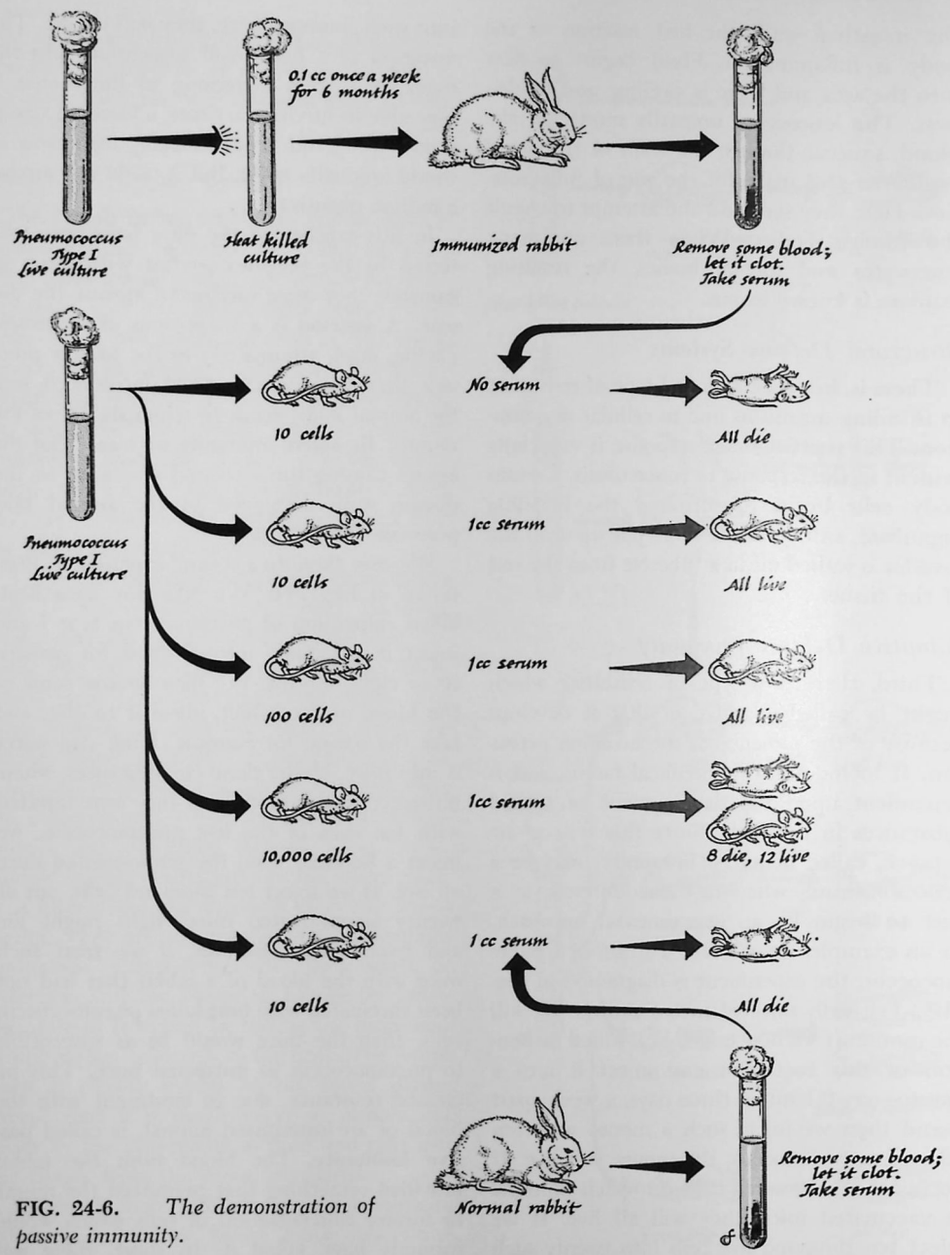


FIG. 24-6. *The demonstration of passive immunity.*

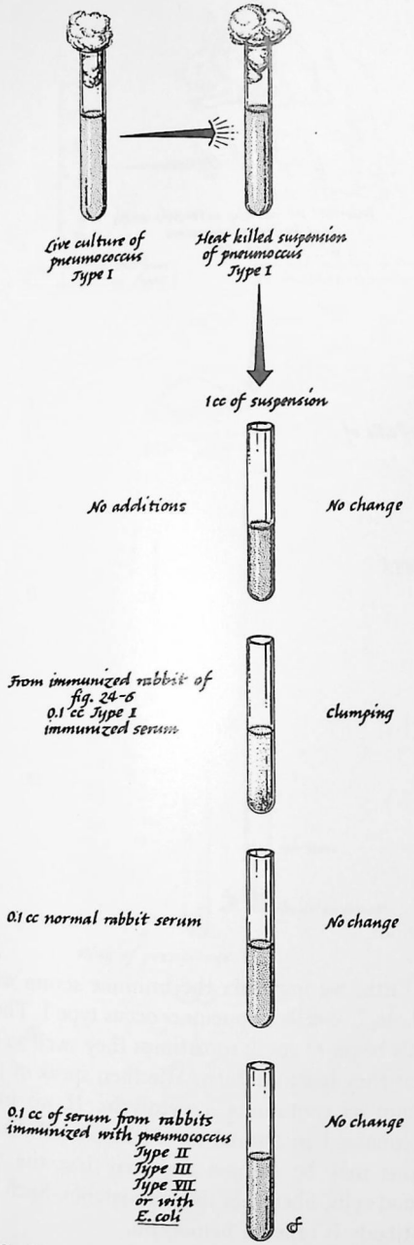


FIG. 24-7. *The demonstration of antibody development by agglutination of killed cells of bacteria.*

mice. This material in the blood is called an **antibody.**

Correlated with this protective power, the immune blood or serum can often be made to react in the test tube in a fashion that non-immune blood would not. Suppose we take some of the immune rabbit blood from the preceding experiment (Fig. 24-6) and we mix this immune serum with a heat-killed suspension of pneumococcus cells. We observe (Fig. 24-7) that the cells clump or agglutinate. Normal rabbit blood serum from an animal that has not been vaccinated with a heat-killed suspension of pneumococcus does not cause this agglutination. But if we immunize a rabbit with type II pneumococcus or type III or type VII or with *E. coli* and mix this blood serum in the same fashion with pneumococcus type I cells we will obtain no agglutination.

The material which causes the formation of the antibody is called an **antigen,** and the antibody formed is specific for the antigen which made it. If we vaccinate a rabbit with a type I pneumococcus, the antibody formed in the blood will react with type I pneumococcus but not with other antigens. If we vaccinate a rabbit with *E. coli,* then the blood antibody formed will react with *E. coli* but not with pneumococcus (Fig. 24-8).

To repeat, an antigen is any substance which will stimulate the formation of antibody in the host. The term is broader than vaccine since it does not imply that the material is an organism or that it necessarily has anything to do with disease. An antibody is a substance produced in the body of the host in response to an antigen.

Let us review the experiment of the rabbit vaccinated with a pneumococcus suspension. The blood from this rabbit did things that blood from a normal rabbit would not do. First, it protected a mouse against an otherwise fatal number of pneumococci (Fig. 24-6). Second, it agglutinated or clumped a heat-killed

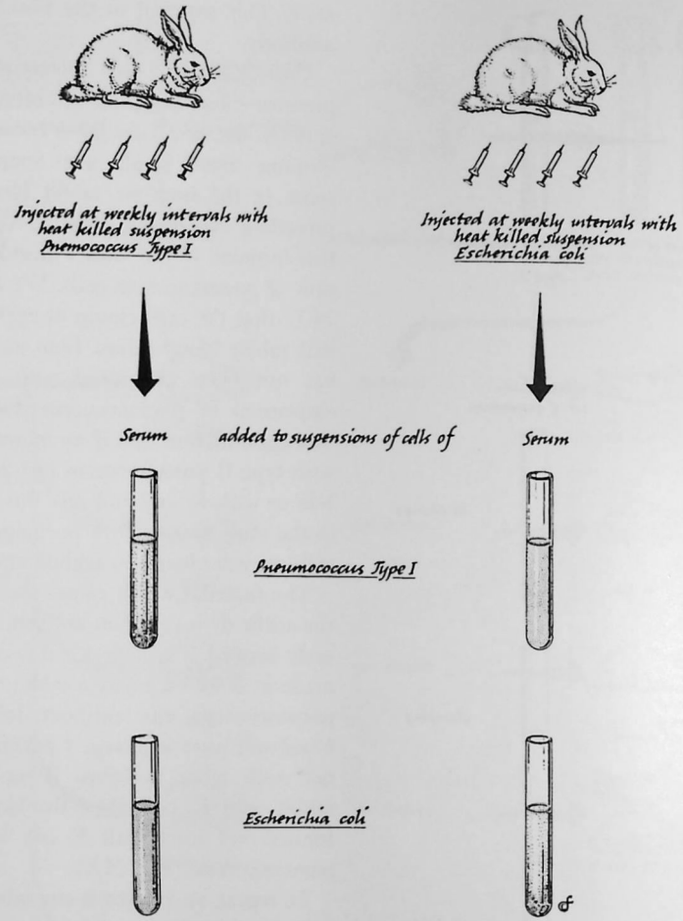


FIG. 24-8. *The specificity of antigen-antibody reactions.*

suspension of pneumococcus of the same type as that used as the vaccine (Fig. 24-7). Third, it would not agglutinate heat-killed or live cell suspensions of other pneumococci nor of *E. coli* (Fig. 24-8). There are still other unusual things this immune blood will do.

Fourth, we may mix the same serum from an immune rabbit with an extract of the soluble proteins of pneumococcus type I. Under these circumstances, we will get a precipitate of these proteins, for the blood contains an antibody which will cause this precipitation.

Fifth, we may mix the immune serum with whole, live cells of pneumococcus type I. These cells begin to swell; sometimes they swell so far that they lyse and burst. We then speak of the serum as containing a **cytolysin.** If we have immunized an animal to red blood cells, such lysins may be formed and may lyse the red blood cells, liberating the hemoglobin. Such an antibody is called a **hemolysin.**

Sixth, we may mix this same serum from a rabbit treated with type I pneumococcus with pneumococcus type I cells and phago-

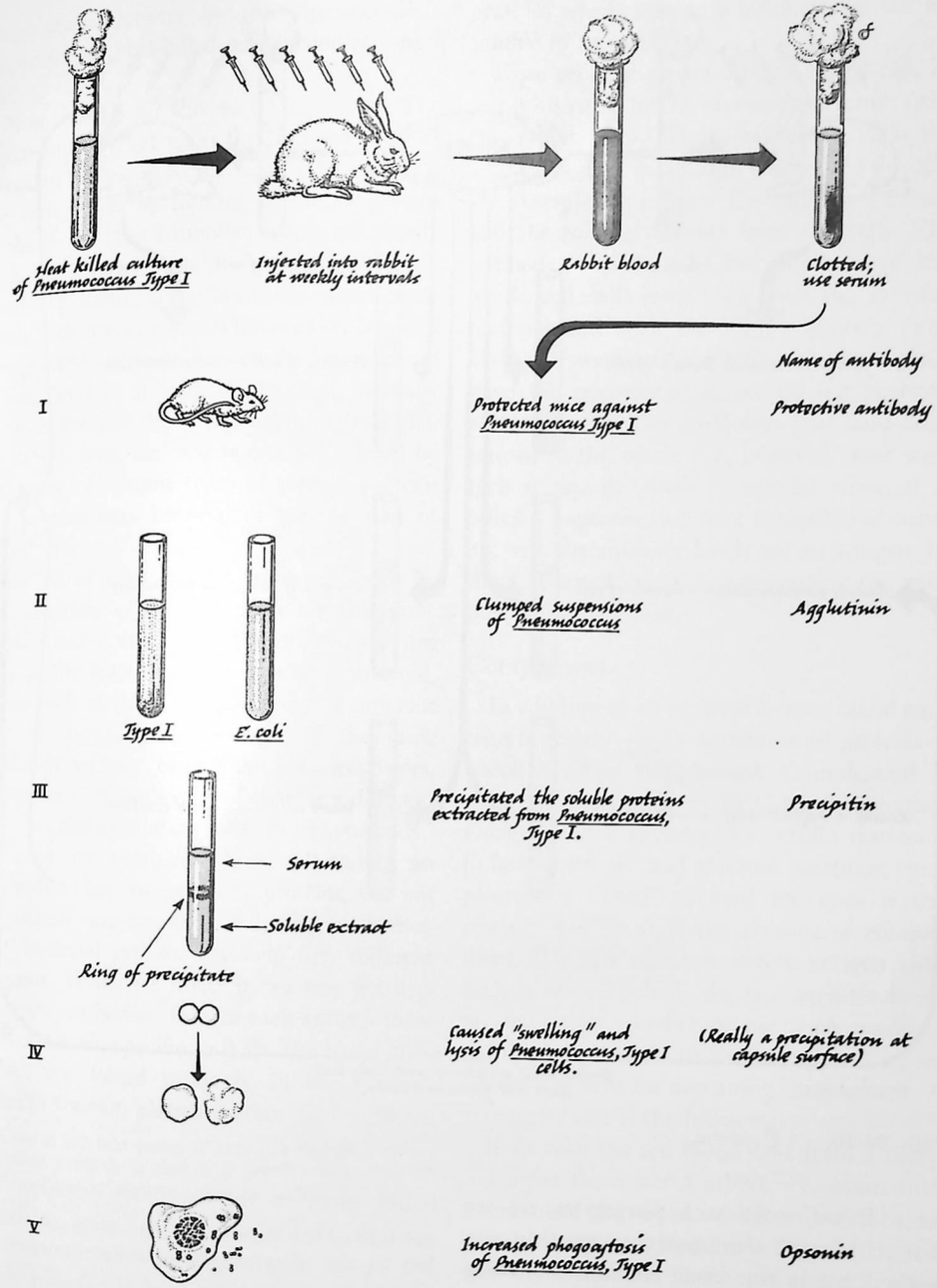


FIG. 24-9. *Some types of methods for demonstrating antibody.*

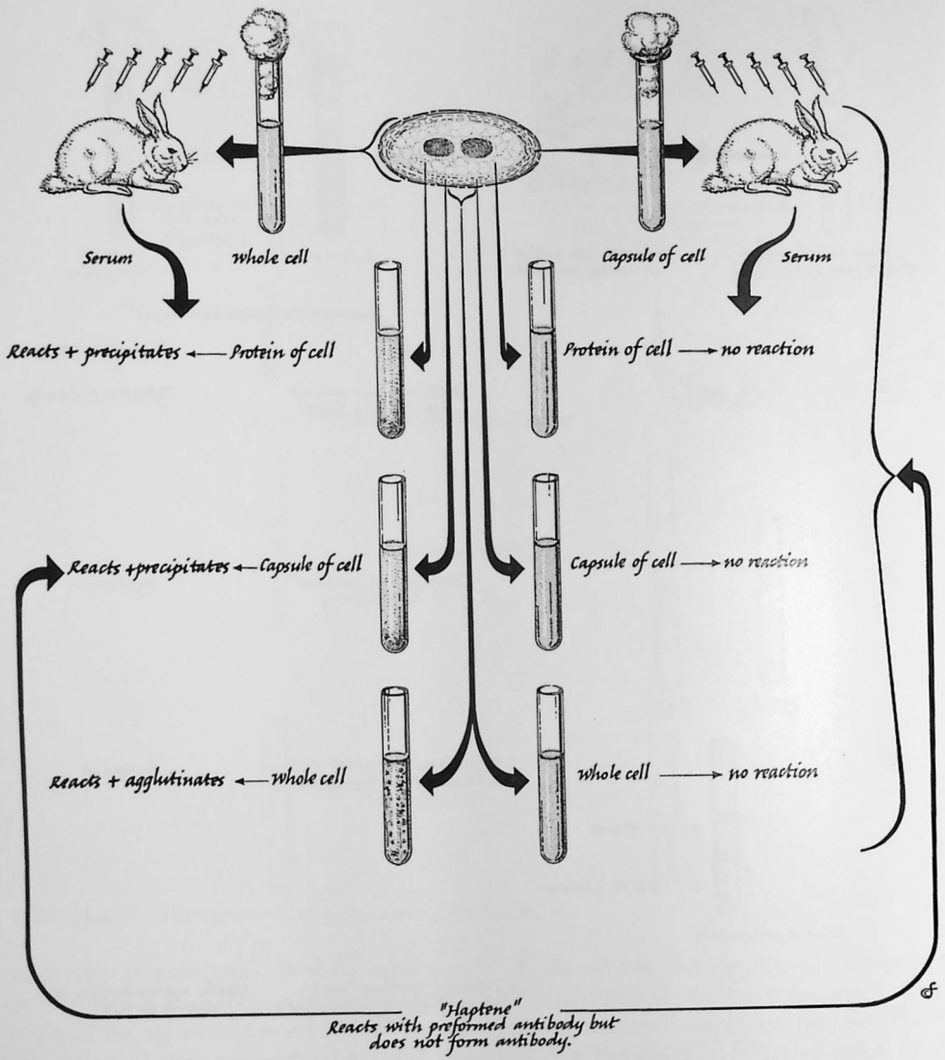


FIG. 24-10. *Haptene.*

cytes. If we watch such phagocytes, we see that they engulf more pneumococci than normally. That is, the serum contains something which increases phagocytosis.

We have summarized these experimental results in Fig. 24-9, where we have listed the names given to the "antibody" causing the reaction. This is probably the same antibody, but we are measuring it in different ways. In this case we have called forth the development of all of these different manifestations: agglutination, precipitation, lysis, and stimulation of

phagocytotic activity by the injection of a suspension of heat-killed pneumococcus type I cell.

Now, how is all this to be explained? The explanation is that upon the intimate entry of a foreign protein into the body (the antigen), the body reacts by forming a reactive protein in the blood (the antibody) which reacts only with the foreign protein which originally entered and, within some limits, discussed later) reacts with nothing else. This reaction between antigen and antibody may confer immunity to disease, and/or it may be manifest in many ways in the test tube, i.e., agglutination, precipitation, lysis, etc. Antibodies are formed by the entry of certain types of foreign protein; this protein may be dead or may be part of living cells.

Are all of these antibodies called forth by the injection of a pneumococcus the same thing? That is, is there a different antibody for each of the reactions which we have observed, or are each of these different things a different way of showing the presence of the same antibody? So far as our present knowledge goes, it appears as though each of these is a different way of demonstrating the same antibody.[1] Antigens are anything which will induce an antibody. They are generally proteins, but not all proteins are active in inducing antibodies. The bacterial cell may contain fifty different antigens. When we inject it, we may get fifty different antibodies. But for each antigen there is an antibody specific to it. In the blood antibodies are found primarily in the gamma-globulin fraction plasma protein. Globulins are proteins which are insoluble in water but are soluble in salt solution.

If we take the pneumococcus type I cell, inject it into a rabbit, and remove the serum from the rabbit, such serum will react with the whole cell. It is possible to fractionate the cell; for example, to prepare the capsular material and the soluble proteins from the cells. The antibody, obtained by the injection of the whole cell, will react both with the capsular material and with the soluble protein (Fig. 24-10). If we inject a rabbit with the capsule from the pneumococcus, we do not generate any antibodies. The antibodies that have been formed to the whole cell, however, react with such a capsule. Such a capsular material is called a **haptene;** that is, it is capable of reacting with the antibody but is not an antigen. In short, it is not capable of generating the antibody in a given host.[1]

Complement

In addition to all of these factors, blood contains a protein—really a mixture of proteins—which is called **complement.** Complement is destroyed by heating to 56°C for 30 minutes. Complement is necessary for certain reactions; in fact, in antigen and antibody reactions, complement is "fixed" or used up, even if the reaction will occur in the absence of complement. We can therefore detect antigen antibody reactions which do not agglutinate or precipitate by whether or not such reactions use up complement. In this case, however, we require a system for measuring complement. A very useful one is the following.

If we take the red blood cells from a sheep and inject them into a rabbit, we obtain antiserum from the rabbit which will dissolve or lyse the sheep's red blood cells (Fig. 24-11). So with the sheep's red blood cells plus antiserum from the rabbit, we will obtain lysis. If we heat

[1] One of the best pieces of evidence that they are all the same antibody is that it is possible to use up all the agglutinin in a given serum by adding the specific bacterial cells. These clump and use up the agglutinin in the process. We can add enough cells so that the supernate will no longer agglutinate the cells; we call this process "antibody absorption." When we have removed all the agglutinins by such specific absorption, we have also removed all the specific precipitins, all the opsonins, and all the hemolysins which may have been present in the original serum.

[1] Capsular polysaccharides can generate antibody in mouse and man, but not in the rabbit.

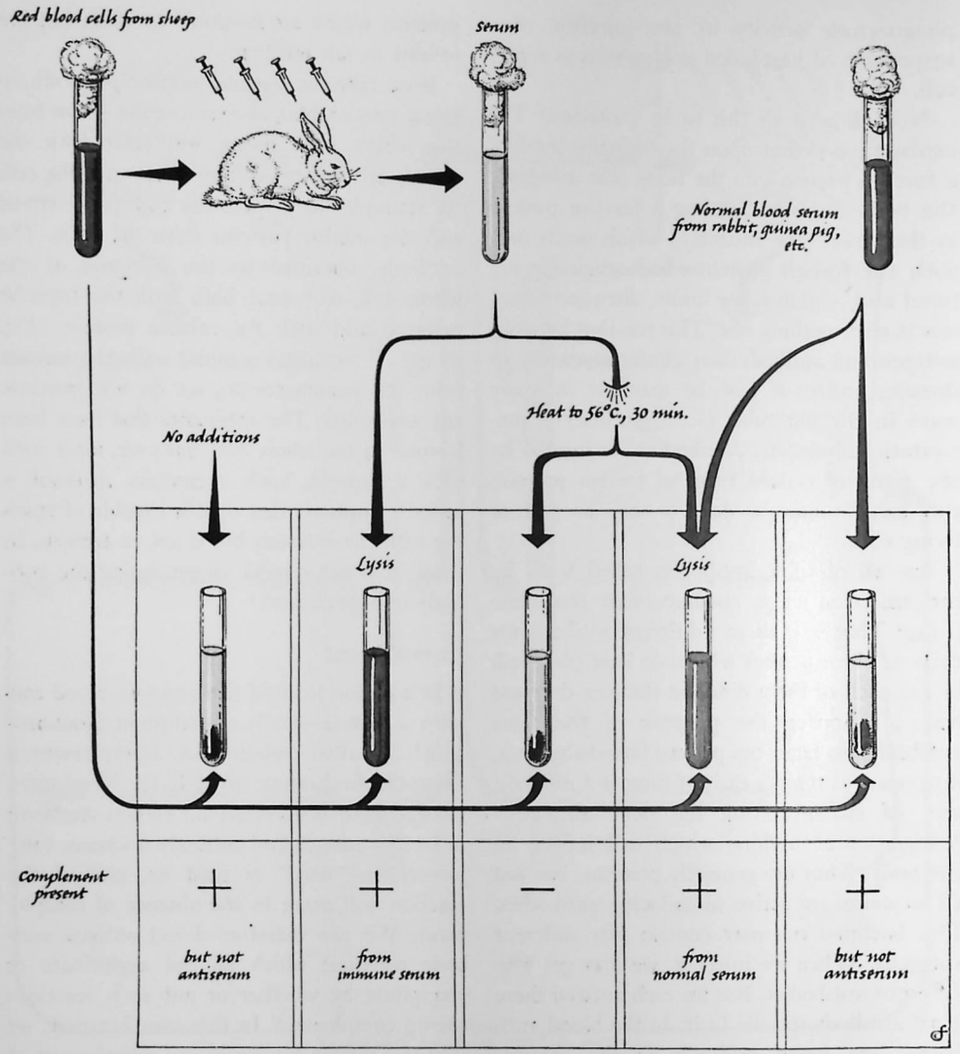


FIG. 24-11. *Hemolysin and complement.*

such serum from the rabbit to 56°C for 30 minutes, there is no lysis. If to this sheep's red blood cells plus heated rabbit antiserum we add fresh nonimmune serum or some other source of complement, lysis is restored. This can therefore be used for a system of detecting the complement. If there is no lysis, there is no complement. If lysis has occurred, complement is present.

For example, we have an antigen and a heated antibody and a known amount of complement mixed together, and we wish to determine whether a reaction has occurred among them. After a suitable incubation period to

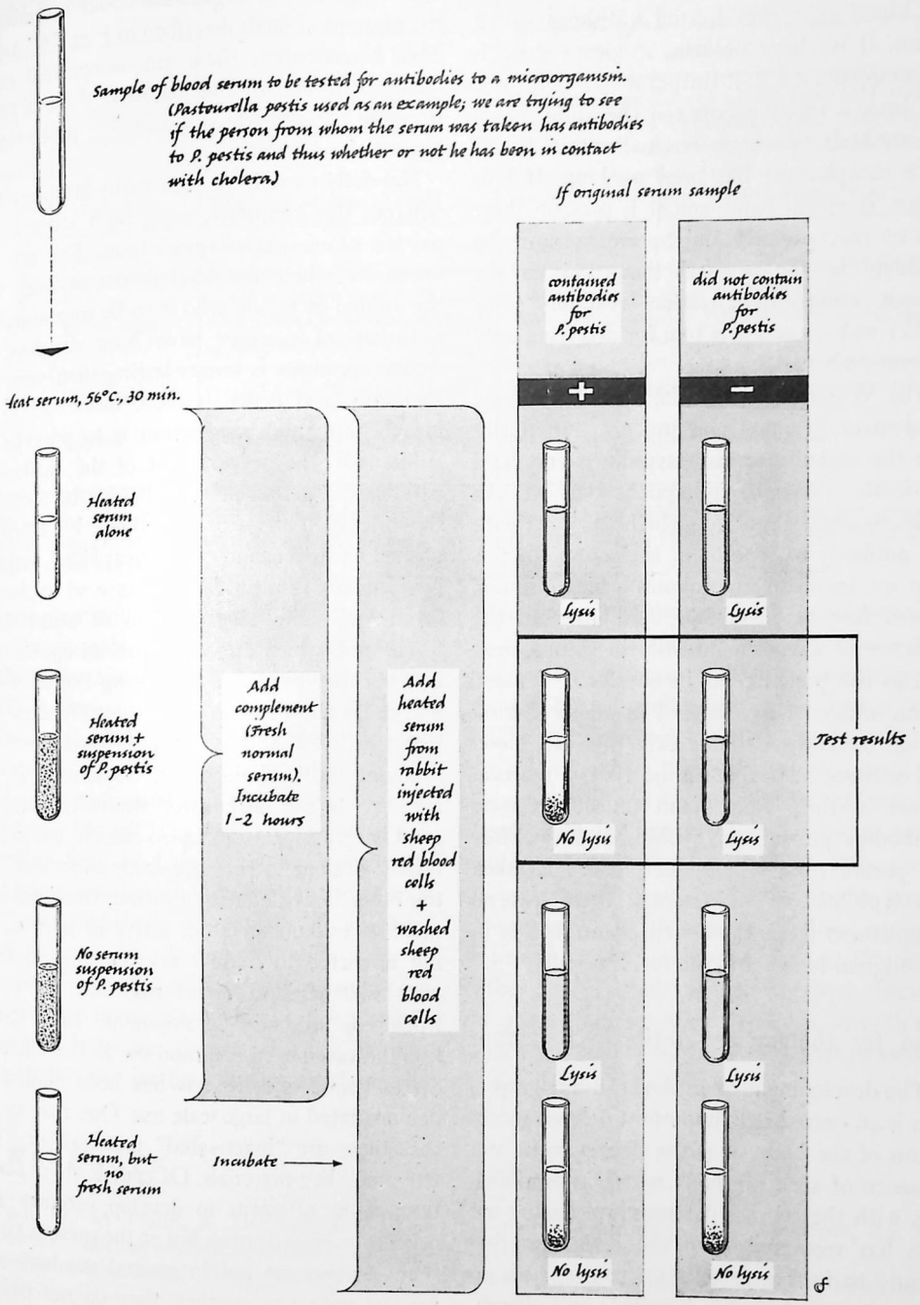


FIG. 24-12. *The complement fixation reaction.*

permit such a reaction to go on, we add sheep's red blood cells plus heated antisheep rabbit serum. If we have no lysis, it means there is no complement left; it further means that there has been a reaction between the antigen and the antibody which we originally started with since complement has been used up. If lysis occurs, it means complement is present; there was no reaction between the antigen and the antibody in the first reaction. This is the famous **complement-fixation reaction** (Fig. 24-12) widely used as a tool for detecting antigen-antibody reactions.

