Chapter 21 Viruses of Animals and Plants

OUTLINE

In the last chapter we discussed the viruses of bacteria, the bacteriophages. Viruses also exist that infect other living cells. There are viruses that infect fungi, algae, and insects. And of course there are viruses that infect animal and plant cells.

Animal and plant viruses vary greatly in size and shape (Figs. 21-1 and 21-2), but they do not have the tadpole morphology characteristic of some bacteriophages discussed in the previous chapter. Size and shape are characteristic properties of each type of virus. Virions range in size from 20 to 350 nm and represent the smallest and simplest infectious agents. Since most viruses measure less than 150 nm, they are beyond the limit of resolution of the light microscope and are only visible by means of the electron microscope.

Much of the basic biology of bacteriophages discussed in Chap. 20 is also applicable to animal and plant viruses. Accordingly, only the special properties of animal and plant viruses are discussed in this chapter.

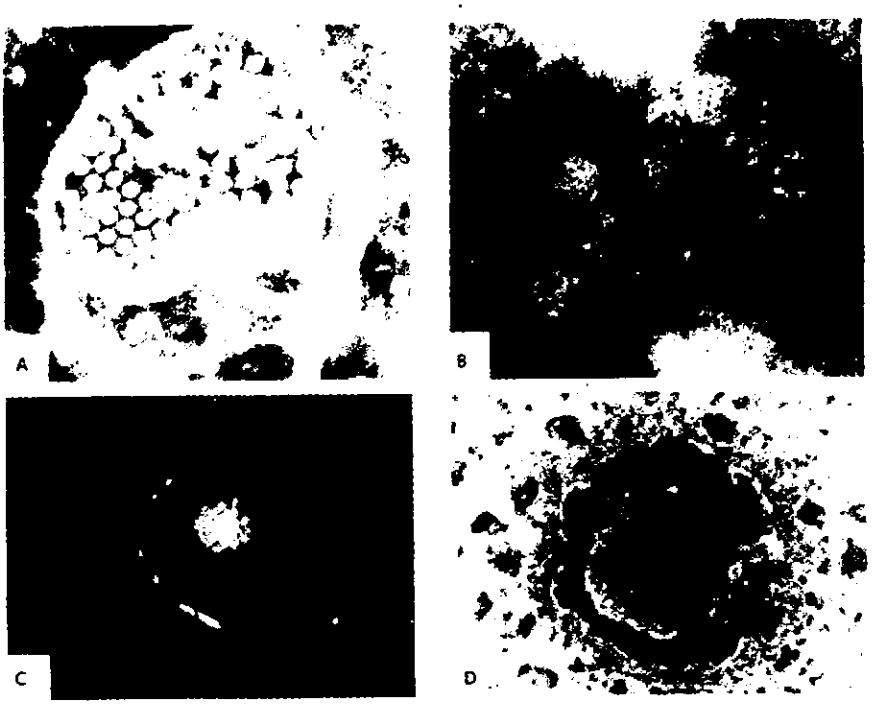


Figure 21-1. Electron micrographs of some animal viruses. (A) A cluster of polioviruses, the cause of poliomyelitis. (B) Rotavirus particles, the cause of acute infectious diarrhea, a major cause of death in very young infants. (C) An enveloped herpesvirus. It is a persistent virus in humans and occasionally manifests its presence in "fever blisters" or "cold sores" of mucous membranes. (A, B, and C are the same magnification, X200,000.) (*Courtesy of Margaret Gomersall, McGill University.*) (D) A negatively stained parainfluenza virus from the nasopharyngeal secretions of a patient with an acute respiratory disease. Within the fringed outer envelope is contained the tightly packed helical nucleocapsid (X125,000). (*Courtesy of Frances Doane, University of Toronto.*)

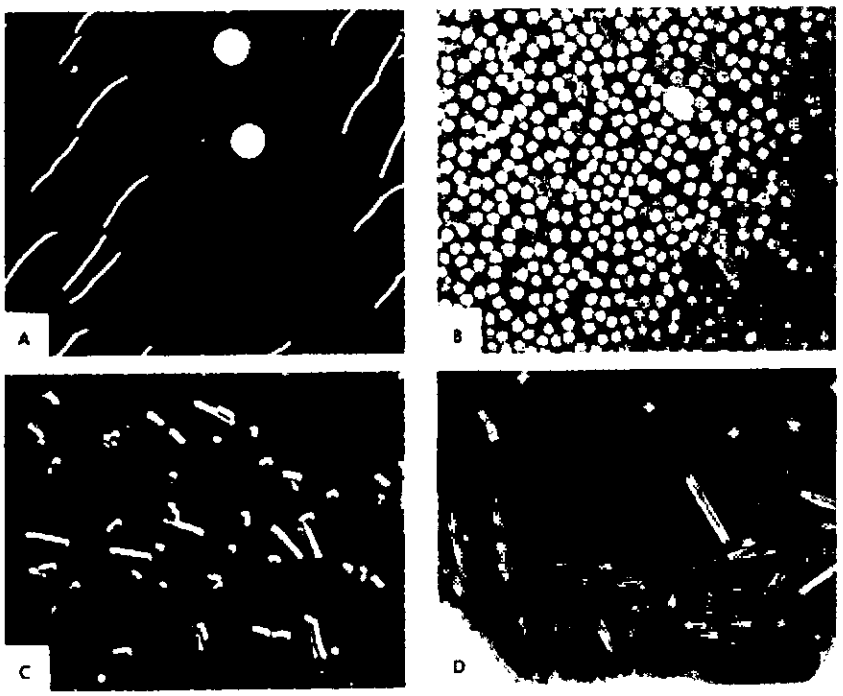


Figure 21-2. Electron micrographs of plant viruses. (A) Potato virus X particles appear as flexous rods 513 nm long (X17,000). Also shown are two latex spheres used in electron microscopy to show relative sizes. (B) Tomato ringspot virus is icosahedral in structure (X150,000). (C) and (D) Tobacco rattle virus particles appear as both long and short rods. Both lengths are necessary to establish infection. (C: X23,600. D: X25,400.) (*Courtesy of M. K. Corbett, University of Maryland.*)

HISTORY

Viruses and Vaccination

Some virus diseases have been known clinically for centuries. Indeed, the first infectious disease for which a practical and effective method of prevention was developed was a virus disease. In 1796 Jenner first vaccinated an 8-year-old boy named James Phipps with material removed from a cowpox lesion on the hand of a milkmaid. Proof that the inoculation gave protection against smallpox was obtained 6 weeks later, when the boy was inoculated with pus from a smallpox victim and did not develop the disease.

Jenner found that persons successfully inoculated intracutaneously with virus isolated from cowpox lesions developed a small scab at the site of application which dropped off after about 2 weeks, leaving a single small scar. Before making his report in 1798, he had successfully vaccinated 23 persons. The material he used came from cows (Latin, *vacca*); hence the term *vaccination*, in contrast to *variolation*, which identified an earlier procedure in which variola (smallpox) virus was artificially introduced into a subject to provide protection against natural smallpox infection. Today, smallpox vaccine is made from virus grown on the skin of healthy calves or sheep or in embryonated eggs, thus eliminating the possibility of transmitting other human diseases in the process. Jenner never saw the causative agent of smallpox; indeed, his discovery and its application came more than half a century before the establishment of the germ theory of disease.

The first American known to have been vaccinated was Daniel Oliver Waterhouse, whose father, Dr. Benjamin Waterhouse, a Boston physician, obtained the cowpox virus from Europe. The 5-year-old Daniel and two servants were vaccinated July 8, 1800; they were later inoculated with smallpox virus and found to be fully protected. Such daring and dramatic demonstrations proved the value of vaccination and brought it to the attention of officials and the public. Recognizing its usefulness, Thomas Jefferson wrote to Jenner in 1806, "Future nations will know by history only that the loathsome smallpox has existed and by you has been extirpated."