The Wassermann test for syphilis is based fundamentally upon this process, but in this case the organism causing syphilis is very hard to obtain; however, it happens that we can make an artificial antigen which will react with the antibody to syphilis in the serum. In this case we incubate an unknown blood serum, heated first to 56°C for 30 minutes, in the presence of a known quantity of complement and in the presence of the so-called "Wassermann antigen." At the end of an incubation period we add red blood cells plus red blood cell antiserum. If there is no lysis, a reaction occurs in the Wassermann test; there were antibodies present for syphilis and therefore the person from whom the blood was taken was syphilitic. If lysis occurs, there was no complement fixed, there were no antibodies in the original blood, and the test is negative.

Uses for the Antigen-Antibody Reactions

The development of antibody to foreign protein is an exceedingly important defense mechanism of the body. It is the development and presence of such antibody which in combination with the cellular defenses give the person who has recovered from the disease an immunity to further attacks. Of course, such an immunity is relative and under some circumstances it may be overcome, just as an overwhelming dose of organisms could overcome the immune animals described in Figs. 24-5 and 24-6. Nevertheless, these are exceptional circumstances, and in the majority of cases the presence of antibody is a valuable protection against the disease.

Naturally there have been many attempts to enhance this immunity, using both active and passive immunization procedures. For active immunity, where one develops the antibody in the animal or person who is to be immunized, a variety of vaccines have been developed. Active immunity is longer lasting than passive immunity and hence is more desirable. The idea behind most vaccination is to provide a stimulus to the development of the antibody without the person actually having to acquire the disease. This may sometimes be accomplished by suspensions of the heat-killed pathogenic microorganism. In the case of typhoid fever, for example, suspensions of heat-killed *S. typhosa*, which are, of course, incapable of invading the body and setting up the disease, will cause the body to generate antibodies to living cells of *S. typhosa* and thus provide immunity to typhoid fever. Sometimes it is necessary to use "attenuated strains" of pathogenic organisms, i.e., strains which are only mildly virulent so they are easily overcome by the other body defenses. Further, the development of immunity, either active or passive, is not restricted to cellular microorganisms; but virus vaccines and toxins may also be controlled by antibody development. Table 24-1 lists the vaccines in common use in the United States whose effectiveness has been clinically demonstrated in large-scale use. One may note that these are "inactivated" materials or "attenuated" live materials. Of course, there have been many attempts to develop vaccines for other infectious diseases but at the present time such vaccines are not in general use because, for one reason or another, they do not prove to be effective clinically. The use of vaccines for the development of active immunity is a primary example of the application of antigen antibody reactions in the prevention (**prophylaxis**) of disease.

Passive immunity is generally most used for therapy. It is, on occasion, useful to supply antibodies preformed and thus active immediately even though they may not persist in the blood as long as actively acquired antibody. The administration of diphtheria antitoxin to a child with diphtheria may save its life. Further, in many cases we do not yet know how to produce an active immunity, thus convalescent serum, i.e., from blood of persons recently recovered from the disease, may be helpful. And there are further situations where the protection is needed immediately, and we cannot wait until the body has had a chance to build up active immunity. Passive immunity is thus the second application of antibodies, especially in the treatment of disease.

If an individual has been infected with a microorganism and has reacted to this microorganism, he will carry within his blood antibodies to the microorganism. We can then use the presence of these antibodies, under some circumstances, to determine whether or not he has or has had a specific disease. We can further study populations to see how disease has spread among them. This is the third application of antibody reactions, that of assistance in diagnosis.

TABLE 24-1. *Vaccines in Widespread Clinical Use*

	DISEASE	VACCINE	REMARKS*
BACTERIA	Typhoid fever	Heat-killed cells phenolized	Generally combined with related *Salmonella* strains
	Whooping cough	Heat-killed cells precipitated with alum	Usually combined with diphtheria and tetanus toxoids, usually given at 2, 3, or 4 months of age, again at 1½ and at 4 years
	Tuberculosis	BCG, live, attenuated strain	Statistical studies show 70–80% effectiveness
VIRUS	Smallpox	Cow pox; low virulence, live form	Oldest form of vaccination
	Poliomyelitis	Formaldehyde-inactivated tissue culture-grown virus	Live virus vaccines claimed to be better, but vaccine not yet in clinical use in United States
	Yellow fever	Live virus of attenuated strain, tissue culture-grown virus	Virus seems similar to yellow fever virus but does not grow to as high a titre
TOXIN	Diphtheria	Toxoid (inactivated toxin)	See Whooping cough } For adults whooping cough is eliminated; the combined toxoids given every 4–5 years
	Tetanus	Toxoid (inactivated toxin)	See Whooping cough }

* In addition, vaccine for scarlet fever has been widely used. Influenza vaccine is also used on occasion but because the virus changes its antigenic character rapidly the vaccine must be prepared from the strains current at the time of use. Measles vaccine has just been developed.

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For background information, see p. 495.

QUESTIONS

1. Define or identify:

exotoxin	constitutive resistance	acquired immunity	Wassermann test
endotoxin	host resistance	vaccine	antitoxin
hemolysin	phagocytosis	active immunity	antigen
coagulase	plasma	passive immunity	antibody
streptokinase	serum	leucocytes	agglutination
reticuloendothelial system	erythrocytes	polymorphonuclear leucocyte	precipitin
	hemoglobin		opsonin
lymphatic system	positional resistance	microphage	haptene
pus	adaptive resistance	histocyte	complement fixation
		complement	attenuated strains

2. Taking the facts given in Figs. 24-5, 24-6, 24-7, 24-8, and 24-9, which form the basis of the study of immunology, it is possible to devise any of a number of theories to explain antigen-antibody reactions. What are some that have been proposed? What is your explanation?
3. Red blood cells contain proteins characteristic not only of the species but also of the individual. In the blood of many people there are also "natural" antibodies which clump certain blood cells and not others. These blood types are of importance in blood transfusions and in many other ways. These antibodies are called isoagglutinins. How could these be distinguished, if only by definition, from bacterial agglutinins?
4. Active immunity does not develop immediately but requires recurrent or continual contact with the antigen. It may begin to be evident in a few days and effective after some weeks. It then lasts for months or years depending upon the nature but may generally be restored by further contact with the antigen (booster dose). Passive immunity is complete immediately but lasts for only a few weeks at most. What would you recommend for a childrens' home which has just discovered a case of diphtheria?
5. Antibodies are highly specific, i.e., within rather narrow limits they will react only with the antigen which formed them. A similar specificity is shown by enzymes. In what ways are antibodies and enzymes alike? How do they differ?

Chapter 25

THE PATHOGENIC COCCI–RESPIRATORY DISEASE

"They have no doctors but bring their invalids out into the street where anyone who comes along offers the sufferer advice on his complaint, either from personal experience or observation of a similar complaint in others. Anyone will stop by the sick man's side and suggest remedies which he has himself proved successful in whatever the trouble may be or which he has known to succeed with other people. Nobody is allowed to pass a sick person in silence; but everyone must ask him what is the matter. They bury their dead in honey and their dirges for the dead are like the Egyptian ones."

HERODOTUS, *On the Babylonians*, Book 5

WE SHALL BEGIN our consideration of the bacteria capable of causing disease with a clear, morphological group: the cocci. We have earlier (pp. 136 and 140) outlined how they were recognized and separated into genera. But we shall now require more detailed consideration, at least of several of the groups; among them some are pathogenic, others not, and it is of the greatest importance to be able to recognize those capable of causing disease. Figure 25-1 shows how the cocci may be separated into seven main genera, four of which contain pathogens. It is with these four genera: *Staphylococcus*, *Streptococcus*, *Diplococcus*, and *Neisseria* that this chapter is concerned. It happens that most of the diseases caused by these organisms (listed in Fig. 25-1) are respiratory diseases, the exceptions being gonorrhea and some of the staphylococcic infections. As a general assumption, we may consider that the organisms enter the body via the respiratory tract; that they grow in the respiratory tract, that they exit via the respiratory tract, and that the principal route of transmission is droplet infection or dust in air. Many respiratory diseases are caused by cocci, but, of course, there are organisms others than cocci which cause respiratory diseases—other bacteria and, especially, viruses.

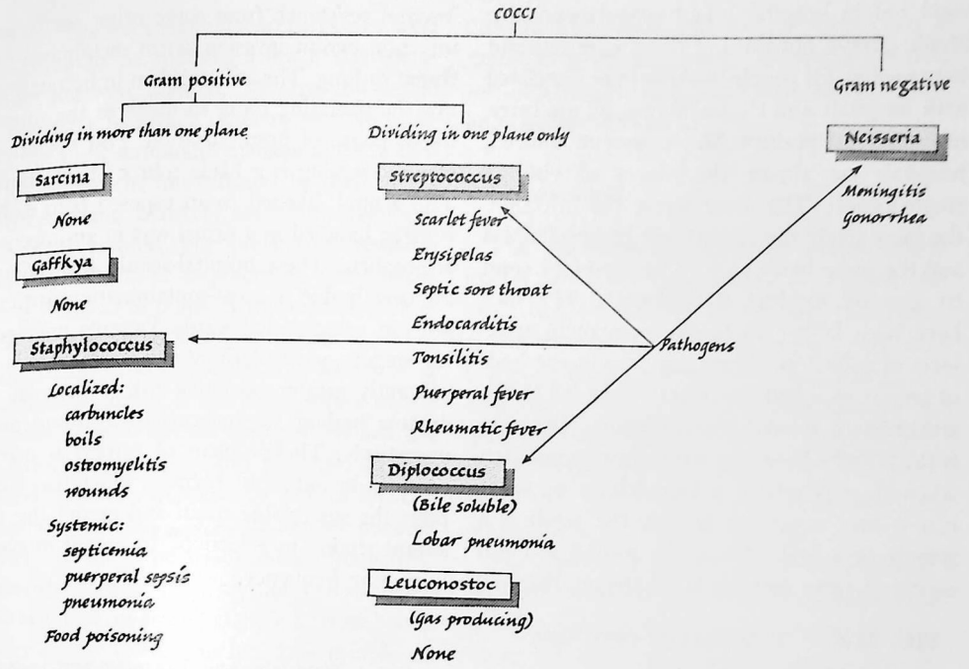


FIG. 25-1. *The kinds of cocci.*

As noted in the introduction to this Part, we are not concerned with the nature of the disease produced, nor with any of the purely medical aspects of the problem of pathogenicity, and for this information you must go to a textbook of pathogenic or medical bacteriology; there is simply no room here. But we will include a short note on diagnostic aids of interest to a bacteriologist because these do have a distinct relation to microbiology and are part of the knowledge he is expected to possess.

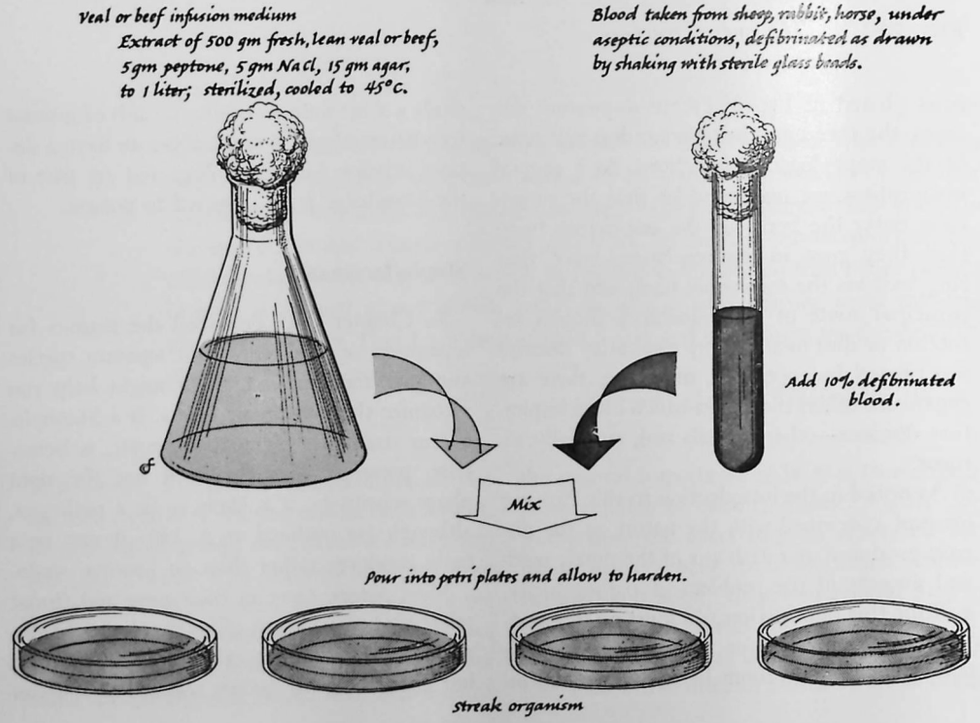
Staphylococcus

In Chapter 7 we described the reasons for separation of this genus into separate species and gave some criteria which might help you recognize the pathogenic forms. If a *Staphylococcus* strain has pigment (orange), is hemolytic, possesses coagulase, and has the right phage sensitivity, it is likely to be a pathogen, although (as outlined on p. 136) it may be a toxin producer rather than an invasive strain.

Most people carry in their nose and throat relatively benign *Staph. albus* or colorless *Staphylococcus* (sometimes called *S. epidermidis*), but about half the people selected at random

(and not in hospitals) had coagulase-positive *Staph. aureus*; one-third of them were resistant to penicillin. Of people working in or associated with hospitals and clinics, almost all are carrying coagulase-positive *Staph. aureus*. Indeed, hospitals are almost the Mecca of virulent staphylococci. The more severe the infection, the more likely the patient will be hospitalized and the more likely he is to be exposed to and to give off virulent staphylococci. Hospitals have been known to be the permanent reservoirs of infection. Of course, present methods of asepsis, especially in surgery, have led to the reduction of wound contamination. The skin is the primary barrier to staphylococci and even when they penetrate a hair follicle or sweat gland, they rarely get farther; the result is a pimple or a boil. Hence, the wound is a real source of entry into the blood stream; that and lowered resistance from some other source or infection permit invasion across membranes of throat or lung. The real problem in hospitals is not the operating room so much as the subsequent phase of hospitalization. Post operative wound dressing is a likely source of infection. The wound, likened to an exposed petri dish, is often handled in a casual way in an infected atmosphere. These hospital-acquired infections are usually due to cross-contamination particularly in overcrowded wards. Patients undergoing surgery, particularly of the chest, incur significantly greater operative risk if their nasal mucosa harbors staphylococcus organisms preoperatively. The problem of carriers is made complex by antibiotic therapy, which may suppress the susceptible strain and permit the resistant strain "by selection" to mount in ever-increasing frequency.

FIG. 25-2. *Preparation of blood agar.*



Streptococcus

If patients with staphylococcus lesions were considered to be suffering from a contagious disease and were isolated, the organism would be less likely to invade other victims. In addition, the most scrupulous of techniques in sterilizing equipment must always be used. Dust, mattresses, and blankets are a few potential sources of trouble, but preventive measures here are difficult or impractical. The human carrier is the focal point. A nurse or other hospital attendant who is a nasal carrier of pathogenic staphylococci, particularly those of a drug-resistant strain, is one likely source of the common hospital nursery epidemic. When the staphylococcus is a hospital problem, as in a nursery epidemic, it would be well to identify the strain and then check the various possible carriers for the same strain. Not infrequently, the newborn becomes an asymptomatic carrier, only to infect his family months later at home. To complicate matters further, there is evidence that the disappearance of nasal staphylococci in a carrier, either spontaneously or by treatment, renders the colonization by resistant strains easier. For the final conquest of microbial disease the reservoir of infection must be wiped out. This means prompt and vigorous treatment of patients with clinical staphylococcus infections, concerted efforts on isolation techniques, separation of carriers from strategic positions, and meticulous handling of all aspects of hospitalized patients. In this way the "ubiquitous golden clump of germs" may be reduced to a second-rate power.

Diagnosis. The isolation of staphylococci is not difficult. Blood agar is the medium of choice since it enhances the yellow-orange color of *Staph. aureus* and permits detection of β-hemolysis (clear zone type).[1] *Staph. aureus* can grow in 7.5%NaCl, and, since fermentation of mannitol is an index of pathogens, a selective medium may be made using salt, mannitol, and an acid-base indicator.

[1] Hemolysis is of two types. One, called α, is a greenish cloudiness usually close to the colony. The other, called β, is a completely clear zone about the colony. Since the reactions are difficult to reproduce on the printed page, we shall rely upon your seeing them in the laboratory.

Streptococcus

The chain-forming cocci are found widely distributed in nature, and this is precisely the problem. Some of them are serious pathogens; others are not only harmless but are even necessary. How, aside from the production of disease, can we detect the pathogens? In the beginning of the problem, it was thought that the pathogens attack blood, causing hemolysis; hence the "hemolytic streptococcus" was considered to be the dangerous group.

The types of hemolysis occurring in blood, especially on blood agar (Fig. 25-2), have come to be recognized characters of certain bacteria. There has been the persistent notion that β-hemolysis is associated with pathogenicity of a rapid acute form while α-hemolysis is associated with pathogenicity of a chronic type. Actually there is no relationship of this sort. The significance of hemolysis is not clear nor is it related to pathogenicity except that among the streptococci all the pathogens are hemolytic (either α or β); but many streptococci are hemolytic which are not pathogens. Many other organisms—most aerobic spore-formers, for example —are strongly hemolytic, but not pathogenic in the least. Therefore, hemolysis is no more a character of pathogenicity than fermentation of lactose or any other criterion; many nonpathogens, indeed many nonpathogenic streptococci, are actively hemolytic. A variety of bacterial products can cause hemolysis, but only some of these are related to pathogenicity.

Certainly some other character than that of action in blood must be used if we are to gain any separation of the streptococci which may be useful for distinguishing the human pathogens. After a great deal of study of strepto-

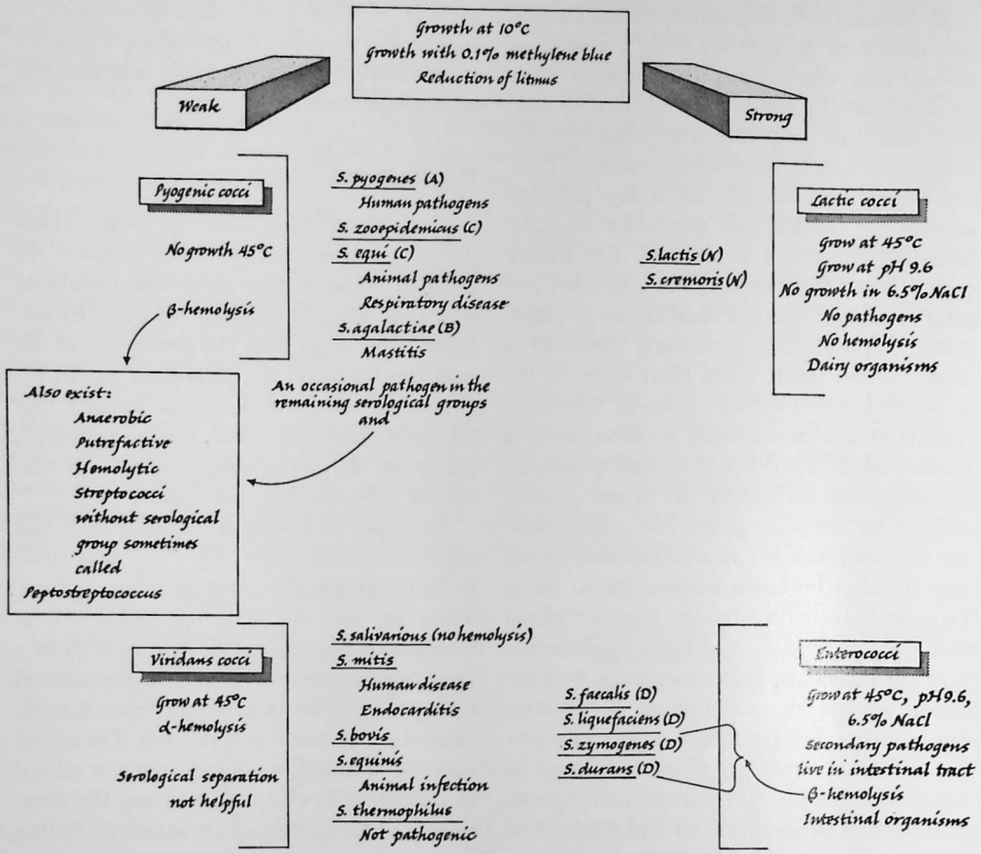


FIG. 25-3. *The streptococci.*

cocci found in milk, in plants, in the intestinal tract of man and animals, and in diseases of man and animals, it turned out that those streptococci capable of causing disease were rather the weaklings of the group; they would not grow at low temperatures, or in the presence of salt, or under other extremes of environment. It therefore became possible to separate the streptococci into several large groups, corresponding in all but a few details to their origin and function in nature. These relationships are outlined in Fig. 25-3. It is only the weak groups, the **pyogenic** (pus-producing) cocci and the viridans group which contain pathogens; but there are many strains—or species, if we wish to separate them—in each of these groups which are not pathogenic. Further separations of the pyogenic group are based upon immunological methods, especially by the use of antisera.

Once we have delineated, on the basis of physiological properties, a group of streptococci that might be likely to be pathogens and separated them from the larger and more rugged groups which are definitely not pathogens, hemolysis now becomes of some importance.

Neisseria

Most of the pathogens of this group fall into the β-hemolytic type of hemolysis; a few, associated with characteristic diseases, are α; any nonhemolytics found within the "weak" organisms are clearly not pathogens. But even in the group of sensitive, β-hemolytic streptococci, there are many nonpathogens living harmlessly in respiratory and genital tracts. At this point, a serological separation is necessary. It happens that there exists in the cell wall of these streptococci a carbohydrate material which can be extracted from the cells by boiling at *p*H 2, and can then be precipitated by antisera prepared by immunizing rabbits against suspensions of heat-killed streptococci. By this means the streptococci can be separated into a number of groups, of which around 10 contain the organisms which physiologically are "pathogen-like" —the "weak" group of Fig. 25-3. And of these ten, four groups—A, B, C, D—contain virtually all of the pathogens. Group A contains all the serious ones for man, while B, C, D contain organisms causing disease in animals, rarely in man. Further separation of group A into "types," of which there are about forty, are not our concern here. A brief summary of the diseases associated with streptococci is given in the list below. Further information must be sought in textbooks devoted to pathogenic microbiology.

Diseases Caused by Streptococci

Erysipelas	Tonsillitis
Septic sore throat	Arthritis
Puerperal fever	Endocarditis
Rheumatic fever	Mastitis
Scarlet fever	

However, while there has been a tendency not to use species names, and to differentiate the streptococci as little as possible, once their serological group was determined, there are many names commonly associated with various kinds of streptococci which it is worth while to mention. We have outlined the main characteristics of the separate species in Fig. 25-3.

Diplococcus ("Pneumococcus")

The cocci of this type occur characteristically in pairs or short chains, but fortunately there is an additional criterion usable if one cannot decide whether the chain is short enough to be a *Diplococcus* or long enough to be a *Streptococcus*. That criterion is that cells of the *Diplococcus* are lysed or dissolved by bile salts and other surface-active agents. *D. pneumoniae*, responsible for at least 80% of human pneumonia is capsulated. There are known to be at least 75 different types of capsules distinguished immunologically. But types I, II, and III are those most commonly associated with pneumonia; the other 70 odd are less frequently pathogenic. We have earlier used capsulation on the pneumococcus as an example of both immune response (p. 419), R ⟶ S ⟶ R variation (p. 207), and transformation (p. 205); hence further discussion is superfluous.

Another organism associated with causing most of the pneumonia not caused by the pneumococcus is *Klebsiella pneumoniae* (Friedlanders bacillus) which is a gram-negative, encapsulated rod, closely related physiologically with *Aerobacter* (see Chapter 17). Since pneumonia means only invasion and inflammation of the lung, any organisms causing such inflammation can cause pneumonia, but *D. pneumoniae* and *K. pneumoniae* cause most of it, especially lobar pneumonia (invasion of the lung tracheae itself). Pneumonia due to *Staphylococcus* is currently increasing.

Neisseria

These are the gram-negative cocci and as a group owe their importance to two members: *N.*

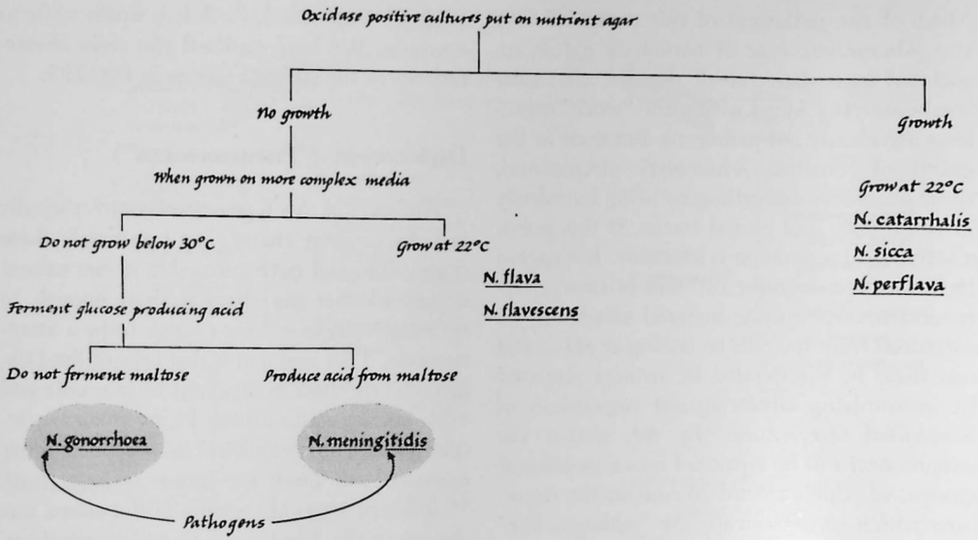


FIG. 25-4. *The important species of* Neisseria.

gonorrhoeae (the gonococcus) and *N. meningitidis* (the meningococcus), sometimes called *N. intracellularis*. The meningococcus causes meningitis, an inflammation of the membranes covering the brain and spinal cord. The gonococcus causes the venereal disease gonorrhea, which was such a common cause of blindness shortly after birth that by law *all* infants have their eyes treated with silver nitrate to prevent any possibility of blindness from this cause.

As a group, these organisms are especially sensitive to heat and drying; the pathogenic members do not grow below 30°C. There are several nonpathogenic species (Fig. 25-4) occurring in the nose, throat, and on the genitals, which sometimes cause confusion with the pathogenic ones. *Neisseria* as a group may be detected by the "oxidase test." This consists of cultivation of the suspected material on heated blood agar—called chocolate agar because of its color—usually in 10% CO_2. After growth, a 1% solution of tetramethyl-p-phenylenediamine is sprayed or dropped onto the plate. The colonies which are high in cytochrome oxidase, as are the *Neisseria*, turn bright purple black because the cytochrome is oxidizing the substituted diamine to a pigment. This itself does not kill the cells and they can be subcultured if this is done within 10 minutes. Such a subculture once grown is tested for temperature of growth, growth on nutrient agar, and glucose and maltose fermentation. But in gonorrhea, and especially in meningitis, a more immediate test is needed. Therefore, smears of urethral or vaginal discharges (for gonorrhea) or spinal fluid (for meningitis) are first examined for gram-negative diplococci *within cells*—generally within polymorphonuclear leucocytes; this, coupled with a positive oxidase test, is generally the basis of laboratory diagnostic procedures.

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For background information, see p. 496.

QUESTIONS

1. Define or identify:

droplet infection	α-hemolysis
coagulase	streptococcus group A
hemolytic	bile solubility
cross-contamination	Friedlander's bacillus
carrier	oxidase test

2. As the officer in command of an Army hospital, you find that a virtual epidemic of "strep throat" is rampant in the hospital, but not generally throughout the base. What would you suspect and what could you do about it?
3. You are given a gram-negative culture in which most of the organisms appear diploid. What further information would you require before you could make an estimate of its disease potential?
4. Scarlet fever is caused by a streptococcus that produces a toxin which causes a red rash. It is sometimes difficult to distinguish from measles, but a test consisting of the injection of a small quantity of antitoxin in the skin is useful in diagnosis. This so-called Schutz-Carlton reaction is positive—i.e., the patient has scarlet fever—if the redness fades. Why does this test work?
5. Some of the α-hemolytic pathogenic streptococci grow in and on the heart valves, causing an endocarditis. When this is acute, the patient dies rapidly. But the disease can also take a subacute form, generally fatal. The organisms are shed into the blood stream. In most cases, the disease seems to be caused by *Streptococcus mitis;* next in number of cases is *S. salivarius* and, rarely, *S. facealis*. How would you differentiate between the first two and the last?

Chapter 26

THE PATHOGENIC GRAM-POSITIVE RODS– DIPHTHERIA, TUBERCULOSIS, LEPROSY

2. When a man shall have in the skin of his flesh a rising, a scab, or bright spot . . . he shall be brought unto Aaron the priest.
3. . . . and when the hair in the plague is turned white and the plague in sight be deeper than the skin of his flesh, it is the plague of leprosy . . .

Leviticus (Chapters 13 and 14 deals with leprosy)

THE LAST CHAPTER considered the pathogenic cocci which were, with two exceptions, all gram positive. This chapter is concerned with the gram-positive rods. (There are no gram-positive spirals.) The gram-positive rods may be first divided upon the basis of spore formation, as illustrated in Fig. 26-1. Of those without spores, *Lactobacillus* and *Propionibacterium* are not pathogens. *Listerella* contains mostly animal pathogens. These gradually shade off into "higher bacteria" which possess the character of pleomorphism with possible branching; of the two genera of this type, *Corynebacterium* contains the pathogens causing diphtheria and *Mycobacterium*, a genus which is in addition acid-fast, contains pathogens causing tuberculosis and leprosy. These shade off further (described on p. 281), by way of branching and longer mycelium, into the actinomyces ("proactinomyces"), and these, by addition of spore

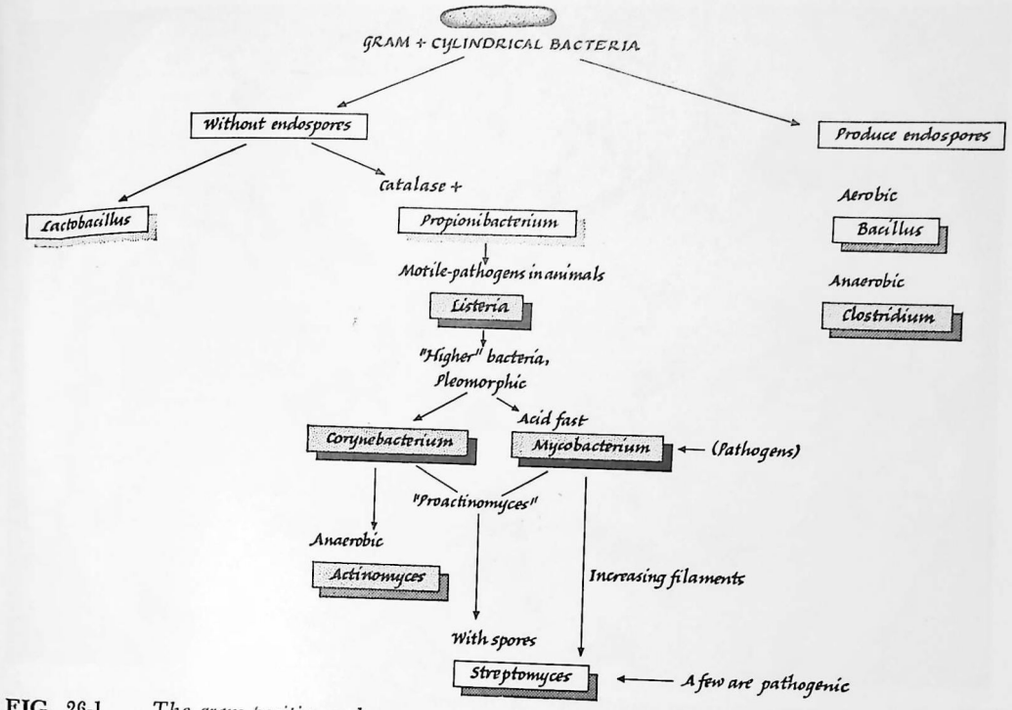


FIG. 26-1. *The gram-positive rods.*

formation (not endospores) into the streptomyces. These higher organisms contain some pathogens—lumpy jaw in cattle, potato scab in plants; but they are probably of minor significance compared to such titans as diphtheria and tuberculosis. All of the human pathogens occur, not in the true bacteria, but in the higher forms.

Of the gram-positive rods capable of forming endospores, most are nonpathogens, but in each genus some pathogens exist. *Bacillus anthracis* is the cause of anthrax. Various *Clostridium* species are responsible for tetanus, gas gangrene, and botulism.

Corynebacterium

The name *Corynebacterium* was intended to convey the idea that one would be likely to find club-shaped forms in culture. The organisms are indeed characteristically pleomorphic (i.e., show many forms) (Fig. 26-2) and stain unevenly with methylene blue. Adjacent cells tend to be at any angle to one another, forming V and L and even Y forms. Most **diphtheroids**, as members of the genus *Corynebacterium* are sometimes designated, grow well on nutrient agar or broth, but growth is better and most abundant on heated serum media (Loeffler's medium). Potassium tellurite (0.04%) added to media tends to inhibit the growth of most bacteria, while having little effect upon *Corynebacterium*, and thus serves as a convenient medium for isolation. Pathogenic strains usually form gray or black colonies, easily distinguished from others.

There appear to be two large habitats of diphtheroids: first, on or associated with man and animals, the skin, the genitalia, the throat;

FIG. 26-2. Corynebacterium diphtheriae. *Methylene blue stain. Note the banded cells, the club-shaped cells, and the very large pleomorphic cells.* [*Courtesy of Professor William Burrows, Dept. of Microbiology, Univ. of Chicago.*]

second, in soil, especially soil to which little organic matter has been added—fallow soil or one in which lignin and humus are the principal organic sources. These latter diphtheroids grow slowly; it takes perhaps a week for them to grow, and they grow less readily at 37°C; hence they are not often mistaken—nor indeed often found—in a search for pathogens. Most of these soil diphtheroids do not ferment glucose, although they oxidize it slowly, and none are believed to be pathogens. The rest of the diphtheroids, living on the body, grow well at 37°C, and ferment glucose and usually maltose with acid production only.

Before going further, it is necessary to describe briefly some of the characteristics of diphtheria, the principal disease caused by the pathogenic body-living corynebacteria. This is an infection of the throat, tonsils, or nose, resulting in marked patches of a grayish membrane from which the diphtheria bacillus is readily cultured. The organism elaborates a toxin which causes the symptoms and introduces toxicity to the body. The typical toxic symptoms, the presence of a throat "membrane" coupled with the isolation of typical colonies, and typically appearing smears from both throat and isolated colonies, are diagnostic. Other diphtheroids having similar cultural properties, but obtained without symptoms of disease, are found on body surfaces, and these can frequently be distinguished only with difficulty from the pathogenic diphtheria organism.

Corynebacterium diphtheriae exists in three distinct types—gravis, mitis, and intermedius,

TABLE 26-1. *Types of Corynebacteria diphtheriae*

	Gravis	Intermedius	Mitis
Morphology	Short; stains more evenly	Barred forms, clubbed ends	Long, metachromatic granules
Nutrient broth	Pellicle or fine turbidity or flakes	Fine granular turbidity, settles to bottom	Uniform turbidity; pellicle late, if any
Colonies	Hard	Intermediate	Soft
Pathogenicity	All strains pathogenic to guinea pig; moderate to mice	10% of strains nonpathogenic to guinea pig; low pathogenicity to mice.	10–20% nonpathogenic to guinea pig; high pathogenicity for mice
Antigenicity	Two main antigenic groups	Antigenically homogeneous	Antigenically diverse

the properties of which are listed in Table 26-1. The gravis type is the most serious. As dramatic as the story of the discovery of diphtheria toxin itself (see, for example, Chapter 6, in *Microbe Hunters* by De Kruif) is the discovery of the gravis type. A diphtheria epidemic, beginning in central Russia, was moving westward in the 1930's. It was more severe and caused by something different than the known diphtherias. While antitoxin helped, it did not stop the epidemic. It rolled westward, across Poland, into Germany, to the borders of France, and was stopped only when antitoxin from the gravis strains, isolated from the epidemic, was employed.

Mycobacterium

The other pleomorphic group of gram-positive rods, the *Mycobacterium*, has the additional and characteristic property of acid-fastness. The two main diseases caused by mycobacteria are tuberculosis and leprosy. The organisms causing tuberculosis were discovered by Koch in 1882, and those of leprosy even earlier, in 1878, by Hansen; this was before their acid-fast property was known. The tuberculosis organisms are of three fundamental types—human, bovine, and avian. These do not all infect all species (Table 26-2).

The disease caused by the tuberculosis organisms is not the same in all species. For example, in the human, tuberculosis is really three diseases simultaneously. First, there are organisms in the blood stream. Second, there are organisms within the cells, especially in the lungs. Third, there are organisms walled off in nodules or tubercles, especially in the lung. In the mouse, the disease is mostly the first one; essentially all the organisms are in the blood stream. The disease in the guinea pig resembles that in man most closely, so that this is the animal of choice for studies. Most of the tuberculosis caused by the human strains is a respiratory disease, especially infections of the lung, transmitted by droplet infection through the air. Most of the tuberculosis caused by the bovine strains is a disease of the intestinal wall, bones, or joints, and is transmitted via milk from in-

TABLE 26-2. *Tuberculosis Organisms*

	Human	Bovine	Avian
Man	++++	++++	—
Guinea pig	++++	++++	—
Rabbit	±	++++	++++
Mouse	++	++++	++++
Cattle	±	++++	±
Swine	±	+	++++
Fowl	—*	—*	++++

* But do infect parrots!

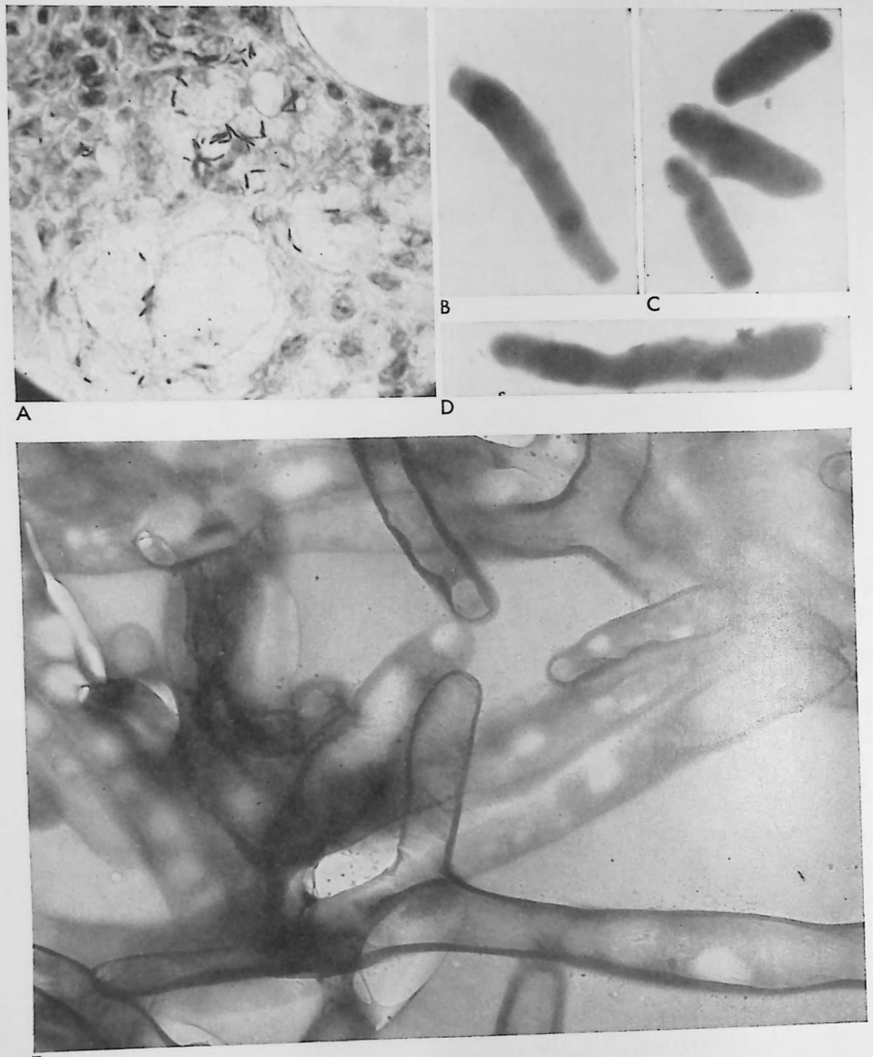


FIG. 26-3. Mycobacterium tuberculosis. *A is the photograph of a tissue section of a mouse infected with an acid-fast tuberculosislike organism and stained with the acid-fast stain. Note how adjacent cells seem to be at angles to each other, as if they underwent some kind of a "snapping" division. Note also how they stand out from the tissue and their somewhat pleomorphic nature. B is the human variety of* M. tuberculosis *isolated from a human patient, photographed in the electron microscope. C is* M. leprae; *D is* M. smegmatis, *a nonpathogenic acid-fast bacterium. E is an electron micrograph of* M. bovis, *which shows vacuoles and branching. However, branching is rather rare among the Mycobacteria and in this case it has been induced by growth on a magnesium-deficient medium. [A is courtesy of Dr. M. Solotorovsky. B, C, and D are from Rosenblatt, Fullam, and Gessler,* Amer. Rev. Tuberc., **46,** 587 (1942), ASM-LS21. *E is from Spitznagel and Sharp,* J. Bacteriol., **78,** 453 (1959); *courtesy of Dr. D. G. Sharp.]*

fected animals. The avian disease is not found in man, but is found in the hog; chickens and hogs spread this type of tuberculosis among themselves rapidly.

Tuberculosis progresses slowly and a body reaction occurs to it, which takes several forms. First,there is the engulfing of the organisms by the phagocytes (Fig. 26-3). However, they are generally unable to digest the tubercle organisms or kill them. Second, when the laden phagocytes collect in nodules, a caseous or cheeselike layer may be laid down around them, forming a tubercle. This walling off of the active cells is an attempt by the body, frequently successful, to dispose of the microorganism and render it harmless. Third, there develops in the blood a kind of antibody. This antibody can be detected by the **tuberculin test:** the injection of a small quantity of the old media on which *M. tuberculosis* has grown, or extracts from the cells themselves, causes a reddening and swelling in infected persons. The tuberculosis infection responsible for the development of sensitivity may have occurred from six weeks to many years earlier. The infection may be inactive, it may be active, it may be of recent origin and not yet evident as tubercles, or it may be a fibrotic calcified nodule resulting from an infection years ago. There is no evidence that a positive tuberculin reaction indicates any protection against the organism; it only means that some time in the past the individual has come into intimate contact with *M. tuberculosis.* In the past, when tuberculosis was widespread, almost everyone had come in contact with the organism and most of the population showed positive tuberculin reactions. Tuberculosis infection was an inevitable consequence of attaining adult life.[1] Because of this, a positive tuberculin reaction had little meaning; today, it means much more. In 1917 in the United States 70% of school children had positive tuberculins; today, it is about 5%. A positive tuberculin test, especially in a child, generally means the beginning of active tuberculosis.

Diagnosis of tuberculosis, bacteriologically, depends on finding the typical acid-fast organism in the sputum or other lesion. More generally chest X-rays are used to attempt to locate tubercles. There is a vaccine, prepared for a living attenuated tubercle organism, BGG (the Bacillus of Calmette and Guerin), which gives immunity demonstrable on a statistical basis when large numbers of people are vaccinated, but does not give a complete assurance to any single individual. The organism is sensitive to, and tuberculosis is treated with, streptomycin, isonicotinic hydrazide, and para-amino salicylic acid.

Leprosy is basically a disease of the skin and nerve. The organism causing it, *Mycobacterium leprae* (Hansen's bacillus), has so far never been cultivated in artificial media, nor even in tissue culture, nor has it been possible to transmit the human disease to animals—who have, incidentally, a leprosy of their own. The organism may invade the nervous tissue and destroy it, thus destroying motor function and feeling, and the isolated organ or limb may simply atrophy without the patient's realizing that this is happening.

Pathogenic Spore-forming Rods

Bacillus anthracis is the only pathogen among the aerobic spore-formers. It is the cause of anthrax, primarily an animal disease, which is transmittable to man. It was known to antiquity: probably one of the plagues of Egypt called down by Moses was anthrax.[2] In man

[1] It still is in underdeveloped countries. Those which have atrociously poor housing and difficult living conditions, have a high proportion of tuberculin reactors.

[2] Exodus 9:9. "The livestock of the Egyptians all died but of the livestock of the Israelites not one dies. But Pharoah remained stubborn. The Lord then said to Moses and Aaron, take two handfuls of dust from a kiln, and let Moses toss it up to the sky in the sight of Pharoah; it shall become a fine dust over all the land of

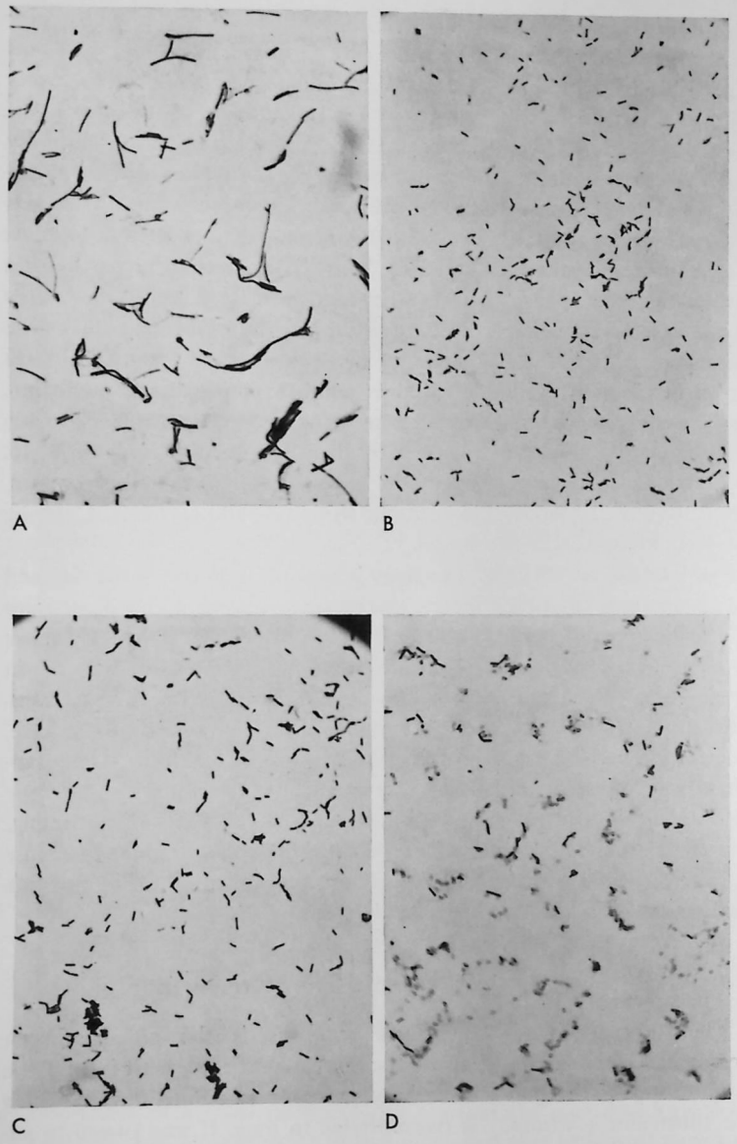


FIG. 26-4. *Various* Clostridium *species. A is* Clostridium tetani *at an early stage of growth. The cells tend to remain attached and spores develop at the end of the cell. These spores, being much wider than the cell, give it a drumstick appearance. B is* Clostridium welchii *(also called* Cl. perfringens*), one of the principle organisms causing gas gangrene. The bacteria are relatively small in size and have central spores no wider than the cell. C is* Clostridium histolyticum*, so named because of its power to liquefy living tissues in gas gangrene. The spores are subterminal and larger than the cell. D is* Clostridium botulinum*, the cause of botulism. The spores are subterminal and swollen. [A, B, and C from Burrows,* Textbook of Microbiology *(17th ed.) (with R. J. Porter and F. W. Moulder), Saunders, Philadelphia (1959). These and D are through the courtesy of Professor W. Burrows.]*

the disease is mostly a surface-entry disease, through cuts or scratches. Anthrax was also the first disease in which a microorganism was definitely *proved* as its cause (by Koch). It was also one of the first in which an immune reaction was demonstrated as protective (by Pasteur). However, killed cells do not provoke an immune response; hence Pasteur and others later had to develop live, attenuated vaccine. This was done by growing the anthrax bacillus for 15–20 days at almost its maximum growth temperature (42.5°C) to a point where it is still virulent for mice but not for guinea pigs or rabbits. Two weeks later the animal is given a second vaccination with a strain not so far along —one which is virulent for guinea pigs but not for rabbits.

For many years no toxin could be demonstrated in *B. anthracis*, yet death seems to be characteristic of a toxemia. It turned out that a toxin is produced when the organism grows in the body and that this appears to be the cause of death.

The important species of *Bacillus* are described on page 143.

Egypt and produce sores that break into pustules on Man and Beast." Is this the first example of bacteriological warfare?

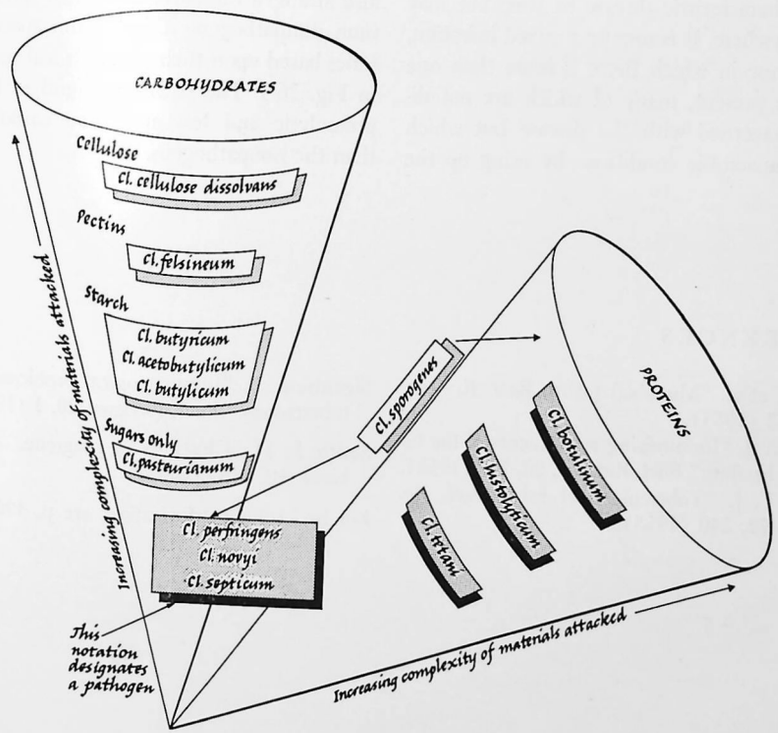
Clostridium

This genus contains the anaerobic spore-forming rods. Among these are the causative organisms of three important diseases as follows:

Botulism	*Tetanus*	*Gas gangrene*
Cl. botulism	*Cl. tetani*	*Cl. perfringens* (*Cl. welchii*)
		Cl. Novyi
		Cl. histolyticum

While there are further a few animal pathogens such as *Cl. chauvei*, the remainder of the *Clostridium* sp. are not pathogens. Most exist

FIG. 26-5. *The properties of* Clostridium *species.*



widely distributed in soil; some fix nitrogen, most produce butyric acid, some butanol. *Cl. botulinum* and the disease botulism have been discussed on page 364, from which it will be recalled that this organism did not grow in the body but in food, and that it caused disease by elaborating a very poisonous toxin. Tetanus or lockjaw is a disease also caused by a toxin but in this case the *Cl. tetani* sets up a local infection generally in a wound, most frequently a puncture wound by a sliver or nail or by abrasion; these produce a toxin which attacks the nervous system. Tetanus toxin is a good antigen and a good antitoxin can be produced, especially in horses. The administration of antitoxin is a protective measure used when the wound may be infected with tetanus. However, active immunization is the best protection.

Gas gangrene is a disease complex which often follows dirty lacerated wounds. As such it is a characteristic disease of war, but may occur anywhere. It is usually a mixed infection, that is, one in which there is more than one organism present, many of which are not directly concerned with the disease but which provide anaerobic conditions by using up the oxygen and thus favor the *Clostridium*. There are four major *Clostridium* species involved: *Cl. perfringens* (also called *Cl. welchii*), *Cl. novyi*, *Cl. septicum* and *Cl. histolyticum*. Each of these grows in the wound, normally on dead tissue in the wound. Most produce a toxin which kills more of the tissue and thus the disease progresses.

These organisms (except *Cl. histolyticum*) ferment sugars and amino acids and produce butyric acid. As noted in Chapter 9, the production of butyric acid requires that the pyruvate be split to acetyl CoA and the *Clostridium species* do this with the liberation of large quantities of H_2 and CO_2. Thus the infected wounds become filled with gas and exudate; hence the name, gas gangrene. The four organisms especially responsible for gas gangrene have a similar morphology, but differ from *Cl. botulinum* and *Cl. tetani*, as shown in Fig. 26-4. We have also arranged these, together with several common nonpathogenic *Clostridium* species, in a series based upon their physiological properties, in Fig. 26-5. The pathogens tend to be more proteolytic and less active on carbohydrates than the nonpathogens.

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For background information, see p. 496.

QUESTIONS

1. Define or identify:

pleomorphism	acid-fast
lumpy jaw	BCG
potato scab	gangrene
diphtheroid	

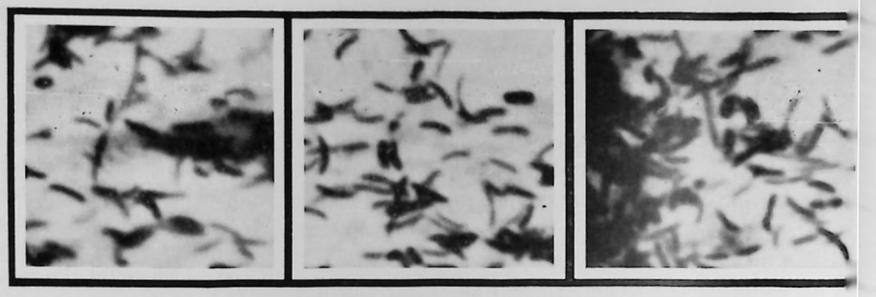
2. Since the leprosy bacillus has never been isolated and grown in artificial culture, Koch's postulates cannot be fulfilled. Why do we put up with this situation? How are we sure that *M. leprae* causes leprosy?

3. In Chapter 24 we did not mention that the interaction of the body with an invader may lead to a different kind of reaction, such as is shown with tuberculosis and tuberculin. This resembles an allergy; indeed, allergy is related to antibody response. How does the tuberculin reaction differ from an agglutination reaction? Would you expect to find agglutinating antibodies to the tubercle organism also?

4. The anthrax vaccine is produced by attenuating virulent strains. What methods can you think of which might also cause attenuation? Pasteur became involved in a controversy (see Chapter 5 of De Kruif's *Microbe Hunters*) over anthrax vaccination and had to set up a public demonstration on 48 sheep, 2 goats, and some cattle. Without really being sure of his vaccine, and using methods which both he and others used later and which later failed, this public demonstration worked. What do you suppose would have happened had it failed?

5. How could you distinguish between the pathogenic and the nonpathogenic *Clostridium?*

Chapter 27



THE PATHOGENIC GRAM-NEGATIVE RODS—ENTERIC DISEASE

"The most terrible outbreak of cholera which ever occurred in this kingdom is probably that which took place in Broad Street, Golden Square . . . , within 250 yards of the spot where Cambridge Street joins Broad Street. There were upwards of 500 *fatal* attacks of cholera in 10 days . . . As soon as I became acquainted with the situation . . . I suspected some contamination of the water of the much frequent street pump in Broad Street . . . but on examining the water . . . I found so little impurity in it *of an organic nature* that I hesitated to come to a conclusion. Further inquiry, however, showed me that there was *no other circumstance or agent common to the circumscribed locality* . . . except the water of the above mentioned pump." (Italics ours.)