Half a century passed before Louis Pasteur became interested in infectious diseases and the role of microorganisms in causing them. His successes in controlling undesirable organisms in fermentation reactions and in diseases of silkworms led him into some problems of human health. One health problem that opportunity dropped on his doorstep was rabies, a disease transmitted to humans by bites of rabid dogs, foxes, wolves, cats, bats, and other animals. Pasteur did not think of this agent as being particularly different from the microorganisms (bacteria, yeasts, and protozoa) he had previously worked with, and he applied his previous experience to the problem at hand. Through laboratory manipulations he was able to attenuate, i.e., make less virulent, the virus from rabid animals. When inoculated into an animal, the attenuated virus produced an active immunity against the disease. It was not until several years later that the viral nature of the disease was established. The method Pasteur used in developing a vaccine against rabies provided the basis for the production of vaccines for other virus diseases.

One of Pasteur's associates, Charles Chamberland, had meanwhile learned that porcelain filters would prevent passage of bacteria but allow passage of the causative agent of rabies. Because in those days the word *virus* was loosely used to describe any toxic substance that caused disease, it was only natural to describe those unseen filter-passing agents of disease as **filterable viruses.**

Tobacco Mosaic Virus and Filterability

In 1892, Dmitrii Ivanowski discovered that the causative agent of tobacco mosaic disease was filterable. By placing some bacteria-free filtrate from ground-up infected plants on healthy tobacco leaves, Ivanowski produced the disease in healthy plants (Fig. 21-3). Beijerinck (1898) confirmed this work, and in the same year Loeffler and Frosch filtered fluid from vesicles in the mouths of cattle with foot-and-mouth disease and transmitted the virus of that disease to healthy animals. These experiments marked the beginning of a new phase of microbiology, the study of infectious agents invisible even through the most powerful microscopes then available. Although they cannot be grown on nonliving culture media, viruses are capable of causing disease in plants and animals.

One of the most important scientific contributions to the field of virology was the discovery in 1935 that the tobacco mosaic virus (TMV) can be crystallized. For this fundamental research, Wendell Stanley, then at the Rockefeller Institute for Medical Research, shared the 1946 Nobel prize in chemistry. Even before Stanley's work, many people had questioned whether viruses are truly living organisms. When it was shown that the "inanimate" crystals of TMV could produce disease in healthy plants, the controversy was renewed with more vigor. One thing these virus crystals (Fig. 21-4) do that other pure toxic chemical compounds cannot do is replicate themselves. In that sense, viruses seem to be alive, because only living things can reproduce and multiply. On the other hand, they seem to be nonliving because they have no intrinsic metabolism and depend on their host for survival and multiplication. The question has never been settled to everyone's satisfaction, and little is to be gained by arguing about it. What is more important is that thanks to the intensive study of microbiologists, chemists, and physicists, these particles we call viruses have given and will continue to give us important information about life processes.

Yellow Fever Virus and Vaccines

Another milestone in virology occurred when Max Theiler, 1951 Nobel laureate in physiology or medicine, found in 1937 that virulent yellow fever virus can be attenuated by serial passage on cultures of chick embryo tissue. Later investigators modified this technique to produce vaccines against other virus diseases. For example, Enders, Robbins, and Weller laid the foundation for the development of effective poliomyelitis vaccines by culturing the virus of poliomyelitis on monkey kidney cells in 1949. This is not attenuation but a method for mass growth of the virus. Indeed, tissue-culture techniques made it possible to cultivate many mammalian viruses in the laboratory (Fig. 21-5) and will undoubtedly lead to the development of methods for controlling the diseases they cause.

Among the virus diseases for which vaccines have recently been developed through the use of tissue cultures is measles (rubeola). The first live attenuated strain of measles virus was isolated in 1962 by Enders after passage of the virus through human kidney cells, human amnion cells, and finally, chick embryo tissue culture. The attenuated live measles vaccine licensed for use in the United States is prepared from chick embryo cell cultures infected with attenuated measles virus. Mumps vaccine is prepared from cultures of chick fibroblasts infected with an attenuated live mumps virus; it has been available since 1968. After extensive clinical trials a vaccine to protect against German measles (rubella) was approved in 1969 by the U.S. Public Health Service for clinical use. Rubella vaccines contain viruses either isolated in African green monkey cells

Figure 21-3. Transmission of a plant virus. By grinding or macerating leaves from naturally infected plants and passing the fluid through a porcelain filter, Ivanowski demonstrated that a virus causes tobacco mosaic disease. The filtrate containing the virus causes infection when placed on the leaves of healthy plants.

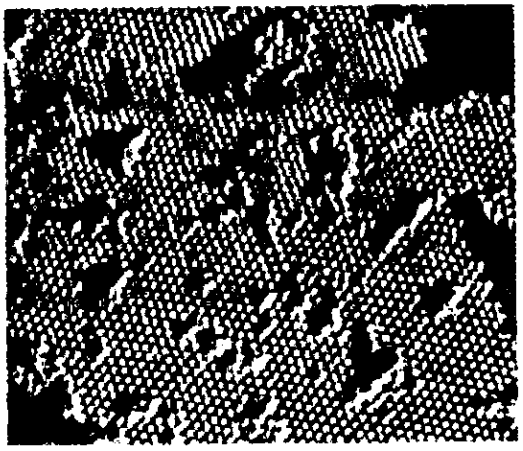


Figure 21-4. Surface of a crystal of tobacco necrosis virus (X55,200). (*Courtesy of M. K. Corbett. University of Maryland.*)

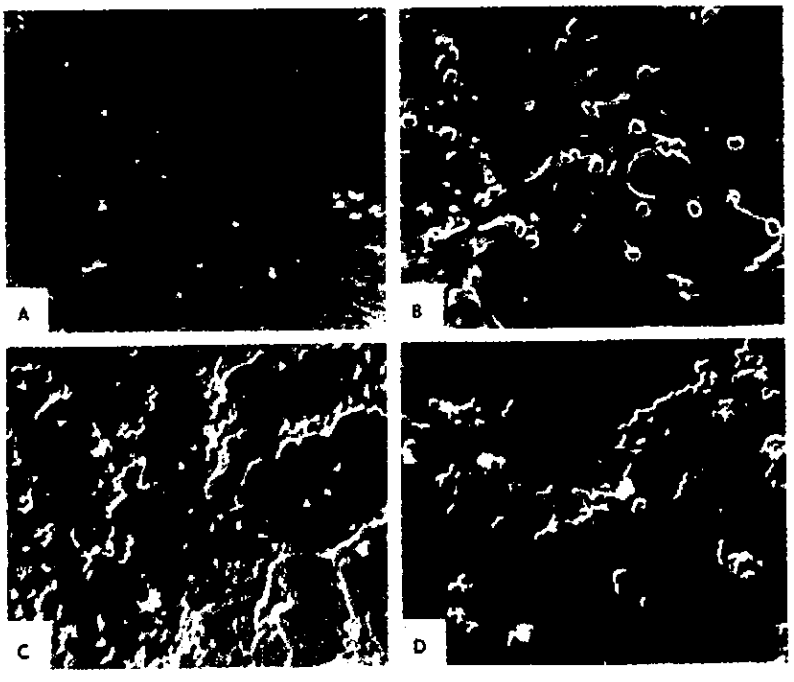


Figure 21-5. Tissue culture of rabbit kidney cells destroyed by vaccinia virus. (A) Uninfected cells appear as smooth flat sheets; 6 h after inoculation, some virus particles are released and lie on the surface of the cells (B). After 24 h of incubation the number of virus particles increases, and the tissue cells are beginning to disintegrate (C). After 48 h the virus appears as clumps dissociated from the cellular material (D). Magnifications are (A) X5,180, (B) X6,280, (C) X3,810, and (D) X4,940. (*Courtesy of John Mathews and the Upjohn Company.*)

and attenuated by further cell passage (in primary duck embryo cells) or isolated and passed in diploid human embryo cells.