SNOW, *On the Mode of Communication of Cholera* (1824)

IN THE PREVIOUS CHAPTERS, dealing with the pathogenic gram-positive bacteria, the diseases encountered were largely respiratory, and the paths of exit, the modes of transmission, and the portals of entry were, with some exceptions, at least similar. It is possible that this somewhat unexpected relation is due to those deep-seated physiological differences of which

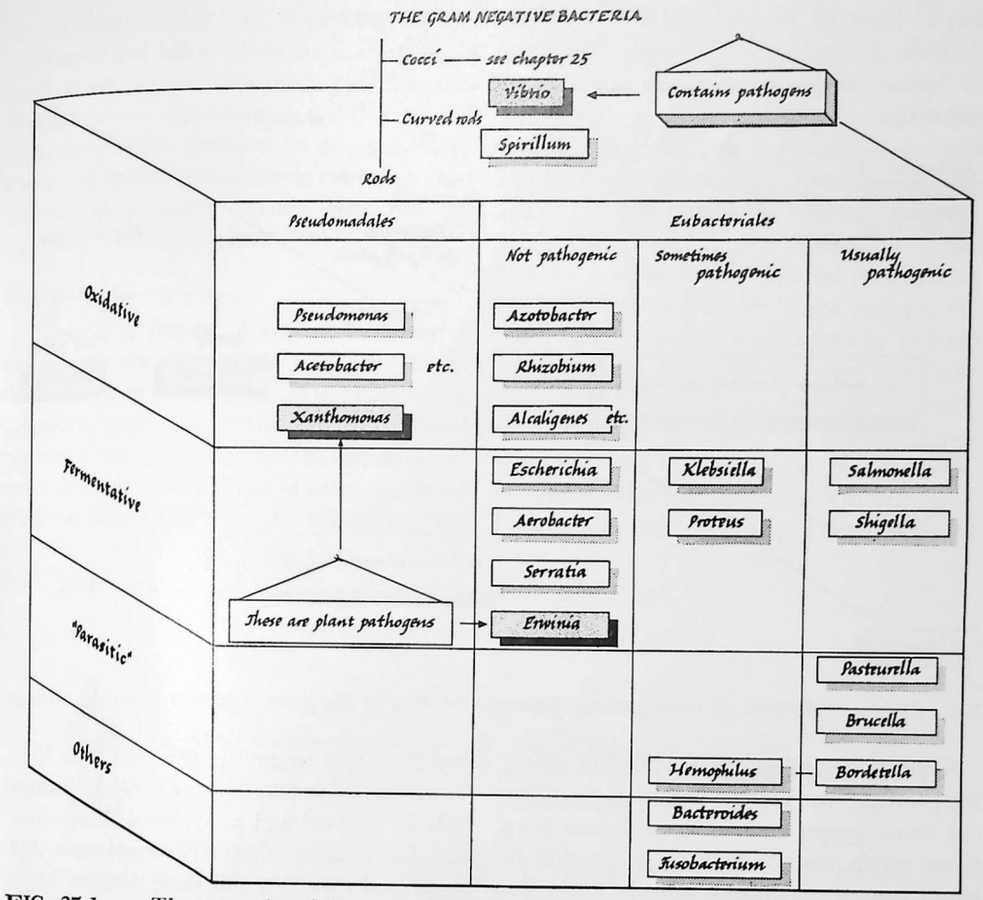


FIG. 27-1. *The properties of the gram-negative rods.*

the gram stain is an index. This notion is strengthened by the examination of the pathogenic gram-negative bacteria, for here are organisms causally related to a group of diseases characteristically involving the gastrointestinal tract—the "enteric" diseases—and close relatives. The path of exit is usually feces or urine. The route of transmission is usually fecal contamination, especially of water and food. The portal of entry is the gastrointestinal tract. Just as the nose, throat, and lung provided environments in which the gram-positive characteristically flourished and were able to cause disease, so do the stomach and the intestinal tract provide environments for the gram-negatives. Outstanding characteristics of the gastrointestinal tract are the high acidity of the stomach, the presence of bile and thus a low surface tension in the intestine, and the anaerobic conditions. These are reflected in the characteristics of the disease producing gram-negative organisms: a general hardiness, certainly compared to gram-positives; the ability to withstand exposure to acid, although being unable to grow below a *p*H of 4.5; an ability to withstand low surface tension; and a facultative relation to oxygen.

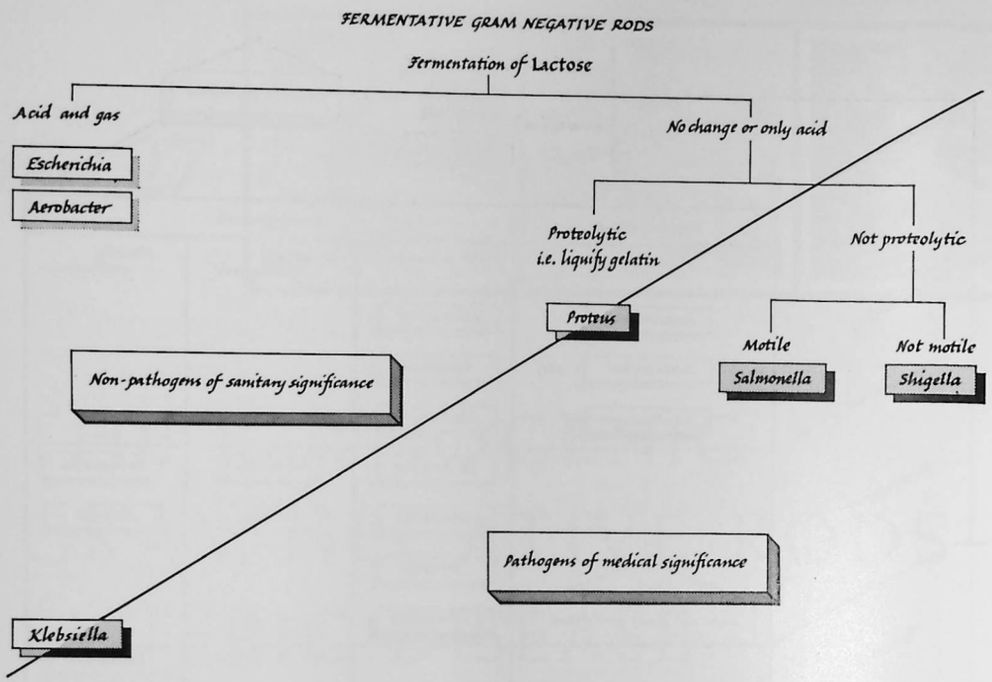


FIG. 27-2. *Methods of distinguishing pathogenic genera in the gram-negative fermentative rods.*

Figure 27-1 outlines the relationship among the gram-negative bacteria and attempts to sort out those genera containing pathogens from those which are not pathogenic. In this attempt, some generalization must be made. For example, *Pseudomonas* is listed as a nonpathogen, but it may sometimes be found in a variety of pus-forming conditions, in some of which it may be the cause. Similarly, some strains of *E. coli* have a degree of significant pathogenicity. In both these organisms, the vast majority of the strains show no pathogenicity or cause disease only under restricted circumstances, usually involving the lowering of body defenses.

Among these organisms are some of the most devastating diseases known to man. Since they leave the body in most cases via the intestinal tract and are spread by water, unsanitary conditions in the past led to widespread epidemics, and a breakdown of sanitary control in the future could lead to similar outbreaks. The *Vibrio comma* is responsible for cholera; *Salmonella* for typhoid and paratyphoid fevers; *Shigela* for bacillary dysentery; *Pasteurella* for plague and tularemia. All these diseases loom large in the tragic history of man when ignorance, carelessness, or disregard permitted their transmission. We shall discuss each major pathogenic genus with concentration on the nature of the bacteria rather than on the nature of the disease produced.

The Fermentative Gram-Negative Rods

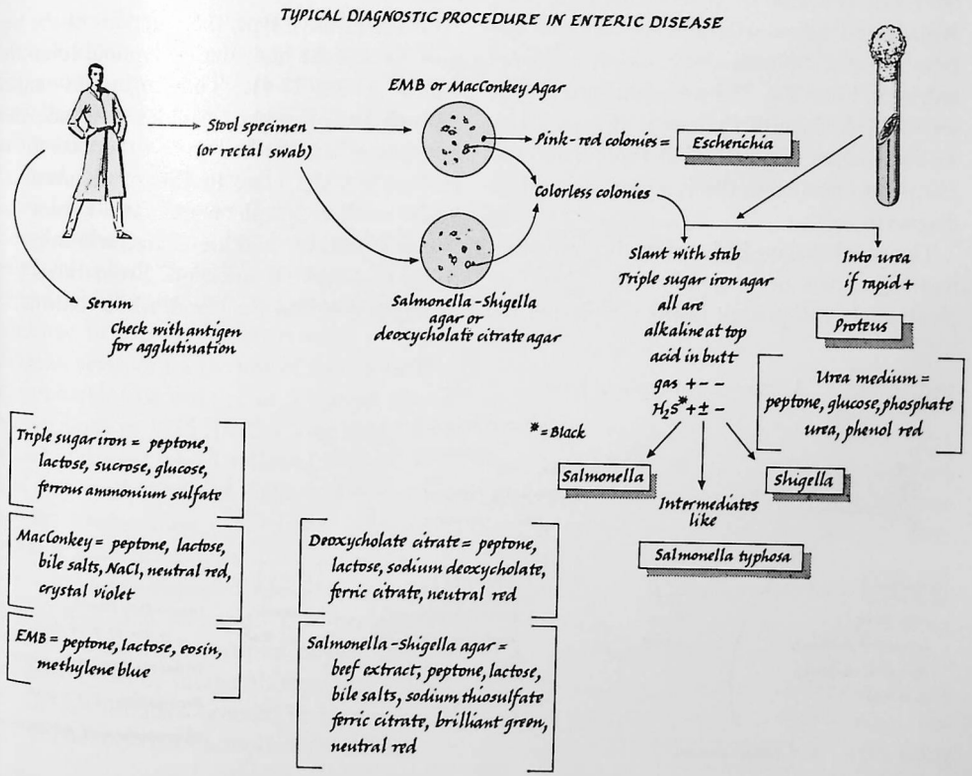
We have earlier encountered these rods in discussions of water (Chapter 17) and foods (Chapter 20) and have discussed the methods whereby the pathogenic genera are distinguishable from the nonpathogens. This information is summarized in Fig. 27-2 and it is so arranged

that one may draw a line, above which are few pathogens and below which are the pathogens with which we are concerned. This line cuts through two genera, *Klebsiella* and *Proteus*, and it is deliberately designed to do so since in these two genera pathogens do exist. We shall discuss the pathogenic genera first, then the "partially" pathogenic ones.

Salmonella

There is a curious, if confused, history of the disease we call typhoid fever and the related family of diseases, the paratyphoid fevers, caused by organisms of this genus. The word typhoid fever is derived from the Greek, meaning a state of irrationality and coma associated with the relatively high fever accompanying the disease. In medical practice the term "status typhosus," originally descriptive of typhoid fever, came to be associated with meningitis or with typhus fever, caused by a rickettsia—see Chapter 30); this also has a high fever and irrational periods. Typhus fever (caused by rickettsia), thus came to be differentiated from typhoid (typhus-like) fever (caused by a bacterium) even in clinical practice, long before the actual causes of either disease were known. The typhoid bacillus was eventually isolated and associated with typhoid fever. But there were found very similar bacteria—generally producing acid and gas from glucose, rather than only acid as did the typhoid bacillus—associated either with diseases similar to typhoid fever or with food poisonings. For a time these

FIG. 27-3. *Diagnostic separation of enteric pathogens from* E. coli.



organisms were separated into two genera: *Eberthella*, to contain the typhoid bacteria, and *Salmonella*, to contain the paratyphoid types; both were named after bacteriologists prominent in their study. Today, general practice is to regard only one genus as valid—*Salmonella*. At about the same time, it was recognized that very similar organisms, but nonmotile, were capable of producing a febrile dysentery; these were placed in a separate genus—*Shigella*.

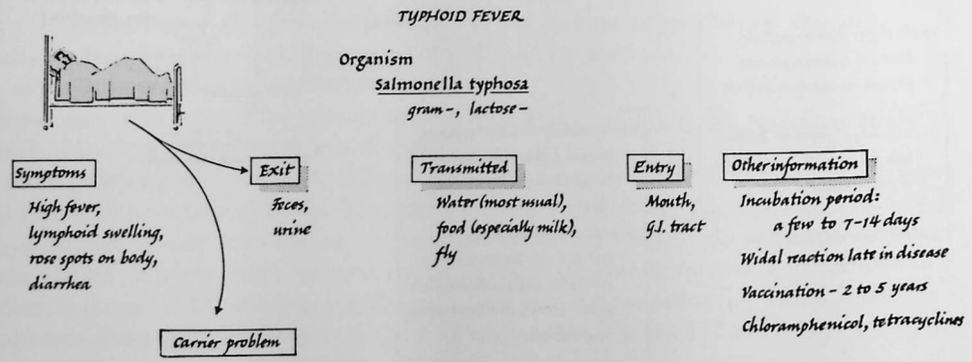
Figure 27-2 also gives something of a notion about how we may go about separating the pathogens from nonpathogens. First, most of the nonpathogens form acid and gas within 24 hours at 37°C, from lactose. The exception, *Klebsiella*, is really not an enteric pathogen (see later) and hence is usually of no concern in the examination of enteric disease. If, therefore, we have organisms producing no gas from lactose, there may be reason to suspect pathogens. Both *Proteus* and *Salmonella* produce acid and gas from glucose; *Shigella* does not—it produces only acid. From Fig. 27-2 we might suspect that we could distinguish *Proteus* at this point, by its liquefaction of gelatin, but *Proteus* also ferments urea readily, so this is a more convenient diagnostic test.

The usual diagnosis, however, begins with a fecal specimen or a rectal swab. There are plenty of *E. coli* present in the sample and the search for the pathogen must begin with selective media which would tend to eliminate the nonpathogens. A typical diagnostic separation is illustrated in Fig. 27-3. We have omitted serological separation, identification, and diagnosis, which is important and indeed critical in this group, as being within the specific province of medical bacteriology.

Salmonella are of three general types: those invading the body from the intestinal tract, causing typhoid and paratyphoid fevers; those growing especially in the intestinal tract but not invading the body and yet causing illness by way of toxin production; and those causing no discernible disease in man although they may cause animal disease. The species of each type are closely related and are separated from each other primarily by serological reactions. There are now over 300 specifically named serological types.

Of the invasive type, the reactions of the organisms and the body during typhoid fever are typical (Fig. 27-4). The organisms enter through the alimentary canal and invade the intestine, where the body tries to localize them in lymph nodes close to the small intestine. Such smaller lymph nodes are known as "Peyer's patches" and are characteristically ulcerated in typhoid infection. From there the organism gets into the blood, while continuing

FIG. 27-4. A *summary of typhoid fever.*



to flourish in the intestine, where it causes ulceration and sometimes diarrhea. The time of ingestion to the outbreak of fever is from 10–14 days, and high fever may last for a week. During the first week the organisms may be isolated from the blood, after the first week from the feces. Rose-colored spots appear usually on the chest or abdomen, usually by the seventh day of fever. About 15% of untreated uncomplicated cases die; the remainder recover after 2–3 weeks, and any time after the second week of the disease the serum will agglutinate cells of *S. typhosa* (the **Widal test**). Vaccination, which is effective in preventing infection, gives a positive Widal within 10 days and it remains positive for two years. Most vaccines are **triple vaccines**, i.e., they contain *S. typhosa*, *S. paratyphosa* (para A), and *S. schottmülleri* (para B).

CARRIERS. Typhoid fever may be transmitted by individuals who to all appearances are normal and healthy but who are dangerous because they harbor the infectious agents. Such persons are carriers. About one-third of the cases of typhoid fever discharge *S. typhosa* in the feces for about 3 weeks. Ten per cent of the cases discharge them for 8–10 weeks; these cases are called "convalescent carriers." Some, fortunately few, may, while appearing healthy, continue to excrete the organisms for several months, years, or for the rest of their lives. The most notable case was that of "Typhoid Mary," whose death in 1938 ended a long series of typhoid outbreaks which followed wherever she was employed. Although held in detention at various times, when paroled she insisted on working in the food industry and on concealing her condition. Repeated fecal examinations, Widal tests, and serological tests are required to detect carriers. Sometimes carriers may be rendered cured by vaccine therapy and removal of the gall bladder—believed to be a reservoir for *S. typhosa*—but chemotherapy has not yet been of much help in curing carriers. If they cannot be cured, they must be carefully isolated, especially from the possibility that they might contaminate food and water.

Shigella

Bacillary dysentery, caused by *Shigella*, does not involve invasion of the blood stream. Rather, the organisms grow in the intestine and irritate the mucosa causing ulceration and bleeding. About 80% of the cases are caused by the mannitol-fermenting strains; while the non-mannitol-fermenters (*S. dysenteriae* proper) cause the most severe disease, they are fortunately rare. Since the organisms do not come in direct contact with the blood stream, there is little development of immunity.

Proteus

A peculiar characteristic of *Proteus* is the tendency of many strains to spread over the surface of agar, which may be prevented by adding tellurite to the agar. Some species, such as *P. vulgaris*, produce colonies that move! They are frequently responsible for urinary and bladder infections, but most species are not pathogenic.

Klebsiella

In 1882, Friedlander, looking at tissues from eight cases of patients dead from lobar pneumonia, saw a micrococcus. In 1883 he studied over fifty cases, in nearly all of which he found these cocci. In the same year he made the first mention of Gram's stain (Gram worked in his laboratory) as a help in identifying this micrococcus; there is little doubt that this was *Diplococcus pneumoniae*.

An attempt was made to isolate the organism —this "Micrococcus"—but the patient did not really have a typical lobar pneumonia and these workers isolated instead a gram-negative rod, heavily encapsulated, which they though was the major cause of pneumonia. By 1886, using

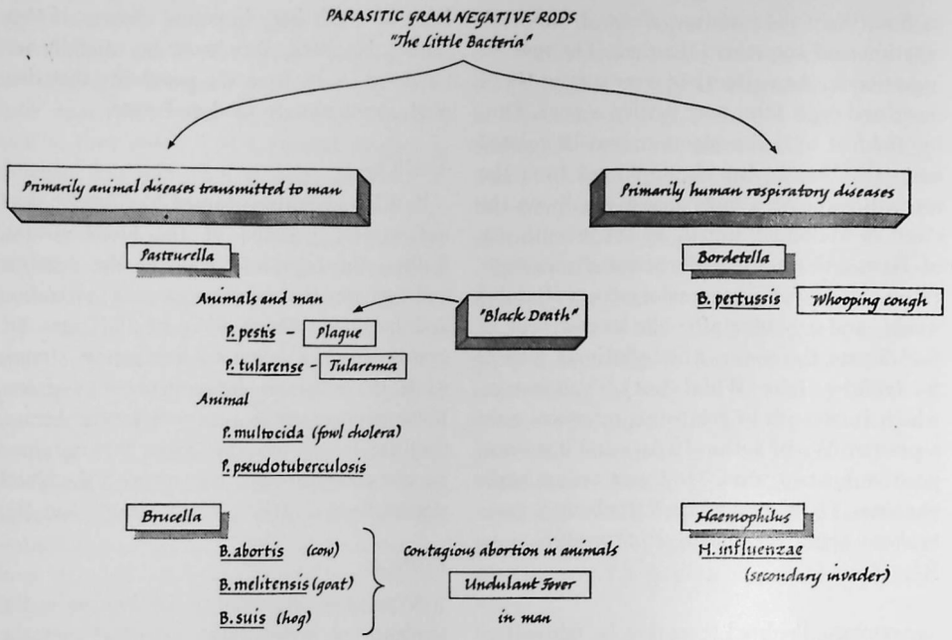


FIG. 27-5. *The "parasitic" gram-negative rods.*

the gram stain especially, Friedlander wrote, "From the foregoing investigations, it emerges, therefore, that the most frequent organism in pneumonia is a capsulated coccus . . . the capsulated bacterium cultivated by me occurs only in a minority of cases; that it has, in fact, caused pneumonia in these cases emerges with greater certainty . . . " In short, some pneumonia and especially bronchial pneumonia can be caused by the growth of encapsulated gram-negative rods, Friedlander's bacillus. Today it accounts for less than 5% of all pneumonia.

The Parasitic Bacteria

This group of gram-negative rods is usually somewhat smaller than the other gram-negative bacteria, and the rods have little fermentative or proteolytic powers. While they appear to resemble the enteric organisms, they are seldom found in habitats outside of the human or animal body. There are four genera of importance here: *Pasteurella, Brucella, Haemophilus,* and *Bordetella,* and, as shown in Fig. 27-5, they are responsible for two general types of disease. *Pasteurella* and *Brucella* organisms cause animal diseases which can, on occasion, be transmitted to man. *Haemophilus* and *Bordetella,* organisms so closely related that they were for years considered to be one genus, are responsible for respiratory diseases.

Pasteurella

There are four species, as shown in Fig. 27-5. The two human diseases, plague and tularemia, are essentially rodent diseases transmissible to man. **Plague** is a severe and highly fatal disease; it is the "Black Death" of the Middle Ages. It occurs in three types: bubonic, in which there are eruptions on the skin; pneumonic, infections of the lungs; and septicemia, in the blood. All three types are caused by the same organism. The disease is transmitted from rat to rat and from rat to man by rat fleas. **Tularemia** ("rabbit fever") is a fever, much like plague in appearance. The organism lives endemically in squirrels and rabbits, and is transmitted both by insect bites and by contact, e.g., skinning infected rabbits.

Brucella

These organisms cause undulant fever in man and contagious abortion in animals. While their distribution is characteristically in the animal named (Fig. 27-5) all three infect man. On first isolation they frequently require extra CO_2 for growth. They are transmitted via milk from infected animals, but also by direct contact with the infected animals. Usually organisms can be found in the blood. In the body they grow within the cells (Fig. 27-6).

Bordetella

Bordetella pertussis, known for years as *Haemophilus pertussis,* is the cause of whooping cough. It is characteristic of this genus, and of the *Haemophilus* genus, that when first isolated the organisms require blood or factors derived therefrom. Fractionation has demonstrated that the blood provides a source of heme (X factor) and of DPN (V factor); if these are supplied, the blood may be dispensed with. Whooping cough is spread by droplet infection as a respiratory disease; hence strict quarantine is required. It is controlled today by early vaccinations of children. Generally pertussis vaccine is mixed with tetanus and diphtheria toxoids (and sometimes poliomyelitis) and routinely given to infants before six months, at two years, and again at four to five years of age.

FIG. 27-6. Brucella *growing within the cell. This is* Brucella abortus *growing in human skin cells in tissue culture. Note adjacent infected and noninfected cells.* [*From Richardson,* J. Bacteriol., **78**, 769 (1959). *Courtesy of Dr. M. Richardson.*]

Haemophilus

There are several species in this genus, some requiring V, some X (see *Bordetella* above), but the main pathogenic species is *H. influenzae*, which was originally supposed to be the cause of influenza. In reality *H. influenzae* is a secondary invader, when resistance is weakened. This organism is responsible for some respiratory infections and for pyogenic (pus forming) infections not caused by cocci. It is transmitted like a typical respiratory disease.

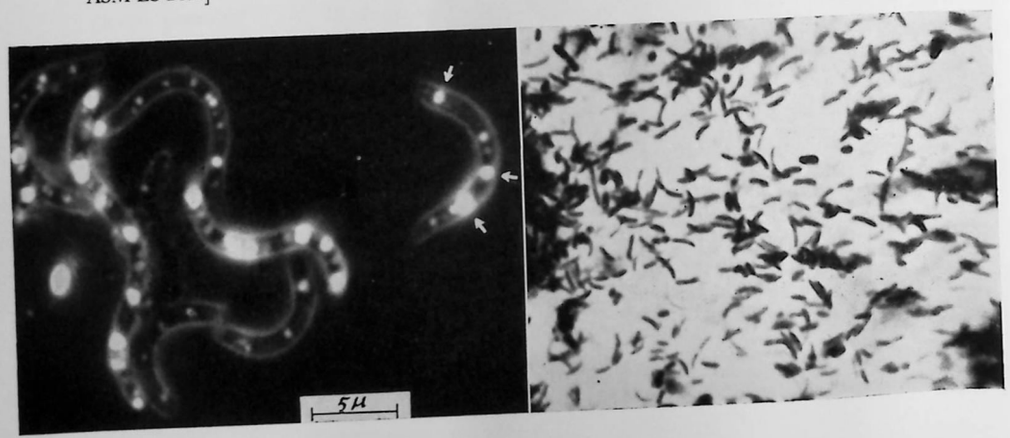
The Vibrio

The gram-negative curved rods are of two types, as shown in Fig. 27-7. One, having large spirals—*Spirillum* and related genera—contains few pathogens, but the other—covering the small comma-shaped organisms—contains a very potent pathogen indeed. This is *Vibrio comma*, the cause of **Asiatic cholera;** the other vibrios are of less importance. We think of cholera as an Oriental disease, but it was first recognized by Snow in London in the epidemic of 1854; he realized that it could be transmitted by water. New York also had serious epidemics of cholera in 1832, 1849, 1854, 1866. Sanitary measures have eliminated cholera from the United States during the last fifty years. It is transmitted via fecal contamination, especially through water.

Two physiological characteristics of V. *comma* are of assistance in its isolation and identification. It is relatively oxidative, hence it grows on the surface of media. It is relatively tolerant of an alkaline *p*H and will grow up to *p*H 9.6; it is usually cultivated at about *p*H 8. The V. *comma* can grow very rapidly and indeed outgrow contaminants on an alkaline medium such as alkaline egg peptone or alkaline peptone broth. There is a reaction called "cholera red," which results from the growth of V. *comma* on peptone nitrate broth; a red color is formed on the addition of sulfuric acid. This color is due to the formation of indole and the reduction of nitrate to nitrite, but while given by most V. *comma*, it is unfortunately given by several other enteric organisms. The only fecal organism growing at *p*H 9 is *Streptococcus faecalis* which is readily distinguishable from V. *comma*.

When V. *comma* is ingested it multiplies rapidly in the intestinal tract and produces an enzyme, **mucinase,** which removes the mucin on the membranes lining the gut. These exposed membranes are then attacked by an endotoxin of the vibrio. This toxin is associated with the vibrio cells themselves and is present in killed cells of the organism. It is quite heat-stable and seems to be a phospholipid. When acting upon the exposed membranes of the intestinal tract, it causes an outpouring of fluid and salts resulting in dehydration of the patient and a diarrhea called typically "rice-water stool." If water and salt are not replaced the death rate is very high. Both the mucinase and the endotoxin are a sufficiently distinct method of causing pathogenicity to warrant mention, although endotoxins are relatively common among the gram-negative bacteria.

FIG. 27-7. Spirillum *and* Vibrio. *The* Spirillum *in the left photo is a dark-field photograph of a time-lapse series showing cell division. The phase-contrast photographs of the same series will be found in Fig. 4-6.* [*From* Williams, J. Bacteriol., **78,** 373(1959). *Courtesy of Dr. Marion* Williams.] *The* Vibrio *is a gram stain of a fecal smear from a cholera patient, showing vibrios with rods and cocci occuring in feces.* [*From Reimann, Chang, Chu, Liu, and Ou,* Amer. J. Tropical Med., **26,** 631 (1946), ASM-LS-282.]



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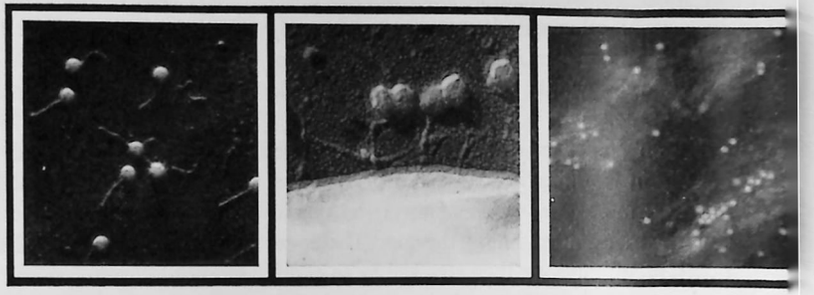
QUESTIONS

1. Define or identify:

enteric	Black Death
gastrointestinal tract	contagious abortion
typhus	X factor
typhoid	V factor
paratyphoid	pertussis vaccine
rose spots	pyogenic
Peyer's patches	cholera red
Widal test	mucinase
carrier	endotoxin
plague	rice-water stool

2. As a Public Health Officer, you see a report from the bacteriology laboratory that in a given city in your district a gram-negative organism has been isolated from the stool of a patient and that this organism gives a positive "cholera red" test. What further information would you demand before you concluded that cholera has broken out? What would you suspect as more likely in the case above?
3. In southeast Australia Dr. Hunterbush reports that an infection of sheep herders known locally as "lazy-man cramps" is due to actual invasion of the blood stream by *E. coli*. Would it therefore be sensible to call *E. coli* a pathogen?
4. In the northern portion of Mexico a revolution has broken out and the rebels have destroyed all the water works in the area. The populace must use the water from streams and rivers for drinking and cooking. What diseases are likely to be encountered? How could you prevent their spread?
5. Tularemia is normally a disease of rodents, and its reservoir appears to be the squirrel, but man contracts it mostly from the rabbit. In Utah the disease "deer fly fever" is, in fact, tularemia, transmitted from rodents to man by the bite of the deer fly. The bacterium is a small gram-negative rod which in young culture appears like a coccus. It has been found in rodents—squirrels, rabbits, mice, etc.—in all states of the union. There are normally about 2000 human cases a year. Why aren't there more?