STRUCTURE AND COMPOSITION

Like bacteriophages, animal and plant viruses are composed of a central core of nucleic acid surrounded by a capsid, which is made up of capsomeres. Most plant and animal viruses exhibit a characteristic symmetry: (1) icosahedral in the case of spherical viruses, (2) helical in the case of the rod-shaped viruses, and (3) complex in the case of a miscellaneous group. This symmetry is a basic criterion of viral classification, which will be discussed later. But in some animal viruses the **nucleocapsid** (nucleic acid and capsid) is covered by an outer membranelike structure called the **envelope**, which is made of lipoproteins and conceals this symmetry. Virions that have envelopes are sensitive to lipid solvents such as ether and chloroform. Their capacity to infect cells is inactivated by these solvents. Nonenveloped viruses are referred to as **naked** virions. Such viruses are not affected by lipid solvents.

Morphology

Icosahedral Symmetry

In searching for a simple, stable architectural structure, R. Buckminster Fuller, the American architect, engineer, and inventor, discovered that an icosahedral shell (discussed in Chap. 20) is easy to assemble and provides an enclosure possessing minimum stress. This is the idea behind Fuller's geodesic domes, the design of which he patented in 1947. These domes usually look spherical and cover more space with less material than any other buildings ever designed. These domes are actually subtriangulated icosahedra constructed of almost

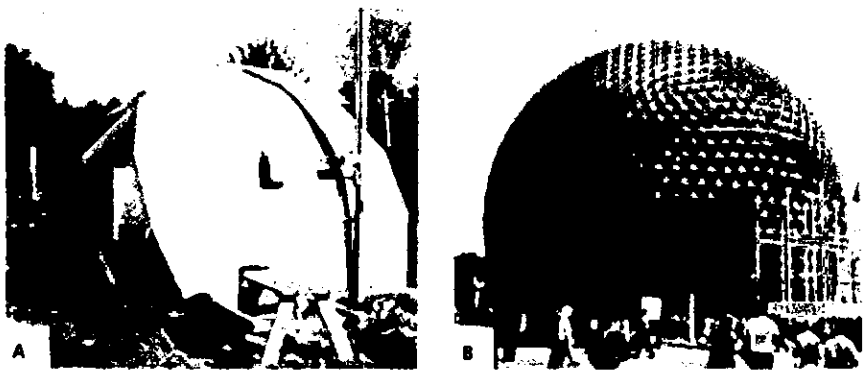


Figure 21-6. Examples of subtriangulated icosahedra constructed from pentamers and hexamers. (a) Geodesic dome in a New Brunswick, Canada, campground. Note the clusters of triangular facets in 5's and 6's. (*Courtesy of Frances Doane, University of Toronto.*) (B) Geodesic dome of the U.S. pavilion in the World Exposition of 1967 in Montreal, Canada. (*Courtesy of E. C. S. Chan, McGill University.*) Thousands of geodesic domes are used as theaters, auditoriums, defense facilities (DEW Line stations in the Arctic), and dwelling places. One of the most recent such structures is the 180-foot-high polished aluminum sphere of Spaceship Earth in Epcot Center at Disney World in Florida.

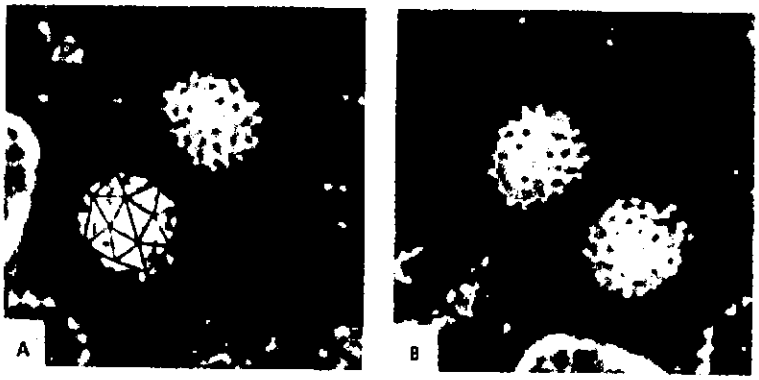


Figure 21-7. Bluetongue virus particles (A) and (B). Part of the icosahedral surface lattice has been drawn on one of the particles (X312,000). (*From H. J. Ehis and D. W. Verwoerd, Virology,* **38**:*213–219, 1969; by permission.*)

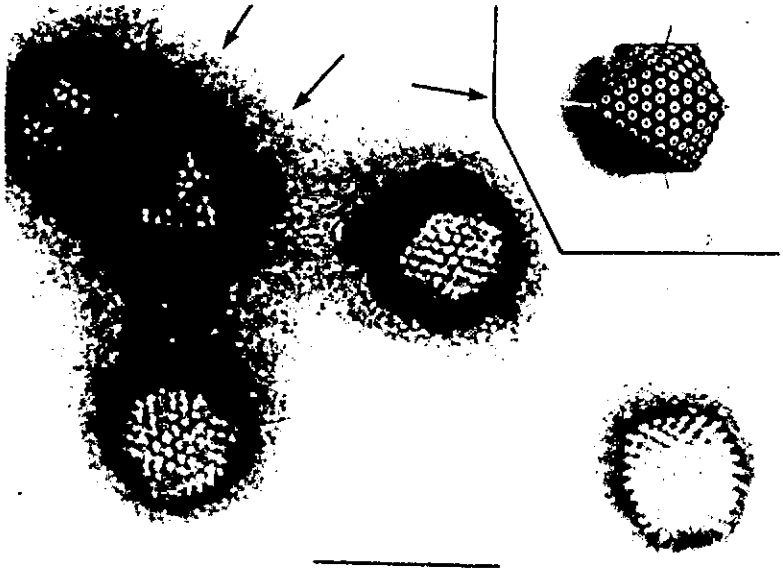


Figure 21-8. A simian adenovirus, SV 15, in an electron micrograph and with a model of an icosahedral particle. The bar equals 100 nm. (*Courtesy of Kendall O. Smith and Melvin D. Trousdale.*)

identical triangular units in clusters of fives and sixes (see triangular outline in Fig. 20-5A and illustrations of geodesic domes in Fig. 21-6). Viruses, too, long ago "discovered" this kind of structure, and so spherical viruses are in reality icosahedral in symmetry (Fig. 21-7). Examples of icosahedral viruses are polioviruses and adenoviruses (Fig. 21-8), which cause poliomyelitis and respiratory infections, respectively.

Helical Symmetry

Plant viruses with helical symmetry are typically rod-shaped (Fig. 21-9). One of the first viruses studied by electron microscopy was the tobacco mosaic virus (Fig. 21-10). Its nucleic acid core is covered by a capsid consisting of closely packed capsomeres arranged in a regular helix (see Fig. 20-5B). Animal viruses with capsids displaying helical symmetry include measles, mumps, influenza,

and rabies. In these viruses, the nucleocapsid is a flexible structure packed within a fringed lipoprotein envelope (Fig. 21-11). The fringes are actually spiked projections made of glycoproteins.

Figure 21-9. Electron micrograph of a chromium-shadowed preparation of cymbidium mosaic virus. Virus particles are flexuous rods 480 nm long (X33,000). (*Courtesy of M. Kenneth Corbett, University of Maryland.*)

Figure 21-10. Tobacco mosaic virus particles partially degraded by phenol, showing protein coat and RNA strand (X120,000). (*From M. K. Corbett, Virology, 22:539–543, 1964; by permission.*)

Figure 21-11. Influenza virus. Note the fringes of spikes on the surfaces of the virions (X55,300). (*Courtesy of Margaret Gomersall, McGill University.*)

Figure 21-12. Vaccinia virus, a poxvirus with complex morphology. (A) Whole (coated) virus showing surface tubules (X200,000). (*Courtesy of Margaret Gommersall, McGill University.*) (B) Immature virion obtained from an infected cell showing a bounding membrane with subunit projections (X160,000). (*Courtesy of K. B. Easterbrook, Dalhousie University.*)