Chapter 28



THE BACTERIAL VIRUS–BACTERIOPHAGE

". . . no fact is so simple that it is not harder to believe than to doubt at the first presentation. Equally, there is nothing so mighty or so marvellous that the wonder it evokes does not tend to diminish in time. Take first the pure and undimmed lustre of the sky and all that it enshrines: the stars that roam across its surface, the moon and the surpassing splendor of the sunlight. If all these sights were now displayed to mortal view for the first time by a swift unforeseen revelation, what miracle could be recounted greater than this? What would men before the revelation have been less prone to conceive as possible? Nothing, surely. So marvellous would have been that sight—a sight which no one now, you will admit, thinks worthy of an upward glance into the luminous regions of the sky. So has satiety blunted the appetite of our eyes. Desist, therefore, from thrusting out reasoning from your mind because of its disconcerting novelty. Weigh it, rather, with discerning judgment. Then, if it seems to you true, give in. If it is false, gird yourself to oppose it."

LUCRETIUS, *On the Nature of Things*

WHEN WE EARLIER DESCRIBED the position of the bacteria in the world of nature (Chapter 15), we pointed out that bacteria were the smallest cells, but that there were even smaller "creatures" whose properties were certainly related to living things. These were the viruses and the rickettsiae. We will begin with the viruses attacking bacteria (this chapter), then

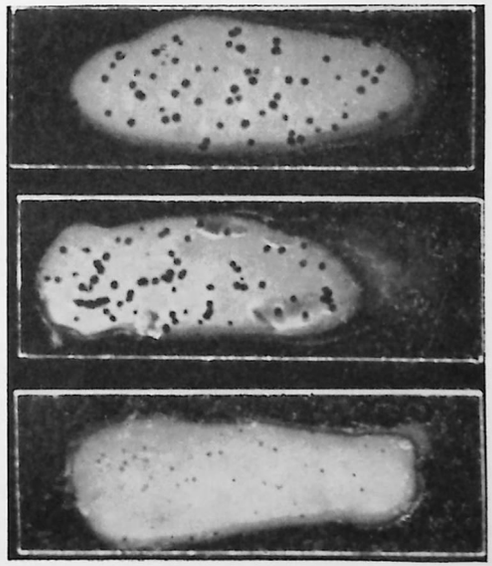
go to the viruses which attack higher organisms (Chapter 29) and finally take up those creatures forming a realm of life between the viruses and the true cells (bacteria) which are known as rickettsiae (Chapter 30).

Viruses function at a level of organization which overlaps that of both living beings and "inanimate" molecules, where the distinction between molecules and living beings becomes uncertain. But these "molecules of virus" also attack bacteria and much can be learned about the nature of all viruses from a study of bacterial virus. We therefore begin at this point in an essentially experimental manner.

The bacterial viruses are called **bacteriophages** or phages. The virus or phage particle —and we will examine the evidence that it is a particle shortly—attacks a susceptible bacterial cell in three stages: first, there is an adsorption to the surface of the cell; second, there is the entrance of at least a portion of the phage into the cell and its replication within the cell; third, there is the release of many phage particles from the bacterial cell, usually with the bursting or lysis of the cell. These processes, adsorption, development, and release, we shall discuss in detail below, but we first must examine the phage particle.

Bacteriophages were discovered between 1915 and 1917 (by Twort and by d'Herelle) in the following fashion. Broth cultures of certain intestinal bacteria would grow to a certain degree of turbidity and then suddenly clear—all the cells in the turbid cultures dissolved! If the clear broth was filtered through filters which would hold back the bacteria and was added to other cultures of the same bacteria, these "normal" cultures would also dissolve. If the dissolved cultures were again filtered and a small sample of the bacteria-free filtrate spread evenly on an agar plate already covered with a crowded film of susceptible cells, after incubation there would appear a number of clear zones or **plaques** where the bacteria had been dissolved (Fig. 28-1). This plaque indicated that phage was a particle and not a solution. If a portion of the clear zone was transferred to a growing culture of the susceptible bacteria, it would cause the culture to dissolve. A moment's consideration will show that the results of these experiments demonstrate that the bacteriophage was composed of

FIG. 28-1. *Plaques formed by bacterophage. This photograph demonstrates phage plaques (the dark "holes" in the bacterial growth) and a method for counting their numbers. In this case an inoculum of susceptible cells, a suitable medium, and a suitable dilution of the phage suspensions are mixed and a known volume spread on a sterile slide, which is incubated in a moist chamber—frequently a pyrex baking dish with moist blotters and a cover. The medium is chosen so that on final dilution the mixture spread on the slide contains 0.3% agar. In the photograph above, the top slide was prepared from a dilution containing approximately 100 phage particles per milliliter; the lower slides have 107 particles of phage— i.e., not all develop.* [*From Ralston and Baer,* J. Bacteriol., **79,** *758 (1960). Courtesy of Dr. D. J. Ralston.*]



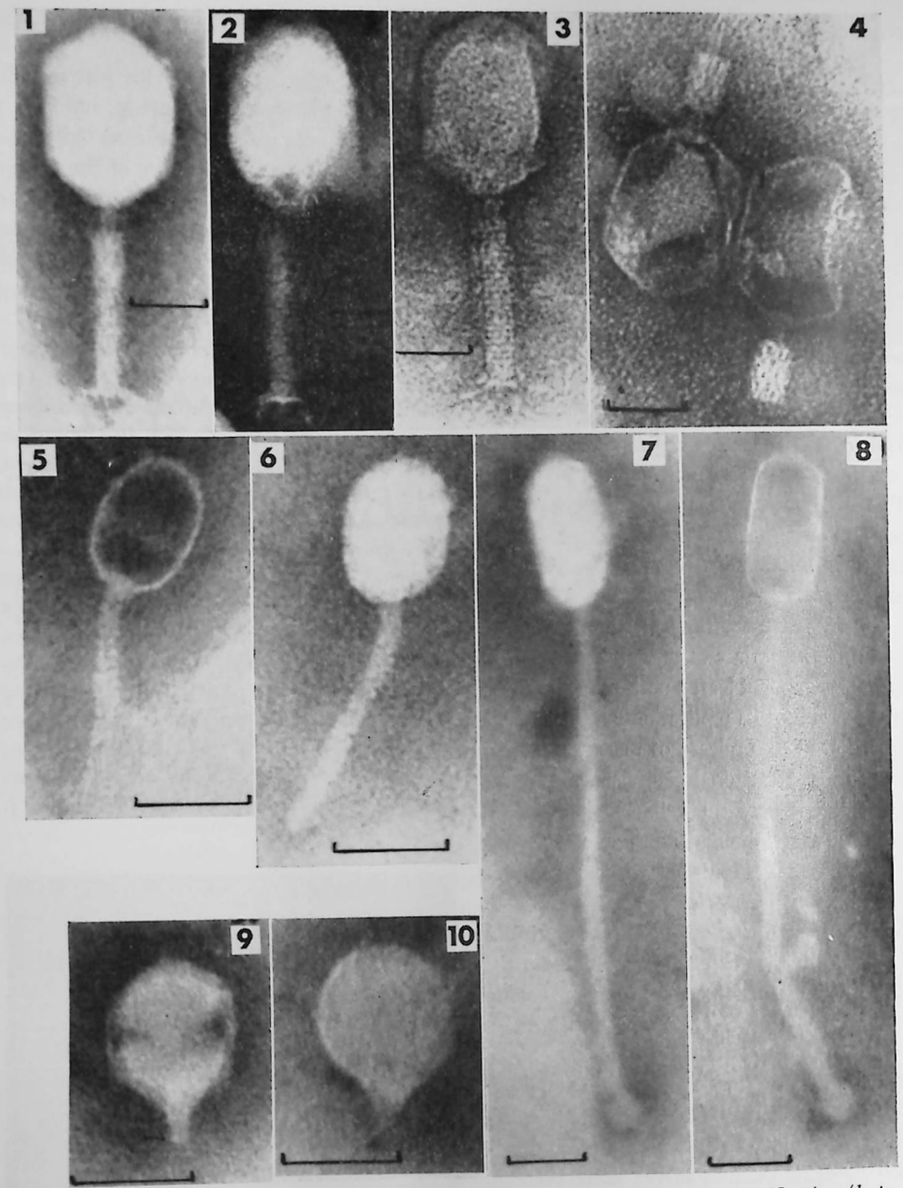


FIG. 28-2. *Phage Morphology. These are photographs at exceedingly high magnification (between 250,000 and 375,000). The line on each photograph represents 0.5 μ. Photos No. 1, 2, 3, 4 are typhoid phage; No. 4, having lost its DNA, shows just the protein coat. Nos. 5 and 6 are* Streptococcus *phage; 5 shows the protein coat. Nos. 7 and 8 are* Staphylococcus *phage (note the very long tails); No. 8 is the protein coat. No. 9 is* E. coli, T_2 *phage; 10 is a* Brucella *phage. On photos 1 to 4, note that the tails have a cross-striated structure and are contractile; compare 3 and 4 which are the same phage with 4 having lost its DNA (presumably by ejection).* [*From Bradley and Kay,* J. Gen. Microbiol., **23,** 553 (1960). *Courtesy of Drs. Bradley and Kay.*]

a filterable (filter-passing) particle which somehow attacked the bacteria. If such filtrates were examined as described above in a compound microscope, no organisms could be seen. There was one further experiment of importance. By spreading known quantities of a filtrate over a susceptible culture on a plate and counting the number of plaques, an estimate of the number of phage particles in a given filtrate could be obtained. If such a filtrate were kept in the cold without access to bacteria, the number of phage particles did not increase and might decrease slightly over the course of time, but the filtrate might keep the same degree of potency for months. If a known number of particles of phage were added to a growing culture of susceptible bacteria, after these had lysed the number of phage particles in the new filtrate would have increased 10, 20, 100, or even 10,000-fold.

Properties of the Bacteriophage

These experiments serve to define the main properties of the bacterial virus, the bacteriophage. It is a particle, smaller than bacteria, passing through filters which retain bacteria, invisible in the optical microscope, and it does not increase in the absence of growing cells. Such particles are visible in the electron microscope and can be isolated by chemical and centrifugal methods. They are virus particles. While there is a wide variety of phages, each differing a little from the other, the principles of virus action and their operation are best seen by concentrating on one good example, with the understanding that other bacteriophages, for other bacteria, may differ slightly in detail but not in principle from the example presented.

For our bacterial example we shall choose a strain of *E. coli* which has been widely studied in many laboratories and is known as *E. coli*, strain B. This strain is attacked by seven different phages, each of which differs a little from the other. These phages have been separated and studied and are known as T1 to T7, T standing for "type." All the T phages, when seen in the electron microscope, consist of a head and a tail. In all T phages the head is a hexagon (Fig. 28-2). The even-numbered phages, T2, T4, T6, are much alike, T1 and T5 are a little smaller, while T3 and T7 have very short tails. All have about 40–50% DNA, the rest being mostly protein. The DNA is concentrated in the head where it is surrounded by protein; the tail is primarily protein. The relationships are such that we can speak of a DNA "core" with a protein "coat." The two parts of the phage have different functions. The protein coat is involved in adsorption onto new cells in the process of "injecting" the DNA core into the new cell, and, to an extent, with the dissolving (lysis) of the cell afterward. The DNA is concerned with the phage reproduction inside the bacterial cell. It is possible to prepare DNA cores separately and protein coats separately. The latter can adsorb to cells, can even adsorb to cell fragments, and can "cause" cell lysis, but no new phage is produced. The DNA, while difficult to insert experimentally into living cells, can, on the rare occasions when this happens, cause the regeneration of the complex phage—head and tail or core and coat.

We may diagram the process of phage adsorption and development as in Fig. 28-3. This applies to the T phages on *E. coli* B, and the times, etc. cited are for these systems, but other bacteriophages on other organisms differ from this picture only in detail; e.g., the time of development (**latent period**) may be 90 minutes rather than 40, the **burst size** may be 20, or 150, rather than 75. But aside from the quantitative differences, Fig. 28-3 represents the process in essentially all bacteriophages.

The first step, that of adsorption, consists of a contact between the growing bacterial cell

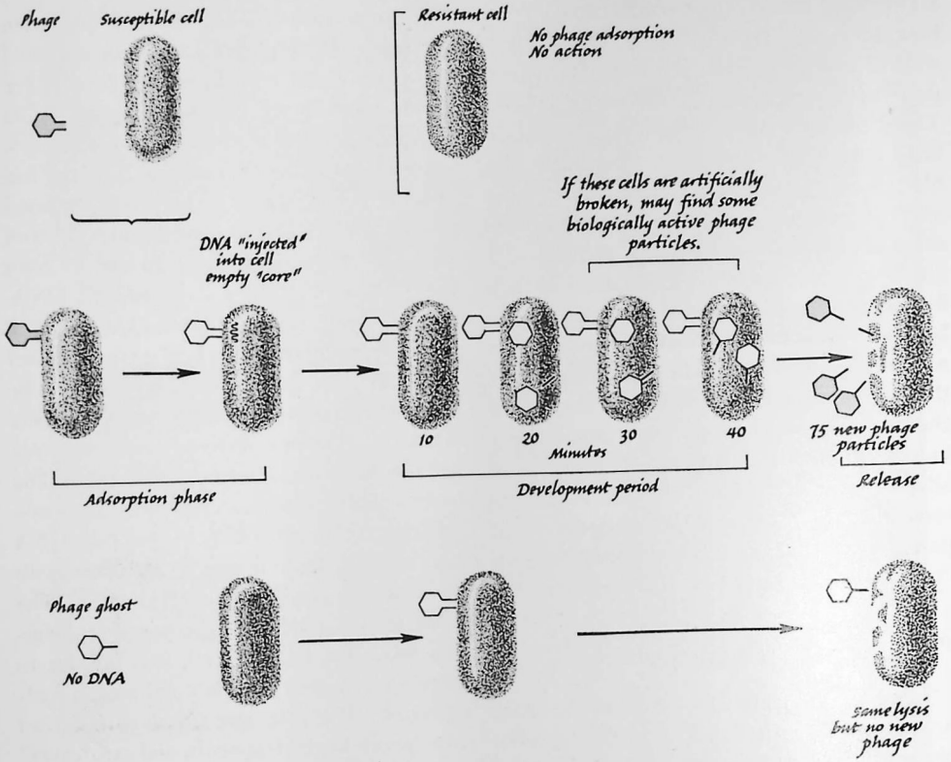


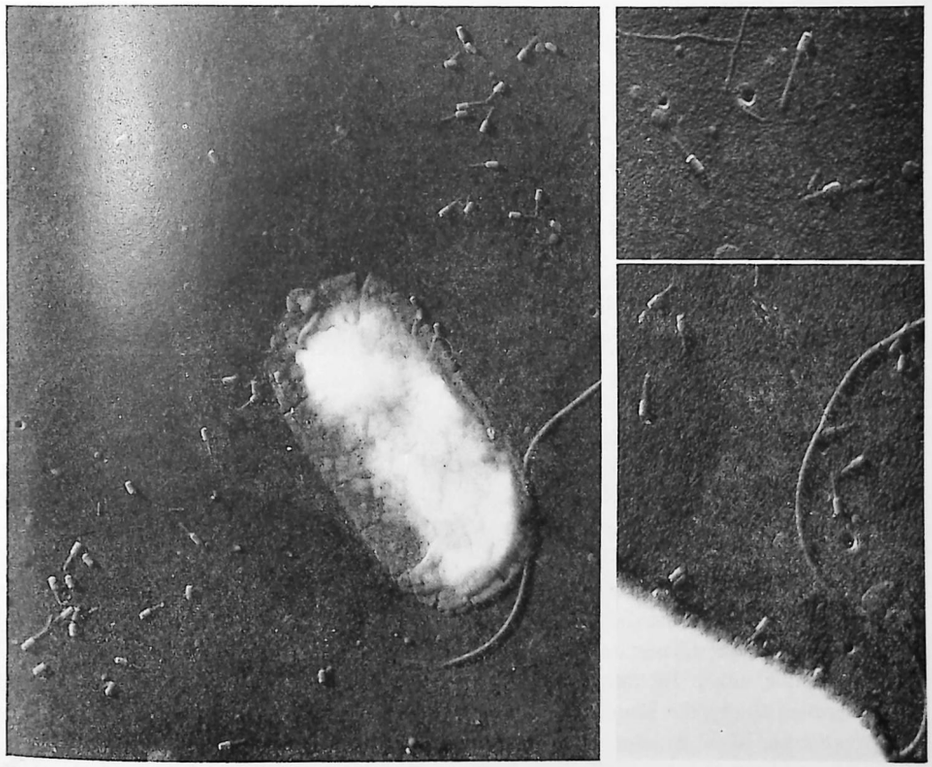
FIG. 28-3. *The Development of Bacteriophage. The phage when adsorbed to the susceptible cell "injects" its DNA and disappears as such (line 2). The coat remains outside the cell, but the DNA organizes the cell to produce, first incomplete, then complete phage. If a phage without DNA (line 3) is used, lysis occurs, but no new phage is formed.*

and the tail of the phage; see electron microscope photograph, Fig. 28-4. On each susceptible bacterial cell there are certain **receptor spots**, which are particular chemical configurations to which the protein of the tail attaches. A given strain of *E. coli* may have such receptor spots for T3 and T5 but not for T7 phage; therefore T3 and T5 but not T7 will adsorb to it. There seem to be several hundred such receptor spots per cell. They appear to be located on the cell wall, since adsorption can occur onto carefully isolated cell wall.

On occasion a bacterial mutation may occur, resulting in the loss of a particular receptor site. Such mutated cells are resistant to the specific phage using the receptor site. When the tail has made contact with its proper and specific receptor site, there is a release of the DNA into the cell interior **(injection)**. At this point, the fate and function of the core and the coat diverge. The DNA goes on to make new phage. The coat remains at the outside. Dead bacterial cells can sometimes adsorb phage and, if conditions are right, this adsorption causes the release of DNA from the phage particle, which, from that moment, is no longer recognizable. There is in this case no new phage; the old phage, now split into coat and DNA, is quite incapable of adsorbing onto any other cell and is incapable of being detected by any biological test.

It is possible to cause the DNA to be ejected from the phage by **salt shock**, i.e., sudden dilution with water of the phage suspension in salt. In this fashion we can obtain **phage ghosts**, the protein part devoid of DNA. Such phage ghosts are capable of adsorbing to living bacterial cells, although only those to which the whole particle would adsorb, and are capable of causing the cell to lyse; however, no new phage is produced. The adsorption and its specificity—i.e., the particular bacterial strain attacked by a phage strain—and the lysis are brought about by the protein coat. The lysis of the cell seems to be brought about by an enzyme, **lysozyme**, in the tail of the phage which breaks links in the cell wall and destroys its rigidity. The cell lyses because of osmotic

FIG. 28-4. *Adsorption of bacteriophage to cells. This is* Pseudomonas *and a curiously shaped phage. Adsorption to the cell by the tail is visible. Note also the curious structure of the tail (C), in the lower right photo. Left photo is 27,000×; Upper right 37,000×; lower right 48,000×. [From Takeya, Mori, Veda, and Toda,* J. Bacteriol., **78**, 332 *(1959) Courtesy of Dr. Takeya.] Phage attachment may also be seen in Figs. 11-12 and 28-5.*



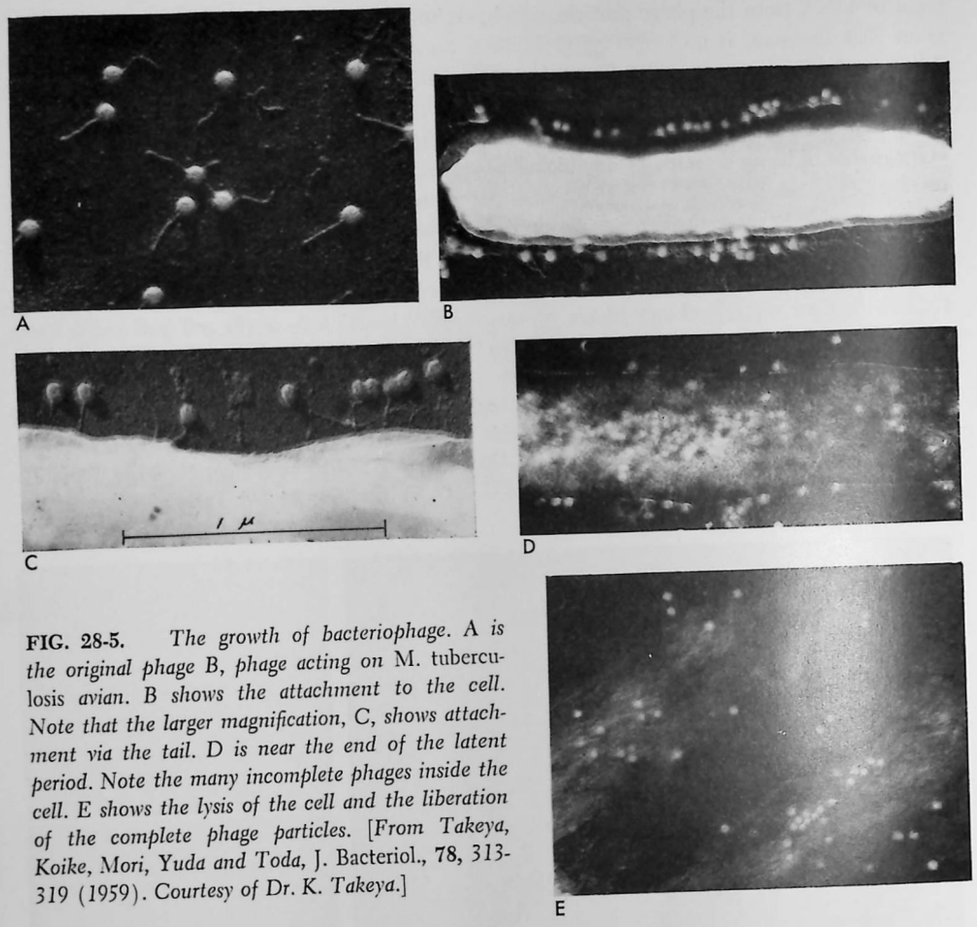


FIG. 28-5. *The growth of bacteriophage. A is the original phage B, phage acting on* M. tuberculosis *avian. B shows the attachment to the cell. Note that the larger magnification, C, shows attachment via the tail. D is near the end of the latent period. Note the many incomplete phages inside the cell. E shows the lysis of the cell and the liberation of the complete phage particles. [From Takeya, Koike, Mori, Yuda and Toda,* J. Bacteriol., *78, 313-319 (1959). Courtesy of Dr. K. Takeya.]*

imbalance and can be protected from lysis after the manner of protoplasts (see Chapter 3). So much for the nature and the function of the protein coat or tail of the phage.

The DNA, once entering the cell, goes about its business and the phage disappears! There are no particles within the infected cells, and the coat we can still see outside, but no phage is visible inside. By measurements of plaque-forming ability, the phage is gone. And it is gone for 10, 20, 30 minutes, or longer. All of a sudden, it appears again—and now, where there was one before, we may have 20, 40, 80, or 100 plaque-forming bacteriophage particles. This period, when the phage disappears, is called the latent period and the number of phage particles per cell is called the burst size, because it is at precisely the point that all the phage particles appeared that the bacterial cell burst and dissolved. A picture of such a cell bursting is shown in Fig. 28-5. A diagram of what is happening over the period is given in Fig. 28-6.

The DNA particle from the original infecting phage splits up; we can no longer see it as a particle, and its components somehow intermesh with the cell's machinery. And well indeed they might, for DNA is the substance of the genes, the substance through which the chain of heredity is passed. For a time we have no detectable phage in the mixture; we can't even find any particles inside the cell, even if we break it open. About half way through the latent period, which will of course vary, depending on the system used, we may find many phage particles released when we break the cell; frequently as many as if we waited for the cell itself to lyse. Sometimes incomplete phages or particles which look like phage but will not infect the susceptible cells are found when the cell is broken. These are called **prophage.** But normally the cell lyses at the completion of the cycle and immediately all the phage particles inside it are released, ready to attack another cell.

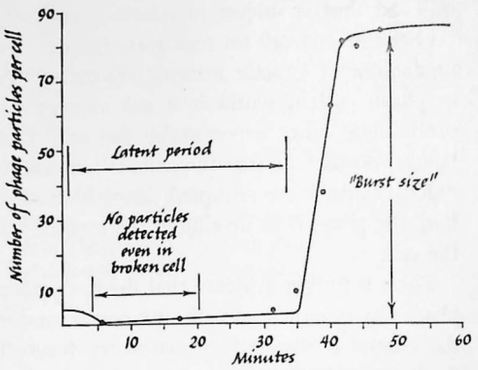


FIG. 28-6. *A one-step growth curve for phage.*

We may summarize the typical situation as follows. A phage particle is adsorbed on a susceptible cell by attaching its tail to a receptor spot on the cell wall. It then sets about breaking down the cell wall and injecting DNA. The DNA, once inside, starts to organize the cell machinery to make, not more cell, but more phage. The new phage particles are released into the environment.

Is the phage alive? It "reproduces," it can mutate, and it has specificity. If these are the characters of life, then it is alive. But it has no metabolism, no respiration, no synthesis of its own. It organizes the machinery of a living cell to its own end. The bacterial cell makes more phage; the phage itself does not multiply. All the properties that phages and other viruses possess which are attributes of living are found in them only when they are in contact with a living cell; hence the life is that of the living cell. What does it mean to be alive as a virus is alive?

This discussion of bacteriophage has had a distinctly genetic flavor. The phage injects DNA and the control of the cell machinery changes. There is an enormous body of knowledge, experimentally derived, about the bacteriophage, its nature, its variation, and its "latency." But this is specialized knowledge to which general microbiology can but serve as introduction. However, there are two matters that a general microbiologist should know something about: phage typing and lysogeny.

Phage Typing

The susceptibility to different races of bacteriophage may be useful in some instances in distinguishing pathogenic from nonpathogenic species or strains. Phage typing is especially useful in distinguishing strains among *Salmonella* and *Staphylococcus.*

Lysogeny

In the previous paragraphs we have described the situation which occurs when a virulent virus infects the host cell and, after an interval, lyses it. There also exist **temperate phages** whose infection, development, and release is somewhat different. When a temperate phage is mixed with a susceptible bacterial culture, part of the infected cells undergo the usual

cycle of phage multiplication and release; the others reach an operational state with the phage whereby the phage is carried in a prophage state from one cell to the daughter cells for generation after generation without developing virulent phage. Such bacteria, which carry a temperate phage in the prophage state, are termed **lysogenic.** A few cells in each generation produce mature or complete phage and lyse, liberating temperate phage into the medium, but most of the cells keep the prophage within the cell. In many cases we can induce the formation of mature phage by agents which tend to stop cell division—irradiation, nitrogen mustard, hydrogen peroxide; in these cases most if not all the cells lyse, liberating phage particles which are still temperate for the strain of bacteria used. However, temperate phages may be temperate for one strain and virulent for another.

While the situation is very complex, it appears as though the phage DNA becomes incorporated into the genetic mechanisms of the cell and thus is subject to whatever controls exist within the cell for regulating the orderly production of genetic material. Phage DNA or phage protein synthesis is not allowed to overbalance other processes in the cell and hence does not destroy the cell. When these orderly controls are disrupted, as with irradiation, the phage does develop at the expense of the cell.