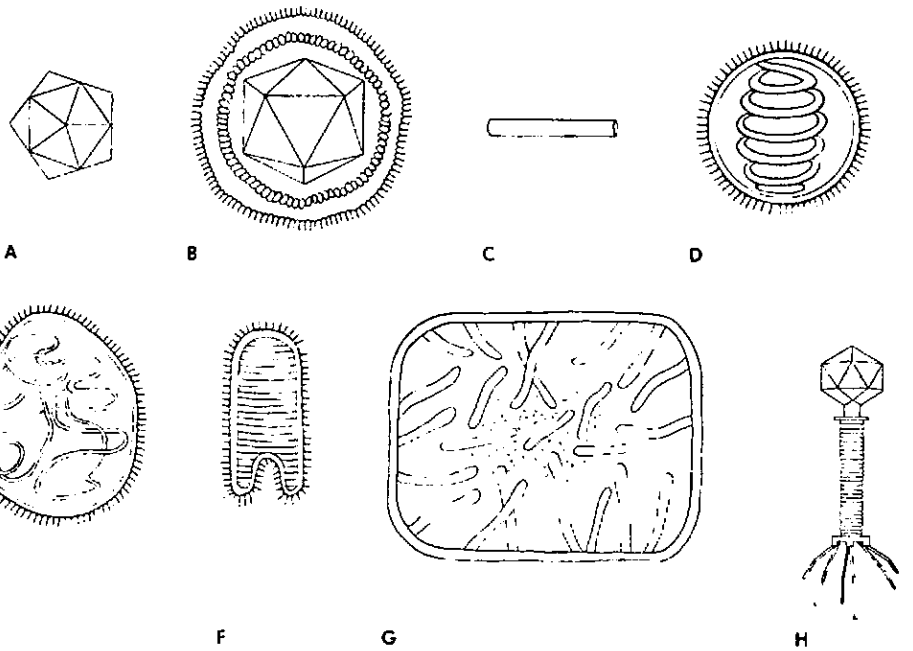


Figure 21-13. Morphology of some well-known viruses. Icosahedral symmetry: (A) polio, wart, adeno, rota; (B) herpes. Helical symmetry: (C) tobacco mosaic; (D) influenza; (E) measles, mumps, parainfluenza; (F) rabies. Complex or uncertain symmetry: (G) poxviruses; (H) T-even bacteriophages. (*Redrawn by Erwin F. Lessel after a drawing by Frances Doane, University of Toronto.*)

Complex-Structured Viruses

There are viruses with complex or uncertain symmetries. For example, the poxviruses, such as smallpox and molluscum contagiosum, have the most complex virion structure known (Fig. 21-12). They consist of many different proteins and lipoproteins. (The tailed phages discussed in Chap. 20 may be considered to be complex-structured viruses.)

A schematic representation of the morphology of viruses is given in Fig. 21-13.

Nucleic Acids

The viral genome, containing all the genetic information, is composed of nucleic acid. Like bacteriophages, animal and plant viruses contain either DNA or RNA, but never both in the same virion. This, of course, is in contrast to all *cellular* forms of life, which without exception contain both types of nucleic acid in

Table 21-1. Occurrence of Types of Nucleic Acids in Viruses

	Nucleic Acid			
	DNA		RNA	
Virus	Single-stranded	Double-stranded	Single-stranded	Double-stranded
Animal	+	+	+	+
Plant	+	+	+	+
Bacterial	+	+	+	+

each cell. Further, the genome of higher organisms consists of double-stranded DNA (dsDNA). But the genome of a virus can consist of DNA or RNA that is either double-stranded or single-stranded (Table 21-1). All four types of genome have been found in bacterial, animal, and plant viruses.

The proportion of nucleic acid in a virion varies from about 1 percent for the influenza virus to about 50 percent for certain phages. The content of genetic information per virion varies from about 3 to 300 kilobases per strand of nucleic acid. Thus if 1 kilobase is considered the size of an average gene, small viruses (e.g., parvoviruses and picornaviruses) contain perhaps 3 or 4 genes and large viruses (e.g., herpesviruses and poxviruses) contain several hundred. Virions contain only a single copy of the nucleic acid; i.e., they are **haploid**. The exception is the retroviruses; they are **diploid** virions because they contain two identical single-stranded RNA genomes.

In addition, the structure of the nucleic acid in the virion may be either linear or circular. The DNA of most animal viruses is a linear molecule of either dsDNA or ssDNA. In some animal viruses, like the papovaviruses, the DNA occurs as a supercoiled circular dsDNA. (Supercoiling refers to the extra turns in the structure of dsDNA due to the action of the enzyme DNA gyrase.) The terminal bases of some linear DNA viruses, e.g., the dsDNA adenoviruses and the ssDNA parvoviruses, exist as inverted repeat sequences that form hairpins. That is, the sequence of terminal bases on each strand of such DNA may be represented as ABCD . . . D′C′B′A′, where ABCD are complementary to A′B′C′D′. Such sequences are important in replication or in the cyclization of linear progeny strands.

Some plant viruses appear to have a genome of circular dsRNA. But the RNA in animal viruses exists only as linear double-stranded or single-stranded molecules. Unlike the DNA genome, the RNA genome within a virion may exist as a **segmented genome** (divided into several units). Thus reovirus contains 10 different segments of dsRNA and influenza virus has 8 separate segments of ssRNA.

Since the replication of nucleic acids requires that one nucleic acid strand be used as a template for the synthesis of the new nucleic acid strand (see Chap. 12), it follows that both of the original strands must be duplicated so that a virus can replicate its nucleic acid. Except for the RNA tumor viruses, viruses containing single-stranded nucleic acid package only one of the two strands within the capsid. Single-stranded viral RNA molecules which function directly as mRNA in the host cells have been designated as positive, or plus (+) strands. Viruses with negative, or minus-strand (−) RNA molecules must first replicate their RNA (using RNA transcriptase carried within the virion) to form a com-

plementary strand which then acts as the mRNA. RNA tumor viruses have two equal positive-strand RNA molecules.

Other Chemical Components

Protein. Besides nucleic acid, protein is the other major chemical component of the virion. The capsid is made up of protein. Virions also contain internal proteins. Some are basic proteins bound to the nucleic acids. In papovaviruses these basic proteins are regular cellular histones; in adenoviruses they are histonelike but are specified by the viruses. Small peptides and polyamines are found in phages. These cationic compounds are presumed to aid in the folding of the nucleic acids by linking together different loops.

In addition, many viruses have now been found to contain one or more enzymes that function in the replication of their nucleic acid components. The most common viral enzyme is an RNA polymerase. Except for the RNA viruses carrying the single positive strand (mRNA), all RNA viruses must possess their own RNA polymerase. The positive-strand RNA viruses code for their RNA polymerase, which is synthesized by host cell translation of the viral mRNA. This RNA polymerase transcribes the viral RNA, allowing viral replication to proceed. The RNA tumor viruses contain an enzyme (RNA-dependent DNA polymerase, or reverse transcriptase) that synthesizes a DNA strand, using the viral RNA genome as a template.

Lipid. A wide variety of lipid (fatty) compounds have been found in viruses. These include phospholipids, glycolipids, neutral fats, fatty acids, fatty aldehydes, and cholesterol. Phospholipid is the predominant lipid substance and is found in the viral envelope.

Carbohydrate. All viruses contain carbohydrate since the nucleic acid itself contains ribose or deoxyribose. Some enveloped animal viruses, such as the influenza virus and other myxoviruses, have spikes made of glycoprotein on the envelope.

VIRUS REPLICATION

Virus particles outside a host cell have no independent metabolic activity and are incapable of reproduction by processes characteristic of other microorganisms (Table 21-2). Multiplication takes place by **replication**, in which the viral protein and nucleic acid components are reproduced within susceptible host cells.

The entire process of infection can be generalized as follows. The virion attaches to a susceptible host cell at more or less specific sites. Either whole virus or viral nucleic acid penetrates to the inside of the cell. If whole virus has penetrated the cell, uncoating of the virus must take place to release the nucleic acid. Reproduction of the virus takes place in the cytoplasm, the nucleus, or both. The viral protein and nucleic acid components are assembled into virus particles and released from the host cell. The steps of virus infection and replication are therefore: (1) adsorption, (2) penetration and uncoating, (3) component replication and biosynthesis, (4) assembly, and (5) release.

Adsorption

The adsorption process occurs in two steps. The first step involves preliminary

Table 21-2. Comparison of Viruses with Some Bacteria

Microorganism	Multiplication	Diameter, nm	Chemical Composition	Inhibition by Antibiotics
Typical bacteria	In vitro* in fluid and solid media; on cell surfaces; or intracellularly; by binary fission	1000–3000	Complex proteins, carbohydrates, fats, etc.; DNA and RNA; peptidoglycan in cell wall	Yes
Mycoplasmas	Like typical bacteria but by budding rather than fission	150–1000	Like typical bacteria but without cell walls	Yes
Rickettsias	In living cells only and by binary fission	250–400	Like typical bacteria	Yes
Viruses	In living cells only and by synthesis from pools of constituent chemicals	10–300	Either RNA or DNA, plus protein; some may have lipid and/or carbohydrate components	No

* *In vitro* means "in glass," i.e., in laboratory vessels. (This is in contrast to *in vivo*, which means "within a living organism.")

attachment by ionic bonds or charges and is easily reversed by a shift in pH or salt concentration. The second step appears to involve firmer, more specific attachment and to be irreversible. Molecular entities on the surface of cells act as receptors for viruses, interacting with specific proteins on the capsids of naked virions or on the envelopes of enveloped virions. In contrast to the marked specificities of adsorption of animal and bacterial viruses, plant viruses do not seem to require specific receptor sites.

Penetration and Uncoating

The penetration of animal viruses into attached cells occurs by one of two mechanisms. One mechanism consists of engulfment of whole virions by the cells in a phagocytic process called **viropexis**, followed by uncoating or removal of the capsid. This takes place in the phagocytic vacuoles and is due to the action of enzymes called **lysosomal proteases**. The other mechanism occurs in the enveloped viruses; the viral lipoprotein envelope fuses with the host cell's surface membrane. This fusion results in the release of the viral nucleocapsid material into the cytoplasm of the host cell. Uncoating again occurs within the host cell.

Plant viruses penetrate host cells through transient pores (called **ectodesmata**) which protrude through the cell wall at intervals and communicate to the exterior of the cells. These pores function for the purpose of water and nutrient uptake as well as for secretion of substances such as waxes. Whole virus particles are apparently engulfed at these points. Also, insects can inoculate plant viruses into cells during feeding. Sometimes this is a purely mechanical process; at

other times the virus is found in the insect tissue and may even multiply there. Insect feeding is probably the most important means of plant virus transfer in nature. Once the virus is inside the plant cell, uncoating occurs.

Replication and Biosynthesis of Virus-Specific Molecules

Shortly after penetration, there follows an interval of time called the latent period (discussed in Chap. 20). It is during the latent period that uncoating of the virion takes place; the viral nucleic acid is freed from the capsid and is accessible to enzymes required to translate, transcribe, or replicate it. The uncoating process varies from one virus to another. Some viruses are uncoated in the cytoplasm, while others are uncoated in the nucleus.

Transcription of the viral nucleic acid into mRNA is the usual next step for all viruses except those RNA viruses (e.g., picornaviruses) whose viral RNA acts directly as mRNA. (Recall that RNA viruses that carry minus-strand RNA, such as orthomyxoviruses, paramyxoviruses, and rhabdoviruses, must first transcribe their RNA to form the plus strand that can function as mRNA. This transcription is catalyzed by a viral RNA polymerase released during uncoating.)

The biosynthetic processes of virus-specific molecules can be divided into early and late events. Thus in most viruses, only part of the nucleic acid of the infecting virion is initially transcribed into mRNA. Generally, early mRNA codes for early enzymes required for nucleic acid replication and for proteins that inhibit synthesis of cellular macromolecules and break down cellular polyribosomes, making them available for the transcription of viral genes. The exact mechanism for the replication of new copies of the virus genome varies with different types of viruses, i.e., whether their nucleic acid is RNA or DNA, single-stranded or double-stranded, and of positive or negative polarity. This replication process may take place in the cell nucleus or cytoplasm, depending on the specific virus. Generally, the dsDNA viruses include those capable of using host cell polymerases. Viruses with RNA genomes require viral-coded polymerases.

Nucleic acid replication (which follows early protein synthesis) serves as the demarcation line between early and late events in the viral replication process. Since late mRNA is not synthesized until after viral DNA replication has begun, it is transcribed from progeny genomes. Thus late protein synthesis may be defined as taking place after nucleic acid replication. Late mRNA usually specifies the structural proteins (such as those for protomer formation) of the virion. Translation of mRNA into proteins takes place in the host cell cytoplasm and uses ribosomes, transfer RNAs, and enzymes in the cytoplasm. (If the mRNA is synthesized in the host nucleus, it first enters the cytoplasm before translation.)

Assembly

When a critical number of the various viral components has been synthesized, the components (virus-specific molecules) are assembled into mature virus particles in the nucleus and/or cytoplasm of the infected cell (depending on the type of virus). The DNA viruses, with the exception of the poxviruses, are assembled in the nucleus. The RNA viruses are generally assembled in the cytoplasm, as are the poxviruses.

Release

Release of the completed virions from the host cell is the final step in virus multiplication. The mechanism of release varies with the type of virus. In some animal virus infections, the host cells lyse, releasing the virions. Naked virions