There is further evidence that the temperate phage has intermeshed with and comes under the control of the orderly machinery for cell duplication. In some cases lysogenic infection is associated with a particular genetic characteristic. A most interesting case is that of *C. diphtheriae*, in which lysogenic infection with the temperate strain B is accompanied by the ability to produce toxin. Other strains, identical except for having no B prophage, do not produce toxin and are not pathogenic. They can be rendered toxigenic and thus pathogenic by treatment with temperate phage B, which results in lysogenic toxin-producing strains.

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For background information, see p. 496.

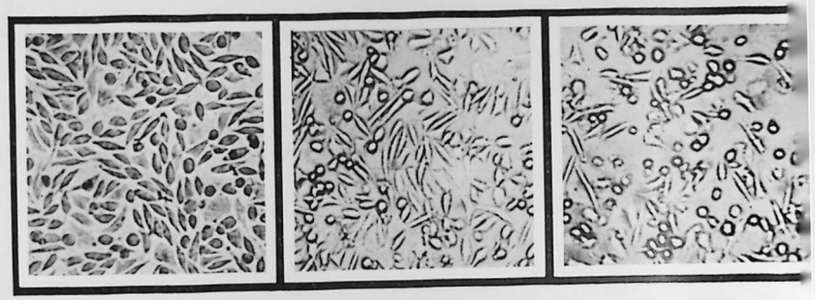
QUESTIONS

1. Define or identify:

virus	latent period
bacteriophage	burst size
phage	receptor spot
rickettsiae	salt shock
adsorption	phage ghost
release	lysozyme
plaque	incomplete phage
filterable	prophage
T phage	phage typing
core, head	lysogenic strains
coat, tail	temperate phage
lysis	

2. What is the evidence that bacteriophage is a particle? What evidence that it is alive?
3. Propose a theory to account for the development of resistance to a virulent phage. What mechanisms might be employed which would permit resistance?
4. If a single particle or a few particles of phage are allowed to act on a susceptible bacterial cell there is a latent period followed by lysis with a definite burst size, i.e., many new phage particles are produced. If thousands of phage particles per cell are supplied there is a shorter latent period and then lysis occurs; not only are no new phages produced, but the phage originally adsorbed to the cell cannot be recovered. Devise a theory to explain these facts.
5. There are genetic changes possible in the phage particle itself. These are primarily of two types: alteration in host range or alteration in plaque type. In the first case, has the phage altered so it now attacks more strains of bacteria than it did previously, or fewer strains? What kind of an alteration has the phage undergone? If the phage can mutate—as it does, as described above—is this not evidence that the phage is alive?

Chapter 29



VIRUSES IN HIGHER CELLS

"16. Ye shall know them by their fruits. Do men gather grapes from thorns or figs from thistles?

"17. Even so every good tree bringeth forth good fruit; but a corrupt tree bringeth forth evil fruit."

"20. Wherefore by their fruits shall ye know them."

St. Matthew, Chapter 7

IN THIS CHAPTER we shall restrict our attention to animal and human viruses, leaving the plant viruses for Chapter 31. We may first point out that the action of viruses in higher cells is similar in some respects to the action of bacteriophage; in other respects the higher cell virus operates differently. There is in higher cells a similarity to bacteriophage in that there is first an adsorption to the susceptible cell, a specific localization in tissues (i.e., adsorption to only certain types of cell in the tissue), a latent period in which the virus disappears, and a release phase. But in many other respects, **virology**, the study of the viruses of higher cells, is faced with somewhat different phenomena. For example, sometimes the virus is released by cell destruction and necrosis, just as it is by lysis in bacterial virus. In some instances bubbles are "pinched off" (Fig. 29-1). In other instances there is a slow continual leakage of virus without untoward evidence of cell damage. In fact, there are examples where the virus can grow in the animal cell without apparent harm to the cell. In general, it is only when the virus is produced in great amount that the cell is injured.

It is such common knowledge that viruses are invisible in the optical microscope, that we

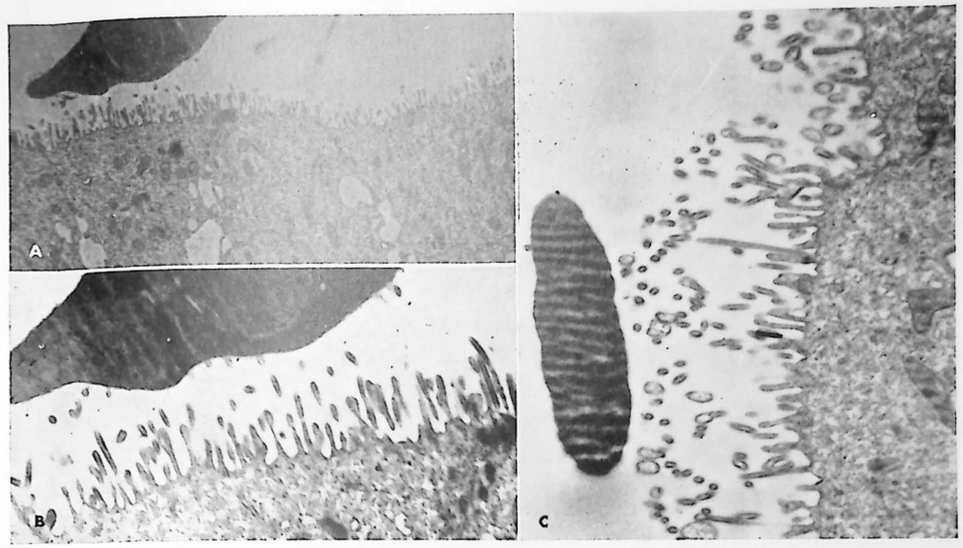


FIG. 29-1. *Virus infection in animal cells. A shows cells of an allantoic membrane 4 days after infection with Newcastle virus, with tremendous surface changes taking place. B is an enlargement of this surface, showing the development of the projections (not evident on noninfected cells). The dark object above the membrane cell in both A and B is a red blood cell (the chick blood cell is nucleated). C shows another membrane cell with the development of projections. Magnification of B and C about 20,000×.* [*From Bang*, Federation Proc., **14**, 619 (1955). *Courtesy of Dr. F. G. Bang.*]

today find it hard to realize how early knowledge of viruses developed and how they were put to very practical use. In 1798, Jenner, after 26 years of study of the diseases of cowpox and smallpox, published a paper which established the feasibility of vaccination; smallpox was known as *variolae* and cowpox as *vaccinae*, hence the word vaccination or vaccine, which has a wider meaning today. By 1799 a Benjamin Waterhouse in Cambridge, Massachusetts, announced in a newspaper that he had inoculated seven children in his family with cowpox vaccine and that they were then "forever after" secure from smallpox; thus, in the day of the sailing packet, vaccination had leaped the Atlantic within a year. In 1806 Thomas Jefferson wrote to Jenner that the future would know him as the man who had "extirpated loathsome smallpox." Still, at this time, nothing was known of the agent of either cowpox or smallpox. In 1892 Iwanowski discovered that the agent of a plant disease—tobacco mosaic—could be transmitted from plant to plant by an "agent" obtained by grinding up the infected leaves and passing the fluid through filters which were small enough to hold back bacteria; i.e., the agent was smaller than bacteria. In 1898 Loeffler and Frosch showed that an animal disease—foot and mouth disease of cattle—was transmitted by a filterable agent. Indeed, the virus of foot and mouth disease is still the smallest virus known. All of this is 20 years before the discovery of bacteriophage.

Methods for Viral Study

It seems obvious that new kinds of methods had to be developed to study these agents

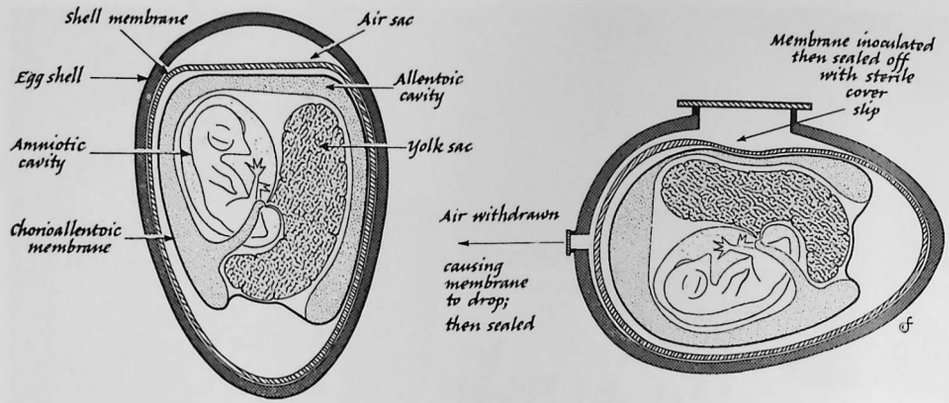


FIG. 29-2. *The chick embryo; main structural features; use with window.*

which were too small to see in the optical microscope. Some of these are hinted at from the history mentioned above. Of course, the development of the electron microscope permitted one to "see" viruses. But at the same time, two other methods were developed for the cultivation of viruses. These are especially useful in virology, but also have a use in other areas of microbiology. These are the chick embryo and tissue culture methods.

Chick Embryo

The embryo of the chick is an easily obtainable living material which is susceptible to many diseases—bacterial, viral, rickettsial—even diseases to which the chick, at the moment of hatching and beyond, is completely immune. A 14-day-old chick embryo looks roughly like the diagram of Fig. 29-2. There are two basic methods for using such an embryo. In one, a window is cut into the egg and the material grown on the chorioallantoic membrane. This method is capable of demonstrating that the virus, even animal virus, is composed of particles. A typical situation is shown in Fig. 29-3. Here a hole is cut in the egg shell as well as a window. Air is withdrawn from the hole, causing the membrane to drop, after which the hole is sealed off. The window, which may be cut by a dentist's drill, then has below it a reasonably large stretch of membrane. A suitably diluted virus preparation is placed on this membrane and the egg is sealed off with a sterile cover slip. The membrane can be watched during incubation and, when development is proper, the membrane can be removed and examined. Photographs of such membranes (Fig. 29-4) show that there are "spots" of tissue destruction entirely comparable to plaques in phage. Here is evidence that animal viruses are particles, just as is phage. We may use this method in order to count the number of infective virus particles in a given preparation.

FIG. 29-3. *Inoculating the chorioallantoic membrane of embryonated eggs with a virus suspension. (Courtesy of Eli Lilly and Company.)*

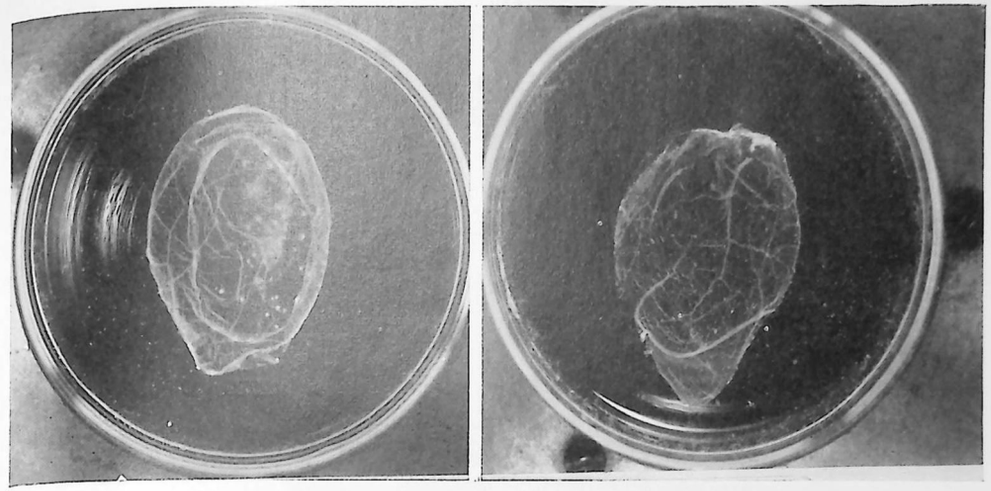


FIG. 29-4. *Pox lesions on a chorioallantoic membrane. Note the pocklike lesions on the infected membrane on the left. There are 62 lesions; hence if this had been inoculated from a 1/100 dilution of the virus, there would have been 6,200 virus particles per milliliter in the original preparation. This is variola virus (smallpox).* [*From Hahon and Ratner,* J. Bacteriol., **74,** *694 (1957). Courtesy Dr. N.* Hahon.]

In a second method, only a small hole is drilled into the egg, and through it a needle may be inserted to reach the yolk sac, the amniotic cavity, the allantoic cavity, or any other part of the embryo, and thus these may be inoculated. Many of the viral and rickettsial vaccines are prepared in this fashion.

Tissue Culture

By tissue culture, which really should be called "cell culture," we mean an in vitro isolated cell complex originating from plant, animal, or human tissue, containing actively proliferating cells which may be transferred from one vessel to another and perpetuated in this state. In a sense these are comparable to bacterial cultures; they may be purified, plated, counted, etc., but there are some aspects in which they differ. First, the animal or plant cell will rarely grow free but generally must be attached to a solid surface, as glass, cellophane,

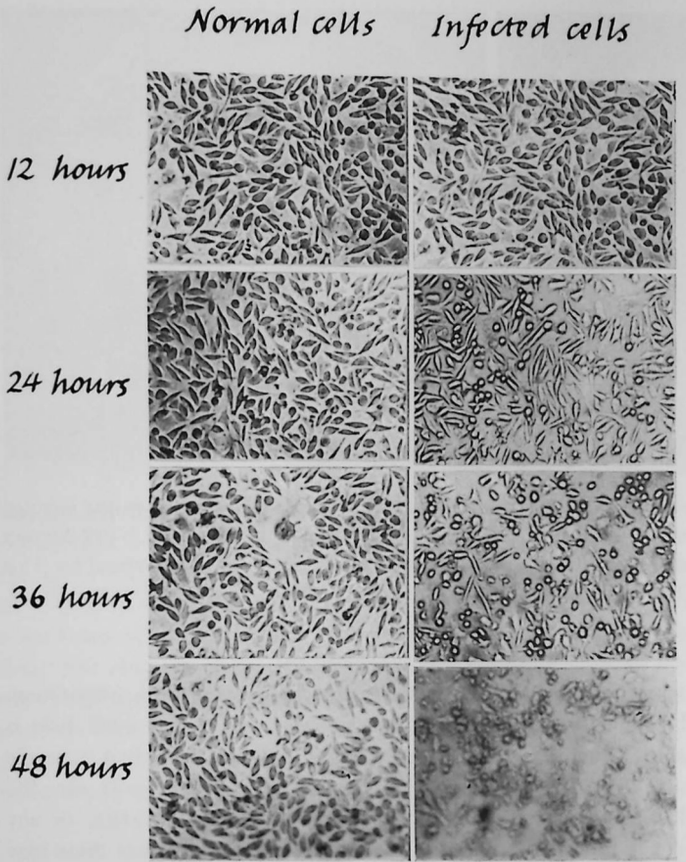


FIG. 29-5. *The effects of virus on tissue culture. This is a culture of "L" cells, a strain that has been carried in tissue culture for several years. The culture on the right has been infected with the virus of Venezuelan equine encephalomyelitis. Note the progressive degeneration of the cells as time goes on. Magnification 100×. [From Hardy and Brown, J. Bacteriol., 80, 20 (1961). Courtesy of Drs. Hardy and Brown; U.S. Army photograph.]*

etc. A few cell lines, carried for many years in culture, have lost the requirement for a solid surface to grow on, and these can be cultivated in suspension; indeed, they may be grown in fermentation tanks like bacteria or yeast, the latter of which they tend to resemble. Second, the media for growth generally requires plasma or serum plus extract of embryo. The factors necessary for growth present in these materials are not yet known. Again, however, there are certain cell lines which can be grown and grown well on a completely synthetic, if complex, medium, without embryo extracts or sera, and possibly all cell lines may eventually be grown in this fashion. The techniques used for growth are similar to those used for growing bacteria, and animal cells may be grown on a thin layer of agar. They cannot be plated in quite the same fashion and we can not make a pour plate; hence, we obtain single cells by what is essentially a streak plate. Tissue culture has now developed to a stage where bacteriological methods are applicable and the best background training for this new and exciting field of study is sound training in microbiology.

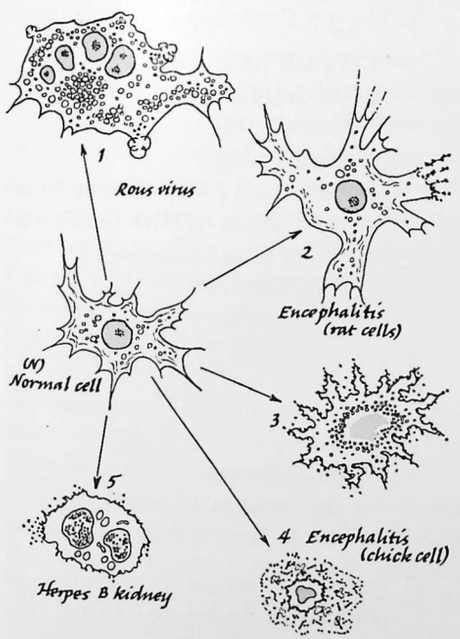
For our purpose here, however, it suffices to know that many higher cells can be grown in pure culture outside of the body. And most importantly, viruses will grow and develop on cells growing in tissue culture. Today this is the

principal method for detecting, cultivating, isolating, purifying, and identifying viruses. Most viruses cause some kind of detectable change in the tissue culture which can be observed. Figure 29-5 shows typical effects. As may be seen, these differ from virus to virus and at times may be sufficiently characteristic of the virus to be useful in identification and diagnosis.

Development of Viruses in Cells

In contrast to the clearly defined adsorption, DNA injection, development, and lysis found in bacteriophage, the entrance of a virus into animal cells and its subsequent development is more obscure, and probably is more varied and complex. There is, however, an adsorption, followed by a latent period. There is generally a specificity; some viruses travel to and develop in nerve, some in the intestinal wall, etc.

FIG. 29-6. *Varied effects of virus infection on tissue culture cells. [Redrawn from Bang,* Federation Proc., **14,** *619 (1955).]*



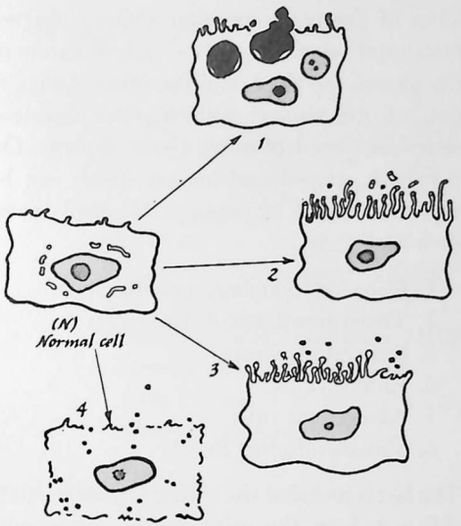


FIG. 29-7. *Methods of virus release from animal cells. [Redrawn from Bang,* Federation Proc., **14,** *619 (1955).]*

In many cases, if we examine the tissues in which the virus is localized, certain cells within the tissue appear to have foreign materials in them, called **inclusion bodies,** which in most cases later prove to be actual aggregates of virus particles. Some of these are so characteristic as to be diagnostic for the disease concerned. Especially is this true for rabies, where such inclusions (**Negri bodies**) in the brain of a suspected dog are diagnostic for rabies.

There are four principal methods for the release of virus from cells, as shown in Figs. 29-6 and 29-7; these are diagrammatic of the types of things seen in electron micrographs, one of which is also shown in Fig. 29-1. In only one is there complete cell disintegration. In most cases the cell remains intact, sometimes hardly damaged.

Types of Viruses

Most animal and human viruses are spherical and classification by morphology is thus not possible; nor can they be sensibly described in

terms of size or composition. Other characteristics must be employed: the general nature of the disease produced, and the interrelations, if any, of the viruses producing this disease—reservoirs, portal of entry, tissue response. On this basis, animal and human viruses can be separated into six large groups. We shall discuss each separately.

1. Psittacosis-lymphogranuloma group
2. Tissue growth-stimulating group
3. Respiratory viruses
4. Enteric viruses
5. Neurotropic virus
6. Viruses infecting insects

The last is included since these viruses are quite different from the other animal viruses and deserve special mention.

Psittacosis-Lymphogranuloma Group

These are a group of serologically related viruses, large (200–300 mμ in diameter), spherical, susceptible to antibiotics (tetracyclines, penicillin, some susceptible to sulfonamides), and they occupy a position close to the rickettsiae. The virus particle contains protein, lipid, carbohydrate, DNA, and RNA, enclosed within a membrane. The general progress of development is the following: after penetration of the host cell, the virus, which is itself just on the limits of optical visibility, "grows" and **elementary bodies** as large as 800 mμ are found in the infected cells. These increase in numbers, form clusters, the clusters tend to coalesce and liberate virus as free individual particles. All have an endotoxin. The following diseases are caused by members of this group.[1]

1. Psittacosis or ornithosis ("parrot fever")
2. Lymphogranuloma venereum
3. Trachoma and inclusion conjunctivitis
4. Mouse pneumonitis
5. Feline pneumonitis
6. Human pneumonitis
7. Opossum meningopneumonitis
8. Bovine encephalitis
9. Ewe abortion

[1] The diseases are listed by name without description. You would not, certainly, be expected to know all of them for general microbiology. However, they are listed and appear in the index; should you run across the name you could look back and see what general kind of virus is involved.

Tissue Growth-Stimulating Group

This group is frequently called **exanthematous** and tumor disease group. It is not especially homogeneous but the viruses are large, some rectangular, most spherical, with some serological interrelations. Among them, however, is the smallest virus, that of foot and mouth disease. They all form inclusion bodies and are not susceptible to usual antibiotics. In many cases in this group, infection stimulates a proliferative response in the host cells, sometimes briefly followed by necrosis, as in a "cold sore"; at other times growth stimulation is prolonged, resulting in tumors. The following diseases are caused by members of this group.

A. The pox (brick-shaped virus particles)

1. Variola (smallpox)
2. Vaccinia (cowpox)
3. Varicella (chicken pox)
4. Zoster (shingles)
5. Animal pox (not pathogenic to man)
   (a) Cowpox (differs from vaccinia in being hemorrhagic)
   (b) Mouse pox
   (c) Fowl pox

B. The herpes group (spherical particle)

6. Herpes simplex (cold sore, fever blister)
7. Animal herpes
   (a) Pseudorabies (mad itch of swine)
   (b) B virus (monkey bite disease)

C. Other (not necessarily related to each other except for numbers 10 and 11)

Types of Viruses

8. Foot and mouth disease
9. Cat scratch fever
10. Rubeola (measles)
11. Rubella (German measles)
12. Infectious mononucleosis
13. Salivary gland disease

D. Tumor viruses

14. Molluscum contagiosum
15. Infectious myxomatosis
16. Infectious fibromatosis (16 may be transduced to form 15, probably by DNA from 15)
17. Rabbit papilloma (Shope papilloma)
18. Mouse carcinoma
19. Avian leucosis
20. Erythromyeloblastosis
21. Fowl sarcoma (Rous sarcoma)

Respiratory Viruses

There is an array of respiratory diseases caused by somewhat related viruses, sometimes called myxoviruses—flu, grippe, colds, etc. In general, these are contracted and transmitted in a fashion similar to the respiratory diseases caused by bacteria. The viruses are relatively smaller, mostly spherical, chemically complex (protein, carbohydrate, lipid, and RNA) with a somewhat variable composition, depending upon the tissue from which it was obtained. Further, while viruses in general can undergo mutation, the viruses in this group seem to have a great predilection for doing so. This gives rise to new strains, such as Asian influenza. Further, while these viruses are as antigenic as any other and vaccines are perfectly possible, the virus strains keep mutating so rapidly that each year finds a "different" virus operating and a new vaccine must be developed for it. This is especially true for influenza, less true for mumps. The viruses and the disease they cause may be divided into three groups.

A. Influenza group

1. Influenza
2. Swine influenza
3. Newborn pneumonitis (type D influenza)
4. Croup viruses (CA or croup-associated virus)
5. Chimpanzee coryza agent (CCA)
6. 2060 viruses
7. Primary atypical pneumonia (PAP)
8. Common cold
   May be caused by more than one virus or combination

B. Mumps group

9. Mumps
10. Newcastle disease of chickens

C. Advenoviruses

11. Acute respiratory disease
12. Pharyngoconjunctival fever
13. Epidemic keratoconjunctivitis

Enteric Viruses

These are small (15–35 mμ in diameter) viruses which are found in the bowel early in infection and are generally transmitted as enteric disease, but may show symptoms elsewhere in the body. For example, poliomyelitis is essentially an intestinal virus and the neurotropic, paralytic form is rare in proportion to the number of people infected. The virus of poliomyelitis has been crystallized as a pure RNA protein. It is the smallest of the human viruses. The diseases caused by this group are the following.

1. Poliomyelitis (infantile paralysis)
2. Encephalomyelitis of mice
3. Encephalomyelitis of swine (Teschen virus)

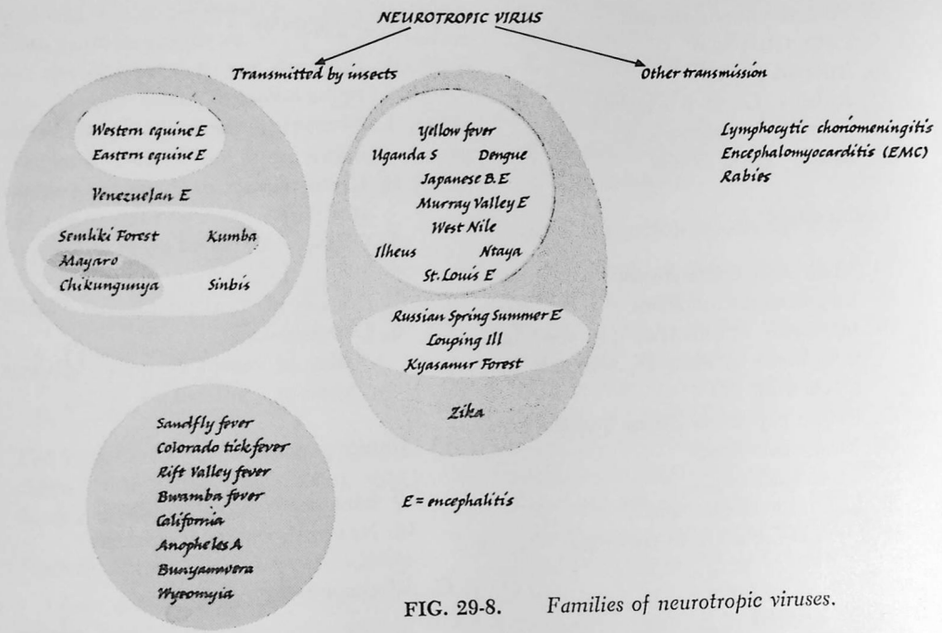


FIG. 29-8. *Families of neurotropic viruses.*

4. Coxsackie viruses
   (a) Herpangina
   (b) Epidemic pleurodynia
   (c) Aseptic meningitis
   (d) Infantile myocarditis
5. Orphan viruses (ECHO)*
6. Viral diarrheas
7. Infectious hepatitis
8. Serum hepatitis
9. Canine hepatitis
10. Marine hepatitis

Neurotropic Viruses

These are small, usually spherical viruses, with a diameter of 40–50 mμ, which have a prediliction for the central nervous system. Many are transmitted by insects and there is reason to believe that, with the exception of yellow fever, human infection is only incidental; that these are primarily animal diseases. The encephalitis ("sleeping sickness") viruses are worldwide but the relationships between them are so complicated and sometimes so obscure that we have thought it proper to organize the information loosely in Fig. 29-8. All viruses within a circle are more closely related than those without and there are groups within larger groups. The names are generally derived from the region where the virus or the disease was studied.

Insect Viruses

Lastly we shall mention viruses which cause disease in insects—bees, moths, etc. These are relatively large viruses, frequently rod-shaped. Two kinds of inclusion bodies are formed by different viruses; one is a large polyhedron (3–5 μ); the other type is a small (1 μ oval body. The viruses themselves contain DNA and protein.

* After tissue cultures were developed and isolation of viruses became easier, a group of viruses were found which caused cytopathogenic effects in tissue culture; i.e., they caused cell disintegration). Some were isolated from man, others from monkeys, cattle, swine, and dogs. However, there is no disease entity associated with them. They have been called "viruses in search of a disease," or enteric cytopathogenic human orphans—ECHO viruses.

General Comments

The study of viruses, virology, in a real sense is a science distinct from microbiology although part of it. The methods, techniques, and background information are quite different than other fields of microbiology. In most cases little can be done to treat the virus disease, but much can be done, mostly via vaccination, to prevent it. The development of polio vaccine, undoubtedly familiar to you, is a case of the application of the knowledge of virology to the needs of man.