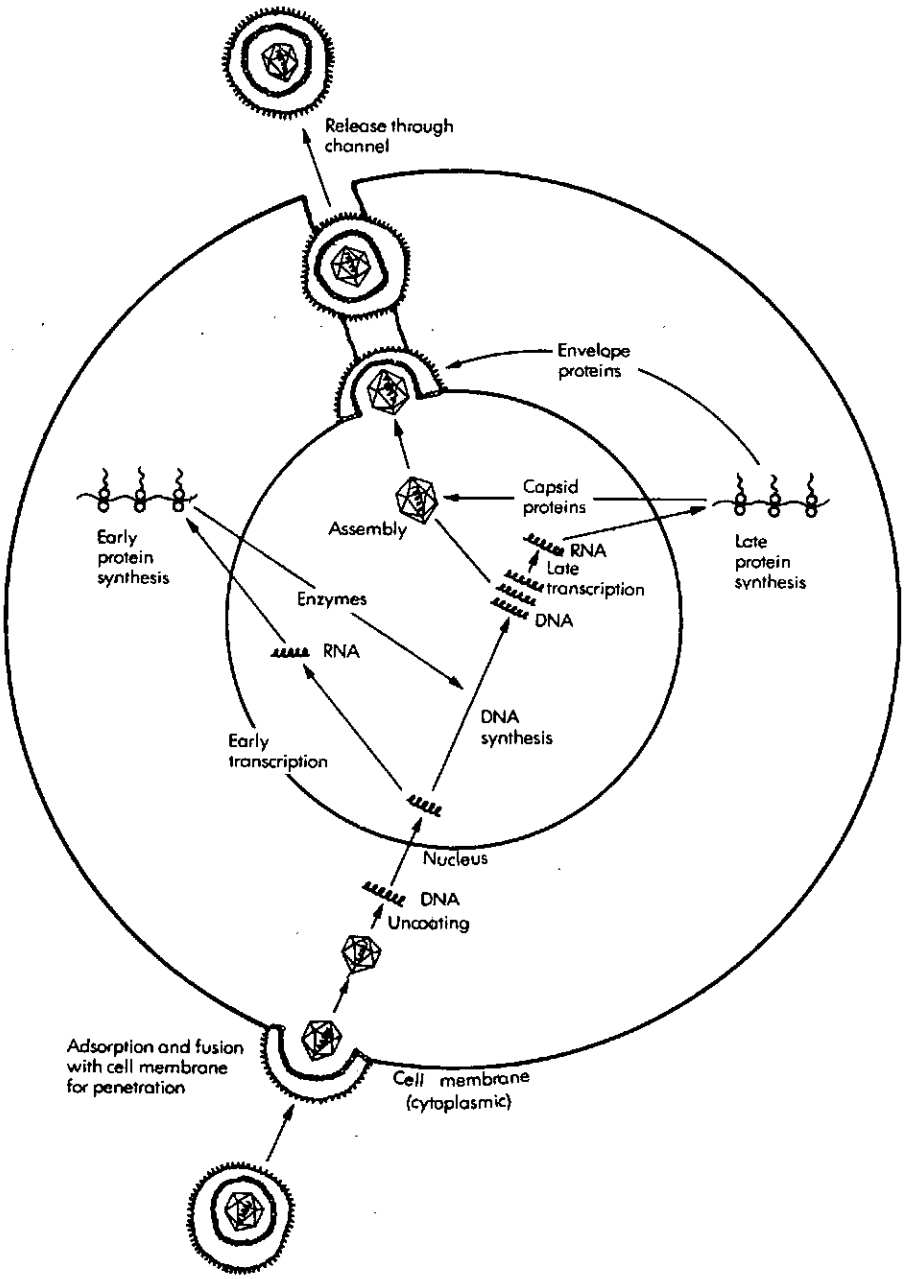

Release through channel
Envelope proteins
Capsid proteins
Assembly
Early protein synthesis
Late protein synthesis
RNA
Late transcription
DNA
Enzymes
RNA
DNA synthesis
Early transcription
Nucleus
DNA
Uncoating
Adsorption and fusion with cell membrane for penetration
Cell membrane (cytoplasmic)

Figure 21-14. Replication of herpes simplex virus. Specific glycoproteins in the viral envelope are essential for optimal adsorption to host cell receptors in the cytoplasmic membrane. Viral envelope and membrane fuse and the nucleocapsid of the virion is released into the cytoplasm. The virion is uncoated and the freed viral DNA is transported to the nucleus. Early transcription and mRNA processing are apparently executed by host cell enzymes. The resulting enzymes are used in viral DNA replication. Nuclear RNA transcripts synthesized after DNA replication are responsible for the synthesis of structural proteins that go to form the capsid and envelope as well as the glycoproteins in both the cytoplasmic and nuclear membranes. The structural proteins enter the nucleus to participate in the assembly of the virion. The nucleocapsids are enveloped by budding through the nuclear membrane. The virus is released from the cell through cytoplasmic channels. (*Erwin F. Lessel, illustrator.*)

are generally released in a burstlike fashion from the cells as they lyse. Alternatively, they may be extruded by a process that is essentially the reverse of phagocytosis. Enveloped animal viruses (and presumably enveloped plant viruses also) are released by budding through special areas of the host-cell membrane coded for by the virus, and in doing so the virions acquire a portion of the host membrane. In a few animal and plant viruses, the host cells are not destroyed. The virions leave the cells by special channels (tubules) over an extended period of time.

The yield of virus particles per cell varies with the virus, the type of cell, and the growth conditions. The average yield of plant and animal virions ranges from several thousand to about a million per cell, compared with a yield of a few hundred bacterial T phages.

As an example of the viral replicative process in the eucaryotic cell, Fig. 21-14 shows the replication of the herpes simplex virus, which is the cause of "fever blisters" or "cold sores." As can be seen in the figure, the events related to biochemical replication occur in both the nucleus and the cytoplasm, with assembly of the virion initiated in the nucleus. The nucleocapsids of these viruses then migrate to the cytoplasmic membrane (after envelopment by budding through the nuclear membrane), where the mature enveloped viruses appear to reach the surface of the cell through cytoplasmic channels.

CLASSIFICATION OF VIRUSES

Many attempts to classify viruses have been made. One of the earliest systems, which still has limited use, established classification according to the kind of host the viruses normally infect (e.g., hog cholera virus, swine influenza virus, fowl plague virus, cucumber mosaic virus, tobacco mosaic virus, and others). Another early means of classification was based on virus tissue affinities, for example, neurotropic (nerve-tissue) viruses and dermatropic (skin-tissue) viruses. Apparently this method was useful for physicians, epidemiologists, and some allied health investigators. However, as methods of measuring physical, chemical, and biological characteristics of viruses have been developed, information has been accumulated that allows formulation of a classification scheme for all viruses on the basis of these properties. Such properties are summarized in Table 21-3.

Table 21-3. Properties Used for Classification of Viruses

Primary Characteristics	Secondary Characteristics
Chemical nature of nucleic acid: RNA or DNA; single- or double-stranded; single or segmented genome; (+) or (−) strand; molecular weight	Host range: Host species; specific host tissues or cell types
Structure of virion: Helical, icosahedral, or complex; naked or enveloped; complexity; number of capsomeres for icosahedral virions; diameter of nucleocapsids for helical viruses	Mode of transmission: e.g., feces
Site of replication: Nucleus or cytoplasm	Specific surface structures: e.g., antigenic properties

Table 21-4. Classification of Viruses on the Basis of Differences in Their Transcription Processes

Class	Type of Virus	Characteristics
I	Viruses containing dsDNA (most DNA viruses)	Viral DNA (except for poxvirus) enters nucleus of host cell, where mRNA is synthesized with no intermediate form; the mRNA then passes into the cytoplasm (poxvirus DNA does not enter the host cell nucleus but remains in the cytoplasm); protein synthesis and assembly of virus take place in the cytoplasm
II	Viruses containing ssDNA (some bacterial viruses and the parvovirus group of animal viruses)	The single-stranded DNA molecule serves as a template for the synthesis of a complementary strand of DNA, resulting in a dsDNA molecule called a replicative form; not known whether mRNA is a transcription product of the replicative form or of the parental ssDNA
III	Viruses containing dsRNA (+ and − strands) (reoviruses; the reovirus genome consists of 10 dsRNA segments)	During the uncoating process not all of the nucleoprotein core is degraded—the viral genome remains enclosed; ten mRNA molecules are transcribed from one strand of the dsRNA and pass into the host cell cytoplasm; this mRNA may be used to produce double-stranded progeny RNA or structural proteins
IV	ssRNA viruses containing the (+) strand (poliovirus)	Following uncoating in the cytoplasm the parental (+) strand RNA acts as mRNA and directs the synthesis of viral proteins; one protein is an RNA polymerase that catalyzes the formation of a (−)RNA strand called a replicative intermediate (RI); the RI is a partial dsRNA since there is a complete (−)ssRNA molecule to which are attached partially synthesized (+)RNA strands; from the RI a complete dsRNA molecule (replicative form) will be produced, consisting of one (+) and one (−) strand; the other (+)RNA strands that were part of the RI may become genomes for new virus or they act as mRNA
V	ssRNA viruses containing the (−) strand (rhabdoviruses, paramyxoviruses, orthomyxoviruses)	Following uncoating in the cytoplasm the (−)RNA strand remains associated with an RNA transcriptase, which catalyzes the synthesis of mRNA from the (−)RNA parental strand; the mRNAs code for structural proteins as well as polymerase and transcriptase; the transcriptase also catalyzes the synthesis of a (+)RNA strand; from the (+) strand more (−)RNA molecules will be transcribed which can act as mRNA or as genomes for future virus progeny

Table 21-4. (*continued*)

Class	Type of Virus	Characteristics
VI	RNA tumor viruses requiring a DNA intermediate for replication	Following uncoating, viral reverse transcriptase (RNA-dependent DNA polymerase) uses the RNA genome as a template to transcribe a complementary DNA strand, resulting in the formation of a DNA-RNA hybrid; from the DNA strand of the hybrid a complementary DNA strand is synthesized using a conventional DNA polymerase enzyme, and a dsDNA molecule called a provirus is formed; the provirus is integrated into the genome of the host—the mechanism for viral transformation of animal cells

Table 21-5. Typical Classification of Animal and Plant Viruses on the Basis of Their Inherent Properties

Nucleic Acid	Capsid Symmetry	Envelope Presence	Virion Size (nm)	Family or Group	Genus or Typical Member
RNA	Icosahedral	–	28	*Tymovirus*	*Turnip yellow mosaic virus*
			70	*Reoviridae*	*Reovirus*
			28	*Picornaviridae*	*Enterovirus*
			30	*Tombusvirus*	*Tomato bushy stunt virus*
		+	35–80	*Togaviridae*	*Alphavirus*
			100–120	*Retroviridae*	*Rous sarcoma virus*
	Helical	–	17.5 by 300	*Tobamovirus*	*Tobacco mosaic virus*
			10 by 1250	*Closterovirus*	*Beet yellows virus*
		+	80–120	*Orthomyxoviridae*	*Influenzavirus*
			150–300	*Paramyxoviridae*	*Mobillivirus*
			60–180	*Rhabdoviridae*	*Lyssavirus; lettuce necrotic yellows virus*
			90–100	*Bunyaviridae*	*Bunyamwera*
			100	*Coronaviridae*	*Avian infectious bronchitis virus*
			50–300	*Arenaviridae*	*Lymphocytic choriomeningitis virus*
DNA	Icosahedral	–	50	*Caulimovirus*	*Cauliflower mosaic virus*
			18–24	*Parvoviridae*	*Parvovirus*
			40–50	*Papovaviridae*	*Papillomavirus*
			70–80	*Adenoviridae*	*Mastadenovirus*
		+	180–200	*Herpesviridae*	*Herpesvirus*
	Complex	Coat	230–300	*Poxviridae*	*Orthopoxvirus*

As can be seen from Table 21-3, the chemical nature of the nucleic acid plays an important role in virus classification. Thus in 1971, David Baltimore (a Nobel prize winner for his work on tumor viruses) proposed a classification of viruses based on how the viral genome is replicated and expressed. All viruses are divided into six classes on the basis of their method of mRNA synthesis or transcription (see Table 21-4). The division is based primarily on (1) whether the genome is a single- or double-stranded nucleic acid and (2) whether the genome is or is not converted to an intermediate form before the mRNA (plus strand) is produced. Although this scheme groups together viruses with similar

replicative steps, it also groups very different virions together in the same class, e.g., bacteriophages and animal viruses. Such a scheme finds favor with molecular biologists who study viruses. On the other hand, biology-minded virologists prefer a more general approach modeled after Linnaeus's classification scheme with nomenclature for families, genera, and species.

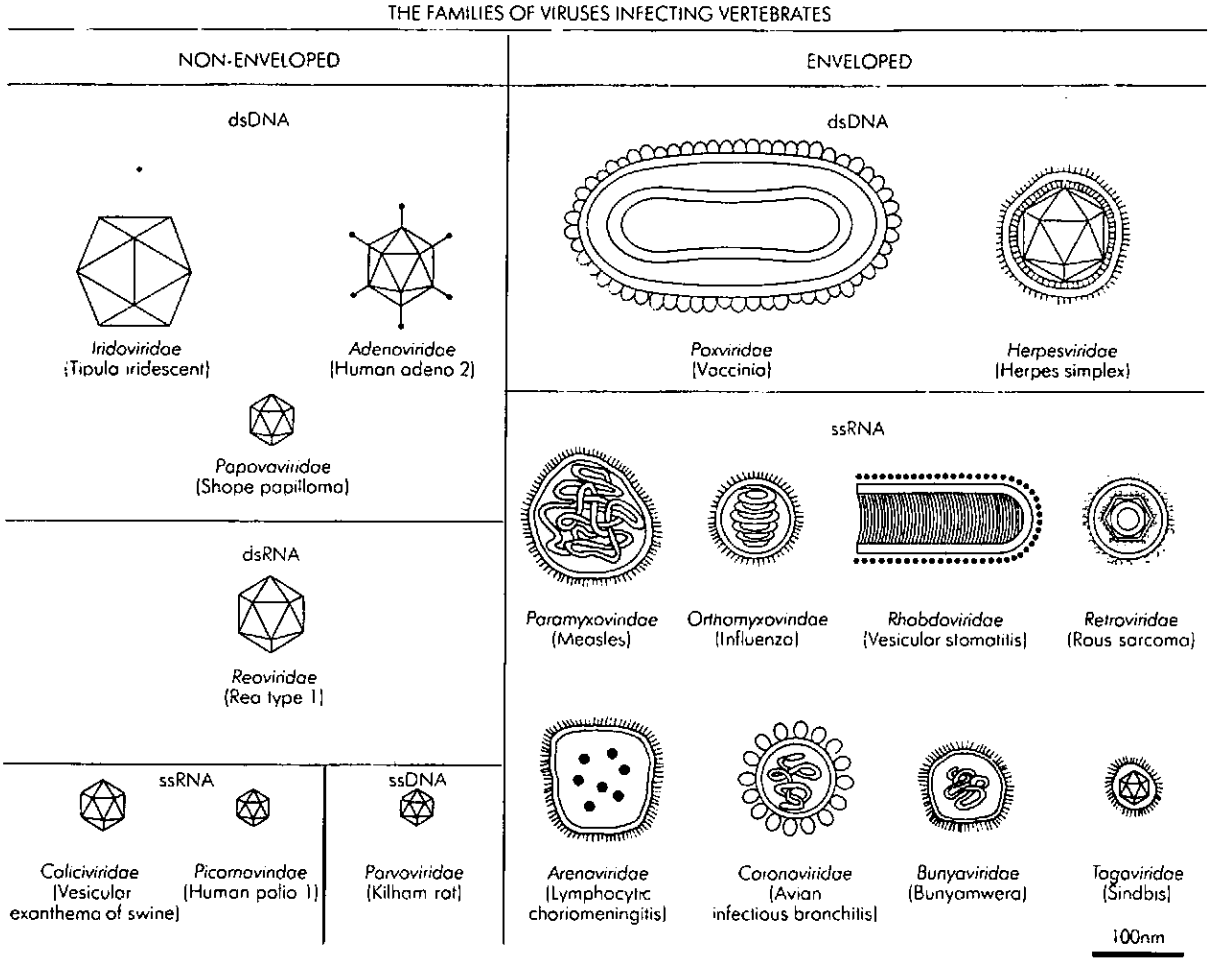


Figure 21-15. Line drawings of the families of viruses infecting vertebrates. All diagrams have been drawn to the same scale. For each drawing the family name is given together with a well-known member of it (but the dimensions and shape used for the drawing may not be exactly those of the virus named). (*Reproduced from drawings by Mrs. J. Keeling in R. E. F. Matthews, "Classification and Nomenclature of Viruses," Intervirology,* **17**:1–99, *1982. By permission from S. Karger AG, Basel, Switzerland.*)