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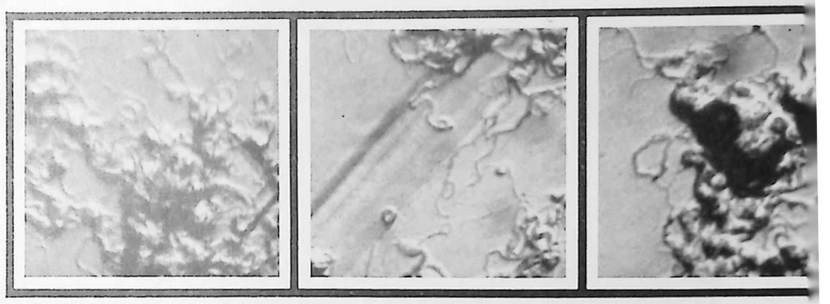
For background information, see p. 496.

QUESTIONS

1. Define or identify:

virus	inclusion bodies
variola	Negri bodies
vaccinia	elementary bodies
chick embryo culture	ECHO virus
tissue culture	

2. You have noticed that the virus causing cold sores is related to those causing tumors. In both cases there is an initial stimulation of cell development, followed, in the case of the cold sore, by a necrosis. If it were possible to induce the tumor virus to produce a "necrotizing principle" or it were possible to isolate one from the herpes virus to be used on the tumor, there would be available a possible way to treat the tumor. As a microbiologist, do you think that these are likely possibilities?
3. If animal tumors, not necessarily benign, are caused by transmissible viruses, and are indeed, infectious diseases, do you regard it possible that human cancer is caused by virus? What evidence could you deduce to favor this viewpoint? What precautions would you need to emphasize before you jumped to this conclusion?
4. It is possible to grow cells from monkey kidney in tissue culture and to grow poliomyelitis on these cells. The polio virus is then inoculated and used as vaccine. But sometimes other viral diseases come through this treatment. How could you demonstrate that a monkey kidney cell line which you were growing was free from encephalitus virus before it was used to grow polio?
5. It is common for people who first learn about animal virus, after having learned about bacteriophage, to regard them as "essentially" similar. What could you cite as profound differences between them? Are these "really" fundamental?



Chapter 30

THE RICKETTSIAE

"The birth of an infectious disease is not as simple a matter as that of man. Gestation is not a mere matter of ten months or so, but represents complex biological interpretations and interactions which cover thousands of years. In this particular case we may say that the *conception* of our diseases took place when the first Rickettsiae became parasitic on insects; and *gestation* lasted through the uncertain but undoubtedly centuries-long period during which parasitism progressed from insect to animal, and finally through other insects to man himself."

ZINSSER, *Rats, Lice, and History*

THE RICKETTSIAE ARE ORGANISMS first observed within the cells of animals afflicted with Rocky Mountain spotted fever. They are exceedingly small gram-negative organisms appearing within cells as either short bacilli (0.3 × 0.5 μ) or cocci (0.3 μ in diameter). They can not yet be grown in the absence of living cells. They must, then, be cultivated in chick embryo in the yolk sac, in tissue culture, or maintained by animal passage or within the arthropod vectors—tick, mite, louse, flea. In this sense they resemble the larger viruses, and just as these are sensitive to some antibiotics, so are rickettsiae.

But on the other hand they resemble the bacteria and can be seen to divide by binary fission within the cells of the host. Further, some rickettsiae can be prepared essentially free of the cells they have grown in. They may be treated as an "ordinary" delicate bacterial cell. They have a rigid cell wall (Fig. 30-1) and do not require osmotic protection. The cell wall contains a hexoseamine, galactose, glucose, and glucuronic acid complex. If the separated

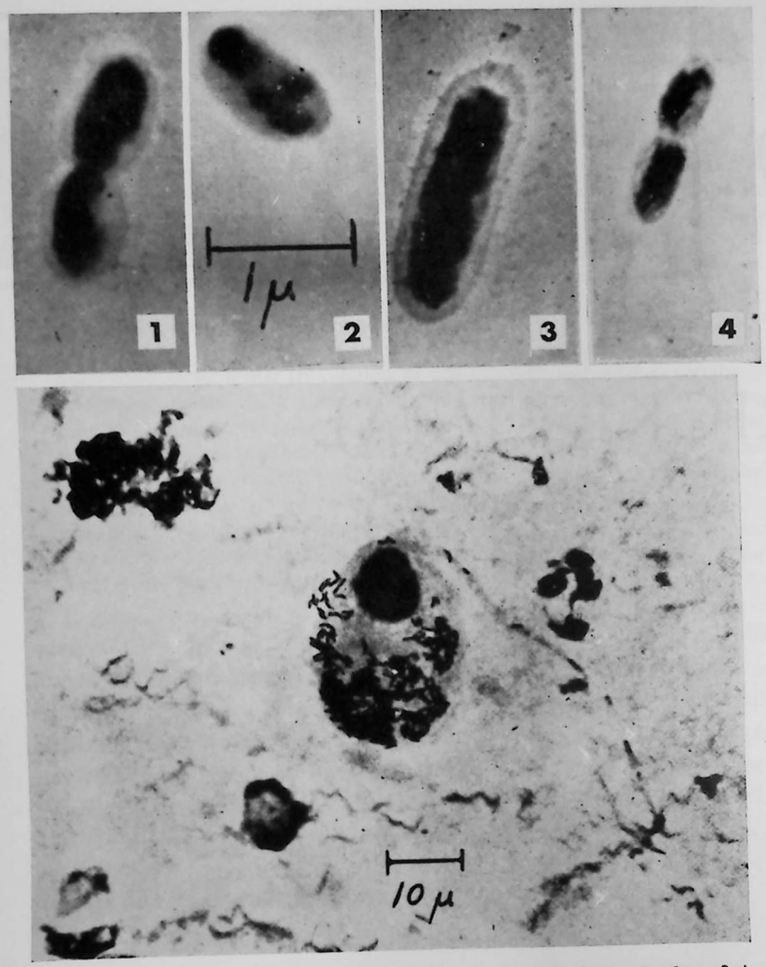


FIG. 30-1. *Rickettsiae. 1 is epidemic typhus, 2 is endemic typhus, 3 is Rocky Mountain spotted fever, 4 is American Q Fever. The lower photograph shows endemic typhus infecting the cytoplasm of a guinea pig cell.* [*From Plotz, Smodel, Anderson, and Chambers,* J. Exp. Med., **77**, 355 (1943). *Amer. Soc. Microbiol., photos LS-25, LS-26.*]

rickettsia are allowed to stand they lose their pathogenicity but this can be restored by the addition of glutamate and DPN. They oxidize glutamate via the citric acid cycle and this oxidation can lead to the formation of ATP. They thus have a metabolism independent of the higher cell in which they were grown. They seem to occupy a position intermediate between the bacteria and the larger viruses.

Relation to Arthropods

There is good evidence that rikettsiae originate primarily in acarids; the spiders, ticks, and mites. These creatures have eight legs, distinguishing them from insects, which have only six. In the acarids the infection with rickettsiae appears to be harmless. The rickettsiae also seem to live in a reasonably balanced and nonpathogenic state in some animals and these tend to be reservoirs of the disease. However, rickettsiae also live in the bodies of insects, lice, and fleas. Here there seems to be some considerable toxicity to the insect. The diseases are transmitted by the bite of the tick or louse. In the human body the organisms are found within the cells; sometimes a phagocyte may be completely filled with them, and they tend also to completely fill the infected body cells.

The Weil-Felix Reaction

It was early discovered that blood taken from individuals suffering from certain rickettsial diseases would agglutinate certain strains of *Proteus*. The laboratory numbers of these strains were X19, X2, XK, etc. While originally this led some to suppose that the rickettsial diseases were caused by *Proteus* species, the peculiar property proved to be a useful diagnostic tool. It is probably caused by the possession of a common antigen in the *Proteus* strains and in rickettsiae. That is, the same protein happens to occur in both organisms, hence there is **cross reaction.**

These various strains exist in two forms: those having flagella and thus spreading over the media—called H-form after "hauch," the German word for spreading; those which lack flagella and do not spread—O-form from "ohne-hauch," without spreading. The O-form is the one reacting with rickettsial sera, hence these are labeled OX19, etc. On the basis of these agglutination reactions, four groups of rickettsiae could be distinguished. These are shown in Table 30-1.

TABLE 30-1. *Rickettsial Diseases*

Group	Human Disease	Vector	Reservoir	Weil-Felix		Where Found
				OX19	OK	
I Typhus	Epidemic typhus European typhus Brill's disease	Louse	Man	++++	−−	Europe, Asia, South America
	Murine typhus	Flea	Rat	++++	−−	Worldwide
II Spotted Fevers	Rocky Mountain	Tick	Rodents	+	−	North America
	Mediterranean	Tick	Dog	+	−	Europe, Asia
	Australian	Tick	Rat?	+	−	Australia
	Typhus	Tick	Rat	−−	−−	North Europe, United States, USSR
	Rickettsial pox	Mite	House mouse	±	−−	Worldwide
Tsutsugamushi	Tsutsugamushi	Mite	Rodents	−−	+++	Asia
Q Fever	Q fever	Tick	Sheep, cattle, goat	−−	−−	Worldwide
	Trench fever	Louse	?	−−	−−	Found only during World War I & II

Rickettsial Diseases

Two diseases are of particular importance, typhus fever and Rocky Mountain spotted fever. Note that typhus fever is a disease of blood and tissue transmitted by lice and fleas contrasted to typhoid fever, which is a disease caused by the invasion of *Salmonella typhi* from the intestinal tract and transmitted by water. Typhus fever has been known since very ancient times and one of its important students wrote a book to illustrate that the disease probably had more influence upon past military campaigns than did the generals (Zinsser: *Rats, Lice and History*). It would pay you to read the book.

In Table I, listed under typhus, is a disease called "Brill's disease," in which the blood shows Weil-Felix OX-19 agglutination, but symptoms of typhus are not noticeable. If such persons are subject to tremendous stress, or if given large amounts of adrenal hormones, they may break out into full-fledged typhus. One-third of the people living in or coming from central Europe or European Russia are likely to have Brill's disease.

Rocky Mountain spotted fever has been known for many years in Idaho, Montana, Wyoming, Oregon, and Washington, but in 1930 it appeared in Maryland, Virginia, West Virginia, and North Carolina. There is a vaccine available. Rickettsial pox was first found in New York City in 1946. Tsutsugamushi disease is found largely in Asia; the name means "dangerous bug" disease. Most rickettsial infections respond to the broad-spectrum antibiotics—tetracyclines and chloramphenicol.

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For background information, see p. 498.

QUESTIONS

1. Define or identify:

rickettsiae	Weil-Felix reaction
arthropod	O Form
vector	H form

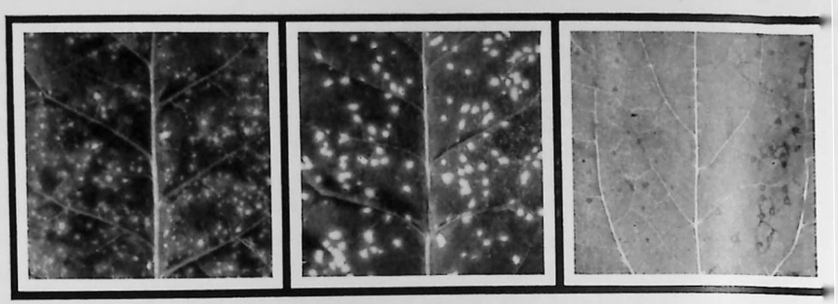
2. What reasons are there for regarding rickettsiae as viruses? as bacteria? Which do *you* think they are?

3. You are in charge of a refugee camp in Austria. Because of tightened border restrictions, most persons newly arrived from Hungary must be placed in quarantine because they break out with typhus, even though you have no typhus in the camp and it is as clean and as louse-free as possible. How could you account for this?

4. The rickettsial body responsible for Q fever is rather smaller than the others and can pass through filters which hold back bacteria and other rickettsia. Why do we call this a rickettsiae rather than a virus?

5. A titer of 1/50 is regarded as negative in the Weil-Felix reaction, with 1/100 possible and 1/200 positive. In routine blood examinations at port of entry at San Francisco, you find OX19 at 1/50 and OK at 1/500. What would you do?

Chapter 31



PLANT DISEASE

"A practical conclusion which everyday experience draws about life, though quiet reflection in the study may challenge it, is that some life is accompanied by mind, and some is not. The whole plant world we recognize as alive, but even to crowning instances of it, such as tree and flower, our practical everyday thinking does not attribute mind. The pragmatic position is then that we distinguish between life and mind. So then we ask 'what is this life,' for instance of flower and tree, devoid as it seems of mind? The answer to which our facts impel us is that this life, for instance of flower and tree, is chemico-physical behaviour with a final cause imbedded in it. Mindless competition as it is it does not connote suffering. Man when he employs such lives, and tasks or sacrifices them, is not doing otherwise than when he employs other machinery, for they are machinery. Life in so far and per se seems no sacred thing."

SHERRINGTON, *Man on His Nature*

A LAST CHAPTER ought to be a summing up—a fitting and—indeed, perhaps a climax to the labor we have borne to reach it. But this is a textbook of microbiology and you do not necessarily read all of a textbook, nor do you read it in any special order. If you have not read the argument in the first chapter, then you have provided direct evidence of the truth of these propositions. We devote this last chapter to plants, not because they are less important, or because we know less about them, but indeed, for the opposite reason. Their importance is such that there has developed a new science, plant pathology.

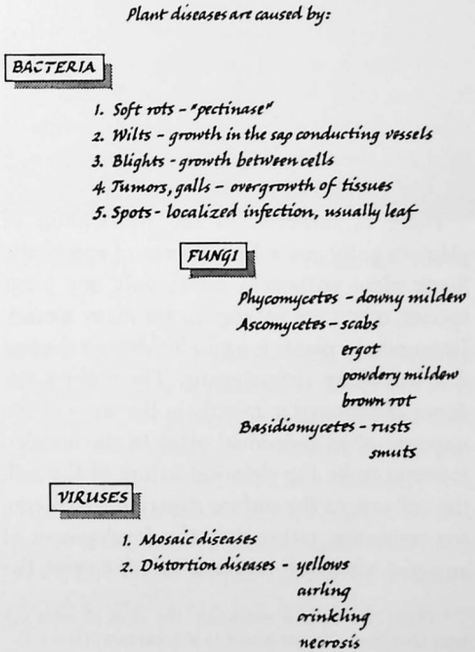
Plant diseases caused by microorganisms were studied by the early bacteriologists, but later studies were carried out by a somewhat separate group of students of microbiology: the plant pathologists. They dealt with a somewhat less dramatic, but nevertheless devastating form of disease. The loss of human life and its protection has appealed to most students of disease more strongly than the blighting of a pear tree or the wilting of a potato vine. Yet from the economic point of view the plant diseases are responsible for enormous losses. We must confess that the average modern microbiologist gives the plant diseases rather less than their just due. There is a good reason for this; there is a science of plant pathology, and microbiology itself is already replete with too much detailed and uncoordinated information. Rather than point out the properties of a few plant diseases, it is, in our opinion, much better to discuss the basic principles of plant disease control, since it is these aspects that a general microbiologist, not specifically a plant pathologist, will need to know.

It is first evident that the creature subject to microbial attack is a distinctly different creature from the animal, and much of the nature of plant disease is necessarily related to the cellulosic nature of the plant. The way of life is different—and this conditions and controls the nature and character of plant disease. Plant pathology is then a somewhat different study than animal pathology, and we shall spend some effort on pointing out these differences.

While, in general, plant diseases follow in many respects the principles outlined for animal disease, in other aspects a completely different relationship holds. Plant diseases are transmitted from infected plants, with a means of exit, a route of transmission, a portal of entry, and once within the new plant there may be a struggle before disease results, although there is little evidence of the development of immune reactions after the organism has invaded. While, further, there has been some attempt to treat the diseased plant, there is relatively less of this than in the animal, and much of what is done is essentially radical surgery, i.e., the infected part is simply removed. This is done, of course, only where the individual plant is of some considerable value —a tree, for example. It is only recently, as we shall describe further on in this chapter, that treatment of the already diseased individual plant by methods other than surgery has even been considered.

Before considering the "public health" aspects of plant disease—i.e., the exit, the transmission, and the portals of entry—it is well to point out that three large groups of organisms are the principal causes of plant diseases—bacteria, molds (fungi), and viruses. Figure 31-1 lists these, with the major types of disease produced. The viruses, of course, can grow only

FIG. 31-1. *Types of plant diseases.*



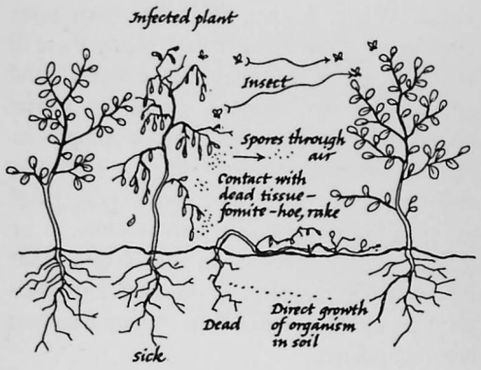


FIG. 31-2. *Transmission of plant diseases.*

in the living plant;[1] the bacteria and fungi, by and large, are saprophytes and will grow on dead tissues. Also the viruses grow within the cell, but most of the bacteria and many of the fungi do not invade the cell at all, but rather grow on the materials between the cells. For example, many of the gram-negative fermentative rods (*Erwinia*) hydrolyze and live on the material cementing the cells together, since they possess a potent **pectinase.** There is rarely within the plant the reaction to invasion that is common in animal infections—indeed, there is no defense by phagocytes or by fever, although there may be abnormal movements of fluid and there are in some cases efforts to wall off the infection.

There is, however, in the relationship of plant to pathogen a high degree of specificity. Some plant pathogens infect only one plant species; others are pathogenic for many species. Immunity in plants is again highly specific and is a hereditary characteristic. There is no evidence of acquired immunity in the sense of the response of an individual plant to the invader. It seems to be the chemical nature of the cell, the cell sap, or the surface structure which confers resistance, rather than the development of an agent after such invasion. In a few cases, the cause of the immunity is known. For example, there are two varieties of wheat, one resistant, the other susceptible to a given root rot caused by a fungus. The resistant variety differs from the sensitive variety in having in the root and the root hair a cyanogenic glucoside, a carbohydrate combined with cyanide. The fungus contains an enzyme hydrolyzing such glycosides, by which it effects its entry to the root. But when it hydrolyzes the one in the resistant plant it liberates small amount of HCN, which kills off the fungus. Thus is the plant "immune" to the disease.

[1] There is a curious exception; the virus of aster yellows also grows in the insect that transmits it.

Transmission of Plant Diseases

It is convenient to begin our consideration of the spread of plant diseases by considering the routes of transmission. For the animal (see Chapter 23), these are air, water, foods, fomites, insects, and direct contact; subsequent discussion demonstrated, for most animal diseases, that air, water, food were perhaps the most important. With plants, transmission by insects is by far the most important, especially by the sap-sucking and winged insects. Infected plants are usually the source of pathogens. From these, usually from the dead or dying parts, the organism may be transmitted by air, water, fomites (implements, animal, human, and plant), direct passage of the organism through the soil, and insects. And the most important of these is insects (Fig. 31-2).

The portals of entry are not so fundamentally important as in the animal. First, much of the plant disease is transmitted by insect vectors; the agent is actually injected into the tissues. Second, many of the plant pathogens, especially the fungi, are capable of growing on and penetrating the outside layer of the plant. In addition to the major portals of entry, there are natural openings (stomata in leaves, pores in stems, nectar ducts in flowers) and wounds,

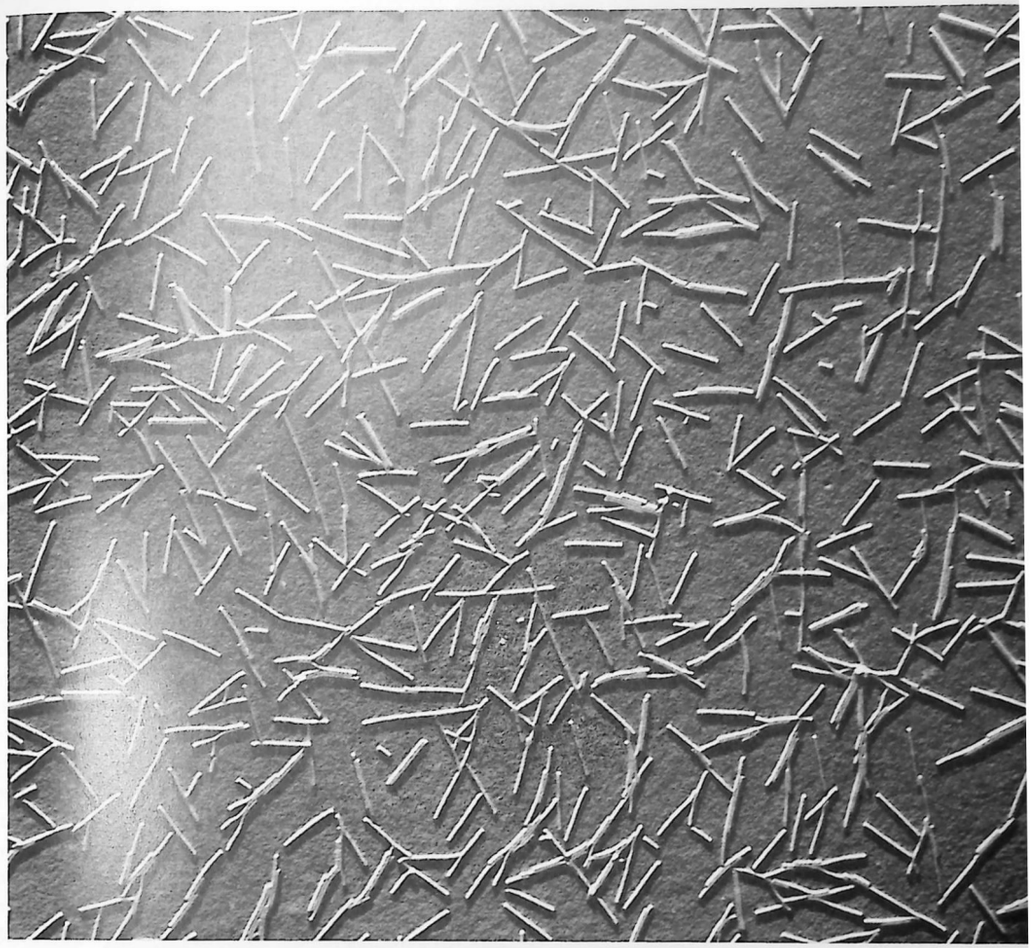


FIG. 31-3. *Crystals of tobacco mosaic virus.* [*From Ribi, Brown, and Goode,* J. Bacteriol., **79,** *142, 1960. Courtesy Dr. E. Ribi.*]

especially from agricultural implements, which serve as additional portals.

Public health measures to prevent the spread of plant diseases are in principle the same as "block means of exit, cut lines of transmission, close portals of entry" used in the animal. However, in addition, there are two further principles applied. First, destroy the infected individual, rather than treat it. Second, keep the pathogen and the plant apart; do not grow the plant in infected places until the pathogen dies out, if this is possible, or lower the insect population by protecting the plant from insect bites. Many sprays are protective to the plant by preventing insect infection. Recently there has been considerable attention given to treatment of the infected plants, mostly with antibiotics. Fire blight of apples and pears (caused by *Erwinia amylovora*) has been effectively controlled in orchards by spraying with strepto-

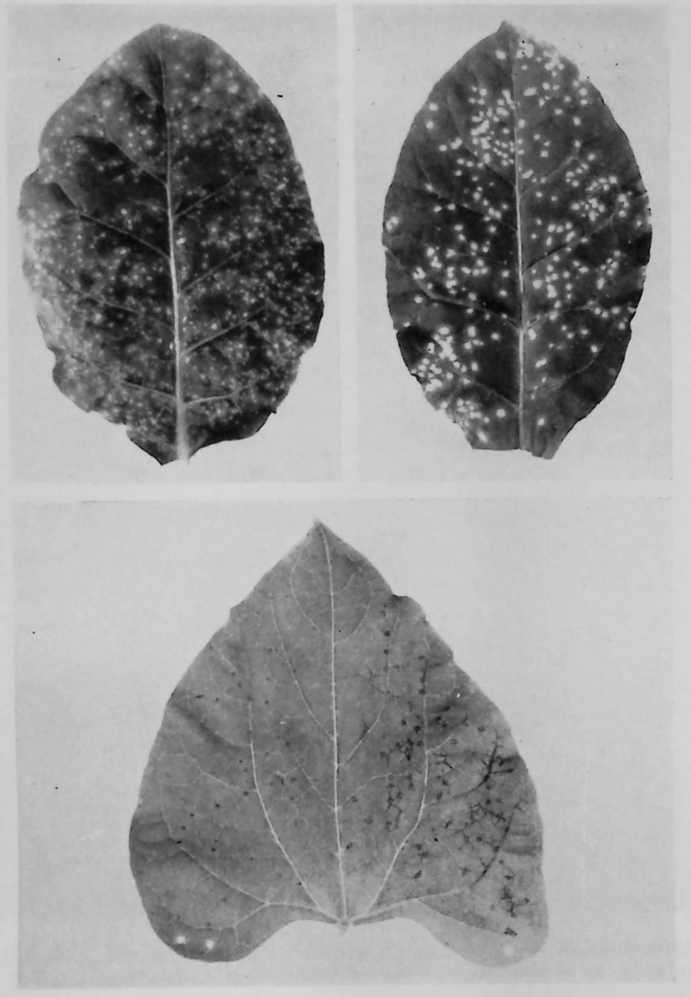


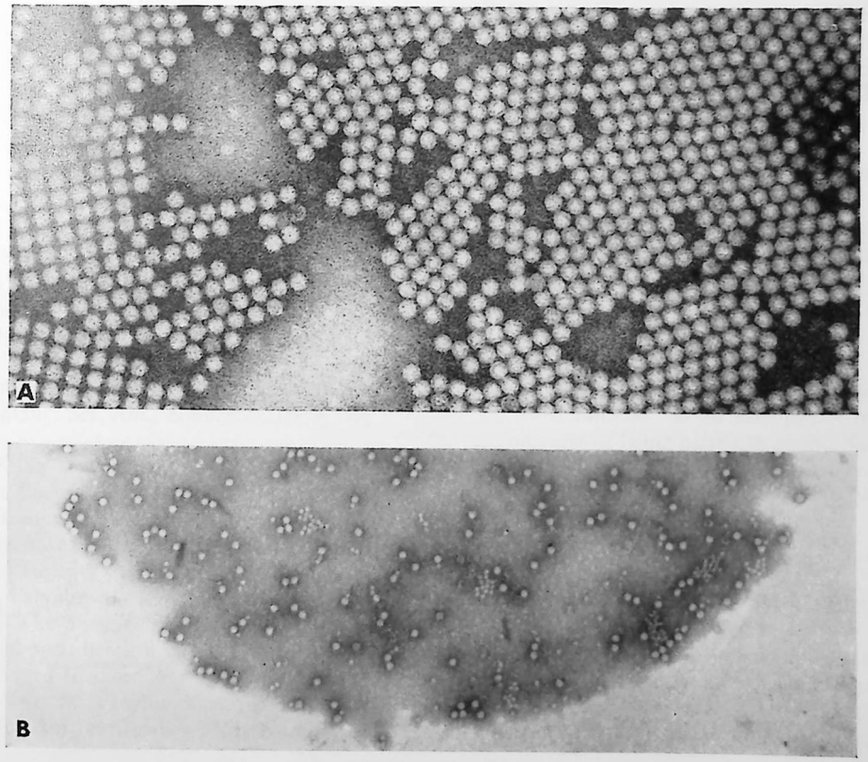
FIG. 31-4. *Plant virus growing on leaves. Top left shows a tobacco leaf inoculated with tobacco necrosis virus. This virus consists of small particles and large particles. Top right is an inoculum of large particles only. Top left is the growth on tobacco leaf of both the large and small particles. Bottom is a bean leaf, the left half of which has been inoculated with the large particles, the right half with the small particles. These photographs demonstrate that the nature of the lesion varies with the nature of the virus (compare top right and left) and with the nature of the plant (compare with the left half of bottom). [From Kassanis and Nixon,* J. Gen. Microbiol. **25,** *459 (1961). Courtesy of Dr. Kassanis and Nixon.]*

mycin. Bacterial canker of the cherry and peach (both caused by *Xanthomonas* sp.), bacterial spot of peach, walnut blight, halo blight of bean, and celery blight has also been controlled by streptomycin. Other antibiotics which have been useful in treatment of plant diseases are actidione, chloramphenicol, penicillin, candicidin, nystatin, tetracycline, and others. This information seems to open up a new approach to plant disease control, and one in which the microbiologist will play an important role.

Plant Viruses

The plant viruses differ in several respects from the bacterial viruses, but in many other respects they resemble them closely. The plant virus is not so highly organized into head and tail as is a bacteriophage. Instead of DNA they have RNA, but this also lies within a protein coat. Several have been crystallized (Fig. 31-3). In fact, the tobacco mosaic virus was the first virus crystallized (by Stanley in 1935). The viruses produce two types of diseases in plants. The first type is characterized by the mottling or spotting of leaves (sometimes blossoms), called the **mosaic disease,** e.g tobacco mosaic, bean mosaic, ring spot, and spotted wilt (Figs. 31-4, 31-5). There is one item of special interest. Certain tulips have flowers of variegated color rather than the same color throughout. This proves to be due to a virus infection. The second type of plant virus disease causes curling, crinkling, yellowing and/or dwarfing of leaves, sometimes excessive branching. Here there is another oddity. One plant virus, which causes aster yellows, is transmitted by an insect (a leafhopper) and there is good evidence that the virus grows in the insect. Normally a virus is restricted to the class of creature it attacks, and even restricted to certain strains within a species, so that for instance, a bacteriophage does not attack a plant, or an animal. But here is a virus growing in both plant and insect!

FIG. 31-5. *Plant virus particles. Plant viruses have varied shapes. Those of tobacco necrosis virus are spherical; those of tobacco mosaic (Fig. 31-3) are cylindrical. A shows the small particles mentioned in Fig. 31-4 at a magnification of approximately 240,000 ×. B shows both the large and small particles at a magnification of 74,000 ×. [From Kassanis and Nixon,* J. Gen. Microbiol., **25,** *459 (1961). Courtesy of Dr. Kassanis and Nixon.]*



The plant viruses resemble the bacteriophage in having a high specificity as to their attack, and this specificity resides in the protein. It has been possible to separate RNA and protein without denaturing either. It has then been possible to recombine them to form an active virus. If the RNA from tobacco mosaic virus is combined with the protein from bean mosaic virus, the new virus attacks the bean, i.e., the attack specificity is dependent on the protein. But in the bean it is the tobacco virus which is produced. The synthesis and properties of the naturally synthesized virus are determined by the nucleic acid, not the protein it is attached to.

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For background information, see p. 498.

QUESTIONS

The value of freedom lies in the ability to exercise it. Since we have survived this far with questions, we shall exercise our freedom to dispense with them.

Appendix

BACKGROUND INFORMATION

Chapter 3

Astbury, W. T., "Flagella"
The whiplike appendages of bacteria are among the smallest living structures. They behave like small muscles; thus they are studied to reveal the basic properties of muscle and other contractile proteins. *Sci. Am.*, January 1951

Acker, R. F. and S. E. Hartsell, "Fleming's lysozyme"
The discoverer of penicillin also found a substance that dissolves bacterial cells. *Sci. Am.*, June 1960

Mazia, D., "Cell division"
Called mitosis, it is the process by which units of living matter duplicate themselves. Its delicate apparatus is now investigated by the remarkable feat of isolating it from the rest of the cell. *Sci. Am.*, August 1953, No. 27

Solomon, A. K., "Pores in the cell membrane"
Molecules and ions pass in and out of the cell through tiny openings in the cell wall. Although the holes are too small to be seen with an electron microscope, their size has been measured experimentally. *Sci. Am.*, December 1960, No. 76

Satir, P., "Cilia"
In a wide variety of organisms from both the plant and animal kingdoms these tiny hairlike projections have the same basic structural plan: two long filaments surrounded by nine others. *Sci. Am.*, February 1961, No. 79

Brachet, J., "The living cell"
Anatomical and chemical views of the cell have now converged to show that it is not a droplet of protoplasm but a highly organized molecular factory. *Sci. Am.*, September 1961, No. 90

Hayashi, T., "How cells move"
A paramecium moves by the beating of its cilia; an amoeba, by the streaming of its cytoplasm; a muscle, by its overall contraction. All these process appear to share an underlying molecular unity. *Sci. Am.*, September 1961, No. 97

Holter, H., "How things get into cells"
It seems that some things passively flow through the cell membrane under various pressures, others are somehow actively pumped across the membrane and others are engulfed when the membrane folds inward. *Sci. Am.*, September 1960, No. 96

Mazia, D., "How cells divide"
All cells arise by division. In the process of mitosis each cell is apportioned a complete set of hereditary instructions when the chromosomes replicate and are separated by the mitotic apparatus. *Sci. Am.*, September 1961, No. 93

Chapter 6

Buswell, A. M. and W. H. Rodebush, "Water"
Although we take its properties for granted, they are most unusual. As an example, it has the rare property of being lighter as a solid than as a liquid. If it were not, lakes would freeze from the bottom up. *Sci. Am.*, April 1956, No. 262

Chapter 7

Clayton, R. K. and M. Delbrück, "Purple bacteria"
Certain microorganisms have a characteristic reaction to the presence or absence of light. They can thus be used as a means of studying the fundamental mechanism by which living things

react to their environment. *Sci. Am.*, November 1951

Chapter 8

Frieden, E., "The enzyme—substrate complex"
Certain transitory compounds play a vital role in the chemistry of life processes. *Sci. Am.* August 1959

Green, D. E., "The metabolism of fats"
Together with the metabolism of sugar it provides the bulk of animal energy. Its key is the oxidation of fatty acids, a full knowledge of which has been obtained only recently. *Sci. Am.*, January 1954, No. 16

Green, D. E., "Enzymes in teams"
The goal of many biochemists has been to isolate individual enzymes and to observe their effects in vitro. In the cell the catalysts of life act in concert. *Sci. Am.*, September 1949, No. 15

Kamen, M. D., "Tracers"
The relatively recent application of labeled atoms in biological research has already brought out some remarkable information about the chemistry of life. *Sci. Am.*, February 1949, No. 100

Chapter 9

Stumpf, P. K., "ATP"
The initials stand for adenosine triphosphate, the substance that provides energy for muscle contraction, for fermentation of sugar by yeast, and a host of other biological processes. *Sci. Am.*, April 1953, No. 41

Huxley, H. E., "The contraction of muscle"
How does muscle turn chemical energy into mechanical work? Recent studies have revealed significant details in the intimate structure of the muscle machine. *Sci. Am.*, November 1958, No. 19

Siekevitz, P., "Powerhouse of the cell"
It is the mitochondrion, a small body which appears to play a central role in the oxidation of foodstuffs. Its structure, as revealed by the electron microscope, mirrors its function. *Sci. Am.*, July 1957, No. 36

Zamecnik, P. C., "The microsome"
This small particle is found in a fraction of living cells which have been broken up for biochemical investigation. It now seems to be a fragment of an intracellular system that makes proteins. *Sci. Am.*, March 1958, No. 52

Lehninger, A. L., "Energy transformation in the cell"
How does the living cell convert the energy of foodstuff into a form that can be utilized and stored? The process involves the sequential action of many enzymes, some of which are part of the cell membranes. *Sci. Am.*, May 1906, No. 69

Lehninger, A. L., "How cells transform energy"
In the chloroplasts of plant cells the energy of sunlight is transformed into chemical fuels. In the mitochondria of animal cells these fuels are oxidized to run the cellular machinery. *Sci. Am.*, September 1961, No. 91

Chapter 10

Green, D. E., "The synthesis of fat"
When the way in which fat molecules are broken down was worked out, it was assumed that they are built up by the same steps in reverse. Now it has been found that the synthetic process is quite different. *Sci. Am.*, February 1960, No. 67

Rabinowitch, E. I., "Photosynthesis"
Life's essential process, performed by stately trees and microscopic algae, is imperfectly understood. But the problem is slowly yielding to a concerted attack. *Sci. Am.*, August 1948, No. 34

Wald, G., "Life and light"
Life depends on a narrow band in the electromagnetic spectrum. This is a consequence of the way in which molecules react to radiation and must hold true not only on earth but elsewhere in the universe. *Sci. Am.*, October 1959, No. 61

Arnon, D. I., "The role of light in photosynthesis"
The primary photosynthetic act, it now appears, is not to make carbohydrates from carbon dioxide, but to convert light energy into chemical energy, which the cell then uses in various ways. *Sci. Am.*, November 1960, No. 75

Allfrey, V. G. and A. E. Mirsky, "How cells make molecules"
The inherited master plan of the cell is contained in the molecules of deoxyribonucleic acid. They direct the manufacture of protein enzymes, which in turn engineer all the chemical reactions of life. *Sci. Am.* September 1961, No. 92

Schmitt, F. O., "Giant molecules in cells and tissues"
How are protein and nucleic acid molecules organized in protoplasm? It appears, largely on the basis of evidence supplied by the electron microscope, that these molecules are monomers of higher polymers. *Sci. Am.*, September 1957, No. 35

Doty, P., "Proteins"
The principal substance of living cells, these giant molecules have identical backbones. Each is adapted to its specific task by a unique combination of side groups, size, folding, and shape. *Sci. Am.*, September 1957, No. 7

Stein, W. H. and S. Moore, "The structure of proteins"
The atom-by-atom structural formulas of these proteins have now been worked out. This knowledge should help in understanding how the structures of proteins are related to their biological activity. *Sci. Am.*, February 1961, No. 80

Linderstrom-Lang, K. U., "How is a protein made?"
These giant molecules are constructed in a fantastic variety out of 22 amino acids. The mechanism of their synthesis is perhaps the greatest challenge facing the modern biochemist. *Sci. Am.*, September 1953, No. 23

Gale, E. F., "Experiments in protein synthesis"
Broken cells shed light on the role of nucleic acids in the making of protein. *Sci. Am.*, March 1956

Hoagland, M. B., "Nucleic acids and proteins"
The nucleic acid molecules inherited by the living cell bear the plan of all the cell's protein molecules. It now appears that another kind of nucleic acid plays a key role in translating the plan into protein. *Sci. Am.*, December 1959, No. 68

Crick, F. H. C., "Nucleic acids"
These polymers appear to carry the pattern of living matter from one generation to the next. Their basic chain consists of sugars joined by phosphates. Attached to the sugars, in turn, are bases. *Sci. Am.*, September 1957, No. 54

Chapter 11

Beadle, G., "The genes of men and molds"
The study of the red fungus *Neurospora crassa* sheds light on exactly how the units of heredity determine the characteristics of all living things. *Sci. Am.*, September 1948, No. 1

Ryan, F. J., "Evolution observed"
Because energetically multiplying bacteria can crowd into a few years generations equal to a million years of man's developments, evolution may be observed. It brings firm confirmation to the Darwinian thesis. *Sci. Am.* October 1953

Crick, F. H. C., "The structure of the hereditary material"
An account of the investigations which have led to the formulation of an understandable structure for DNA. The chemical reactions of this material within the nucleus govern the process of reproduction. *Sci. Am.*, October 1954, No. 5.

Horowitz, N. H., "The gene"
Biologists have yet to see it or to find out what it is made of, but its role in determining the continuity and the variation of living things grows steadily clearer. *Sci. Am.*, October 1956, No. 17

Ingram, V. M., "How do genes act?"
In which the effect of a human mutation that causes a disease of the blood is traced to a change in one of the 300 amino acid units that make up the structure of the protein hemoglobin. *Sci. Am.*, January 1958, No. 104

Taylor, J. H., "The duplication of chromosomes"
It is generally assumed that the basic genetic material is DNA. How are the molecules of DNA arranged in the chromosome, and how do they make replicas of themselves? *Sci. Am.*, June 1958, No. 60

Dobzhansky, T., "The genetic basis of evolution"
The endless process of mutation and combination of genes, now being studied extensively in the laboratory, produces a practically unlimited variety of organisms, including bacteria that thrive on bactericides. *Sci. Am.*, January 1950, No. 6

Mirsky, A. E., "The chemistry of heredity"
We know the factors in the nucleus deeply influence the life of the cell, but exactly how do they do so? The search for an answer proceeds by the chemical investigation of chromosomes. *Sci. Am.*, February 1953, No. 28

Hotchkiss, R. and E. Weiss, "Transformed bacteria"
If DNA is removed from one strain of pneumococci and added to another strain, some of the

cells in the second strain are able to transmit characteristics of the first to their descendants. *Sci. Am.*, November 1956, No. 18

Zinder, N. D., " 'Transduction' in bacteria"
A virus can transfer genetic material, and thereby hereditary traits from one bacterium to another. The phenomenon sheds light on the behavior of viruses and the mechanism of heredity. *Sci. Am.*, November 1958, No. 106

Wollman, E. and F. Jacob, "Sexuality in bacteria"
It has only recently been shown that bacteria have chromosomes and genes. Now it has been found that one bacterium can inject its genes into another by conjugation, giving rise to a hybrid. *Sci. Am.*, July 1956, No. 50

Jacob, F. and E. Wollman, "Viruses and genes"
When a virus infects a bacterium, the genes of the virus sometimes act as genes of their host. The phenomenon has illuminated the mechanism of both heredity and infection. *Sci. Am.*, June 1961, No. 89

Chapter 12

Johnson, F. H., "Heat and life"
Life is limited to the zone between the freezing and boiling points of water, where its processes are synchronized by heat-sensitive enzymes. *Sci. Am.*, September 1954

Hollaender, A. and G. E. Stapleton, "Ionizing radiation and the cell"
The primary site of the biological effects of radiation is in the cell. The specialized structures of the cell, including the genes, are damaged not only by direct hits but also by the chemical products of radiation. *Sci. Am.*, September 1959, No. 57

Crow, J. F., "Ionizing radiation and evolution"
Living things evolve as a result of random mutations in hereditary characteristics that survive when they make the organism more fit. What role does ionizing radiation play in the evolutionary process? *Sci. Am.*, September 1959, No. 55

Muller, H. J., "Radiation and human mutation"
In which the fundamental process of human evolution is discussed, with special reference to trends that may be caused by artificial radioactivity, medical X-rays, and the preservation of harmful genes. *Sci. Am.*, November 1955, No. 29

Puck, T. T., "Radiation and the human cell"
The exposure of single human cells to X-rays indicates that they are far more sensitive to radiation than had been thought. This explains why the relatively small dose of 400 roentgens is lethal to the body. *Sci. Am.*, April 1960, No. 71

Kelner, A., "Revival by light"
Visible radiation can resuscitate microorganisms that have apparently been killed by ultraviolet. The phenomenon opens up a whole new study in the action of radiation on protoplasm. *Sci. Am.*, May 1951

Chapter 13

Roblin, R. O., "The imitative drugs"
The sulfa drugs are actually counterfeit vitamins. The discovery that molecules of similar structure compete with one another in biochemical reactions provides a tool for studying metabolism and drug action. *Sci. Am.*, April 1951

Adams, E., "Poisons"
What is the molecular mechanism by which a toxic substance produces its effect? *Sci. Am.*, November 1959

Chapter 14

Raper, K. B., "The progress of antibiotics"
These substances have saved innumerable lives in the decade since the first penicillin treatments, and production of the drugs has become a major industry. A survey of scientific and industrial progress in the field. *Sci. Am.*, April 1952

Pramer, D., "Antibiotics against plant diseases"
Important blights and other plant diseases may be controlled by antibiotic sprays. *Sci. Am.*, June 1955

Rose, A. H., "New penicillins"
Penicillin molecules with variant side chains are effective against resistant germs. *Sci. Am.*, March 1961

Chapter 16

Milner, H. W., "Algae as food"
Under controlled conditions single-celled plants can be grown to yield proteins and fats at rates that far outdo agricultural yields per acre. Whether such foods will be both economic and palatable is a question. *Sci. Am.*, October 1953

Weiss, F. J., "The useful algae"
Ranging from single cells to treelike colonies, these primitive plants support a whole heirarchy of marine life and also contribute directly to the needs of man. *Sci. Am.*, December 1952

Hutner, S. H. and J. J. A. McLaughlin, "Poisonous tides"
The explosive multiplication of certain marine microorganisms can kill fish by the millions. Studies of these outbreaks are helping to solve a fundamental problem in ecology. *Sci. Am.*, August 1958

Emerson, R., "Molds and men"
The fungi exert a remarkable influence on human affairs, affecting such things as the size of a wheat crop, the control of infectious diseases, the construction of houses, and even the number of Irishmen in the U. S. *Sci. Am.*, January 1952

Maio, J. J., "Predatory fungi"
Some molds are able to trap and devour small animals which reside in the soil. *Sci. Am.*, July 1958

Russell, P. F., "The eradication of malaria"
In 1945 there were 411,600 malaria cases in Italy; in the first half of 1951 there were 392, and no deaths have been reported for the past three years. DDT has made possible almost complete elimination of the disease. *Sci. Am.*, June 1952

Weisz, P. B., "The embryologist and the protozoon"
A one-celled animal is cut to pieces, with curious results that clarify how a fertilized egg gives rise to the specialized tissues of a many-celled organism. *Sci. Am.*, March 1953

Manwell, R. D. and H. P. Drobeck, "Toxoplasmosis"
This little-known disease is surprisingly widespread and sometimes fatal. Although it has been studied for 50 years, it is still a medical and biological enigma. *Sci. Am.*, February 1953

Moscona, A. A., "How cells associate"
The cells of many-celled organisms are marshaled and held together by specific physical and chemical factors. These factors are studied by dispersing the cells of a tissue and allowing them to recombine. *Sci. Am.*, September 1961, No. 95

Lamb, I. M., "Lichens"
A fungus and an alga in combination challenge some of the harshest environments. *Sci. Am.*, October 1959, No. 111

Bonner, J. T., "Differentiation in social amoebae"
Certain amoebae ("slime molds") gather to form a mass of spores and a stalk. *Sci. Am.*, December 1959

Chapter 17

Eliassen, R., "Stream pollution"
The means to control this major threat to one of our most valuable natural resources are at hand or in development, but it will cost the U. S. some $20 billion to restore its watercourses to a reasonable purity. *Sci. Am.*, March 1952

Chapter 18

Kamen, M. D., "Discoveries in nitrogen fixation"
A lucky experimental accident and some shrewd investigation has recently brought to light a surprising multitude of microorganisms with the ability to take nitrogen from the air and fix it in useful chemicals. *Sci. Am.*, March 1953

Chapter 22

Rose, A. H., "Beer"
Its manufacture from grain is now understood in terms of enzymes and microorganisms. *Sci. Am.*, June 1959

Chapter 23

May, J. M., "The geography of disease"
Knowing where a disease is prevalent, and where it is not, may furnish important clues as to its cause and prevention. Presenting some of the first results of a project for mapping man's illnesses over the globe. *Sci. Am.*, February 1953

Yoeli, M., "Animal infections and human diseases"
Diseases carried by animals and insects have plagued mankind through the ages. *Sci. Am.*, May 1960

Chapter 24

Wood, W. B., Jr., "White blood cells vs. bacteria"
Some new observations have settled an old argument as to whether phagocytes are primary or secondary antagonists of infection. It seems that in acute infection they are the first line of defense. *Sci. Am.*, February 1951, No. 51

Zweifach, B. W., "The microcirculation of the blood"
The primary purpose of the circulatory system is served by the microscopic vessels in which the blood flows from the arteries to the veins and thereby nourishes all the tissues of the body. *Sci. Am.*, January 1959, No. 64
Burnet, Sir M., "How antibodies are made"
How do cells manufacture the molecules that neutralize foreign substances? The answer is not known, but the author discusses an hypothesis that accounts for some curious new observations. *Sci. Am.*, November 1954, No. 3
Burnet, Sir M., "The mechanism of immunity"
How does an animal make an antibody that neutralizes a single foreign substance, or antigen? The evidence favors the theory that cells able to make the antibody are "selected" by the antigen and then multiply. *Sci. Am.* January 1961, No. 78
Williams, C. A., Jr., "Immunoelectrophoresis"
This formidable word and the technique it identifies are derived from immunochemistry and electrophoresis. These methods have been combined into a precise tool for study of protein chemistry. *Sci. Am.*, March 1960, No. 84
Singer, S. J., "The specificity of antibodies"
How is it that an antibody molecule joins with only one kind of antigen molecule? *Sci. Am.*, October 1957
Gitlin, D. and C. A. Janeway, "Agammaglobulinemia"
It is a disease in which the blood lacks gamma globulin to fight bacterial infection. *Sci. Am.*, July 1957
Gross, J., "Collagen"
The main constituent of connective tissue, it accounts for a third of the protein in the human body. Its nature has been clarified by dissolving it and allowing its molecules to reassemble into fibers. *Sci. Am.*, May 1961, No. 88
Dubos, R. J., "Second thoughts on the germ theory"
When the late George Bernard Shaw mischievously suggested that "The characteristic microbe of a disease might be a symptom instead of a cause," he was not far from being right. It is useful to consider it as a bit of both. *Sci. Am.*, May 1955
Crowle, A. J., "Delayed hypersensitivity"
Slowly developed allergies play a role in a variety of pathological conditions. *Sci. Am.*, April 1960

Chapter 25

Mudd, S., "The staphylococcus problem"
The rise in infections due to this microorganism has renewed interest in its biology. *Sci. Am.*, January 1959

Chapter 26

Pappenheimer, A. M., Jr., "The diphtheria toxin"
A study of the mechanism by which the poison from one such bacillus interrupts a vital process of the living cell. *Sci. Am.*, October 1952
Long, E. R., "The germ of tuberculosis"
Although the tubercle bacillus has been studied more intensively than almost any other microorganism, and although medical measures against it are increasingly effective, the reason for its virulence is a mystery. *Sci. Am.*, June 1955

Chapter 28

Stent, G. S., "The multiplication of bacterial viruses"
The organisms that infect bacteria provide a means of studying the mechanism of heredity. Some new tracer experiments reveal that their reproduction has several rather unexpected features. *Sci. Am.*, May 1953, No. 40
Lwoff, A., "The life cycle of a virus"
What happens in the time between the infection of a cell by a virus and the appearance of a new generation of the viruses? The evidence of bacteriophages suggests that the process involves the concept of the provirus. *Sci. Am.*, March 1954
Luria, S. E., "The T2 mystery"
T2 is a virus which dissolves bacteria. Normally its attack is followed by the appearance of a generation of new viruses. But sometimes the viruses seem to be missing. Why? *Sci. Am.*, April 1955, No. 24

Chapter 29

Puck, T. T., "Single human cells in vitro"
When a suspension of bacteria is spread on a layer of nutrient, each bacterium gives rise to a colony of genetically uniform descendants. The same can now be done with cells of various human tissues. *Sci. Am.*, August 1957, No. 33
Moscona, A. A., "Tissues from dissociated cells"
How the cells of embryonic organs can be separated and made to come together. *Sci. Am.*, May 1959

Rustad, R. C., "Pinocytosis"
The living animal cell ingests many substances by enfolding them in its outer membrane. *Sci. Am.*, April 1961
Melnick, J. L., "Viruses within cells"
A recently developed technique for slicing tissue into ultrathin sections makes it possible for the electron microscope to look into a cell infected with virus and to photograph some of the stages of viral reproduction. *Sci. Am.*, December 1953
Pollard, E. C., "The physics of viruses"
The structure and function of the smallest living things are presently studied by physicists, using a wide variety of physical methods and tools, among them the cyclotron. *Sci. Am.*, December 1954, No. 32
Burnet, Sir M., "Viruses"
They are defined as microorganisms that are much smaller than most bacteria and can grow only in the cell. They are studied not only for the treatment and prevention of the diseases that they cause but also for their intrinsic interest. *Sci. Am.*, May 1951, No. 2
Knight, C. A. and D. Fraser, "The mutation of viruses"
Viruses provide a simple example of the basic process of biological change. Some recent experiments suggest that it may soon be possible to alter their inborn characteristics predictably by chemical means. *Sci. Am.*, July 1955, No. 59
Fraenkel-Conrat, H., "Rebuilding a virus"
The tobacco mosaic virus is composed of protein and nucleic acid, which may be separated by gentle chemical treatment. When the two components are mixed together, they form infective virus particles. *Sci. Am.*, June 1956, No. 9
Stewart, S. E., "The polyoma virus"
This recently discovered organism causes a variety of malignant tumors in laboratory animals. Studies of its action lend weight to the idea that viruses may be responsible for human cancers. *Sci. Am.*, November 1960, No. 77
Gray, G., "Unknown viruses"
Viruses usually reveal themselves through infection, but now virologists have come up with some new ones for which no known diseases have been found. Some viruses are now thought to fall into kinship patterns. *Sci. Am.*, March 1955
Fenner, F., "The rabbit plague"
Myxomatosis is a virus disease nearly 100% fatal for the common European rabbit species. Its deliberate introduction into Australia in 1950 and more recently in Europe is viewed as a two-edged sword. *Sci. Am.*, February 1954
Burnet, Sir M., "The influenza virus"
The organism that causes the disease which periodically sweeps through large sections of the human species is curiously changeable. It is this property that makes it difficult to prevent the epidemics with vaccines. *Sci. Am.*, April 1953
Burnet, Sir M., "The structure of the influenza virus"
The behavior of the virus is clarified by studies of its physical and chemical nature. *Sci. Am.*, February 1957
Andrewes, C. H., "Viruses of the common cold"
Investigators have succeeded in cultivating a number of viruses that cause colds. *Sci. Am.*, December 1960
Melnick, J. L., "A new era in polio research"
The discovery that polio virus could be grown in test tubes as well as in the spinal cords of living monkeys was the first big break in a long campaign. Now we can hope for more rapid progress against the disease. *Sci. Am.*, November 1952
Hammon, W. M., "Gamma globulin in polio"
How a fraction of blood plasma offers short-term immunization against the paralytic disease. Unfortunately the utility of the substance is limited by its short supply, and medicine still awaits a successful vaccine. *Sci. Am.*, July 1953
Salk, J. E., "Vaccines for poliomyelitis"
We shall soon know the results of last year's large-scale test of the new vaccine against poliomyelitis. Some of the work leading up to the present vaccine has thrown new light on the mechanism of immunization. *Sci. Am.*, April 1955
Melnick, J. L., "Enteroviruses"
It is now apparent that the three viruses which cause polio belong to a family of more than 50 viruses which ordinarily dwell in the human alimentary tract without causing disease. *Sci. Am.*, February 1959
Isaacs, A., "Interferon"
This natural product of animal cells protects them against attack by a variety of viruses. If it proves to be effective in humans, it should have

considerable medical importance. *Sci. Am.*, May 1961, No. 87

Chapter 30

Bovarnick, M. R., "Rickettsiae"
Widely known as the cause of typhus, these primitive organisms now hold the stage for other reasons. Midway between viruses and bacteria, they may help solve the evolutionary puzzle presented by viruses. *Sci. Am.*, January 1955

Chapter 31

Pramer, D., "Antibiotics against plant diseases"
A number of economically important blights and other plant diseases may soon be controlled by antibiotic sprays. The study of ways in which the drugs are taken up has revealed new facts about plant physiology. *Sci. Am.*, June 1955

Niederhauser, J. S. and W. C. Cobb, "The late blight of potatoes"
This fungus disease has been successfully combated by genetic studies in Mexico. *Sci. Am.*, May 1959

Maramorosch, K., "A versatile virus"
China asters are affected with a searing disease called aster yellows, caused by an organism which is on the borderline between plant and animal viruses. Its study is linking up the two fields once separated. *Sci. Am.*, June 1953

Maramorosch, K., "Friendly viruses"
Many viruses, like many bacteria, help rather than harm the organisms they live in. *Sci. Am.*, August 1960

Braun, A. C., "Plant cancer"
Crown gall is a tumorous growth in plants which is similar in many ways to animal cancers. The disease is now being intensively studied for the light it sheds on the general problem of tumor origin and formation. *Sci. Am.*, June 1952

Biddulph, S. and O. B., "The circulatory system of plants"
Substances required in the metabolism of plant cells not only travel up from the roots but also move about in other ways. This circulation has recently been clarified by experiments using radioactive tracers. *Sci. Am.*, February 1959, No. 53

Butler, W. L. and R. J. Downs, "Light and plant development"
Events in the life of a plant such as flowering are responses to a change in the length of the night. The light-sensitive enzyme that mediates these responses has now been discovered. *Sci. Am.*, December 1960, No. 107

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