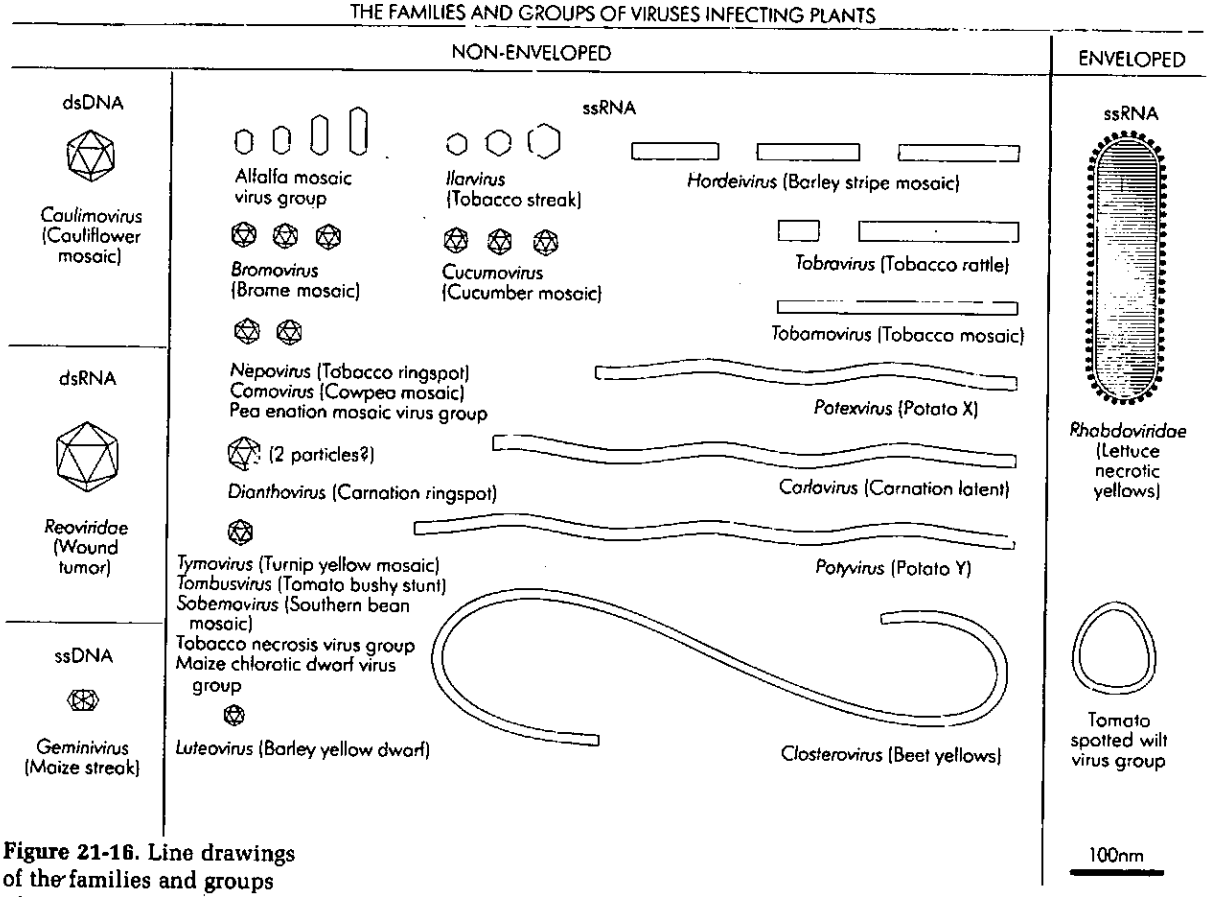


Figure 21-16. Line drawings of the families and groups of viruses infecting plants. All diagrams have been drawn to the same scale. For each drawing the group name is given together with a well-known member of it (but the dimensions and shape used for the drawing may not be exactly those of the virus named). (*Reproduced from drawings by Mrs. J. Keeling in R. E. F. Matthews, "Classifications and Nomenclature of Viruses," Intervirology, 17:1–99, 1982. By permission from S. Karger AG, Basel, Switzerland.*)

In order to prevent chaos in the naming of new viruses, an International Committee on Nomenclature of Viruses (ICNV) of the International Association of Microbiological Societies was formed at the Ninth International Congress for Microbiology in 1966. Since that time, this committee, now called the International Committee on Taxonomy of Viruses, has worked constructively towards a meaningful and practical scheme of viral classification. So far, the animal and bacterial virologists have subscribed to a nomenclature for various taxa. Family names agreed upon end in *-viridae*, subfamily names in *-virinae*, and genera, like species, in *-virus*. However, the plant virologists have not classified all their viruses in terms of families and genera. They use groups of viruses that share similar properties. Names for these groups are usually derived from the name of the prototype of the group. For example, the name of the group of viruses related to tobacco mosaic virus is the tobamo group or tobamovirus. Table 21-5 shows the manner in which the criteria of Table 21-3 are used for the classification of viruses. Figs. 21-15 and 21-16 are line drawings of the families of animal viruses and of the groups of plant viruses, respectively.

ISOLATION AND IDENTIFICATION OF VIRUSES

Isolation and identification of viruses from clinical specimens or materials for research purposes can be accomplished by a number of different methods, but no single technique is satisfactory for all viruses or every kind of specimen. The first step in laboratory identification of a virus is the proper collection and care of specimens until susceptible animals, tissue cultures, embryonated eggs, or other appropriate media are inoculated. This includes making the specimen bacteria-free by filtration, differential centrifugation, or treatment with bactericidal agents.

If a virus is present, characteristic antibodies, i.e., hemagglutination-inhibiting, complement-fixing, or neutralizing viral antibodies, may be produced. Techniques for the conduct and interpretation of tests to identify these antibodies are described briefly in Chap. 34.

CULTIVATION OF ANIMAL VIRUSES

Embryonated Chicken Eggs

Since viruses can grow only in living cells, one of the most economical and convenient methods for cultivating a wide variety of animal viruses is the chick-embryo technique (Fig. 21-17). The discovery that viruses could be cultivated by this simple technique was made in 1931. Fertile chicken eggs incubated for 5 to 12 days can be inoculated through the shell aseptically. The opening may be sealed with paraffin wax and the egg incubated at 36°C for the time required for growth of the virus.

Chick embryos contain several different types of cells in which various viruses will replicate. By using embryos of various ages and different methods of inoculation (Fig. 21-18), it is possible to grow the type of virus desired. The cells to be inoculated are found in the various embryonic membranes and tissues of the egg. For example, vaccinia virus can be grown on the chorioallantoic membrane and produce lesions or pocks. The yolk sac and the embryo also can be used to grow viruses. Also, the fluid from the egg can be harvested and assayed for the presence of virus. The chick-embryo technique has been used in the production of vaccines against smallpox, yellow fever, influenza, and other diseases and in immunologic tests and other studies whenever large amounts of virus are required.

Tissue Cultures

Cell cultures are today the method of choice for the propagation of viruses for many reasons. Among them are convenience, relative economy of maintenance compared to animals, observable cytopathic effects, and choice of cells for their susceptibility to particular viruses. On the basis of their origin and character-

Figure 21-17. Embryonated hen's egg is used for the cultivation of many mammalian viruses.

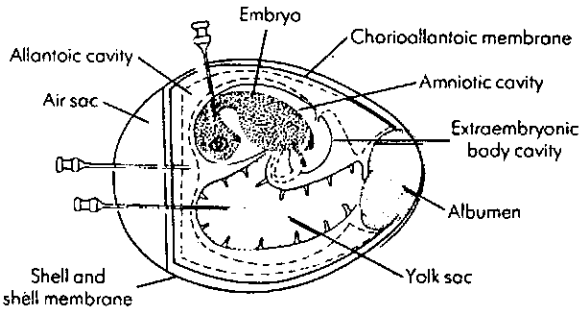


Figure 21-18. Diagrammatic representation in sagittal section of the embryonated hen's egg 10 to 12 days old. The hypodermic needles show the routes of inoculation of the yolk sac, allantoic cavity, and embryo (head). The chorioallantoic membrane is inoculated after it has been dropped by removing the air from the air sac.

istics, cell cultures are of three types: primary cell cultures, diploid cell strains, and continuous cell lines. Primary cell cultures are derived from normal tissue of an animal (such as mouse, hamster, chicken or monkey tissue) or a human (e.g., gingival tissue). When cells from these tissues are processed and cultured, the first monolayer is referred to as a primary culture. (A monolayer is a confluent layer of cells covering the surface of a culture vessel.) The cells from subcultures are called secondary cultures. (See box for the technique for processing such primary cell cultures.) Cell cultures prepared from fresh tissue resemble more closely the cells in the whole animal than do the cells in continuous cell lines. Unfortunately, cells derived in this manner can be subcultured only a limited number of times before dying. For some types of cells only a few divisions are possible. For others, 50 to 100 divisions occur. Cell cultures derived from embryonic tissue are generally capable of a greater number of divisions in vitro than those derived from adult tissue.

Diploid cell strains are derived from primary cell cultures established from a particular type of tissue, such as lung or kidney, which is of embryonic origin. They are of a single cell type and can undergo 50 to 100 divisions before dying. They possess the normal diploid karyotype (appearance of the set of chromosomes). Such diploid cell strains are the host of choice for many viral studies, especially in the production of human vaccine virus. Vaccines prepared from tissue cultures have an advantage over those prepared from embryonated chicken eggs in minimizing the possibility of a patient developing hypersensitivity or allergy to egg albumen. The Salk poliomyelitis vaccine, which is produced in tissue culture, was developed after basic research had shown that the poliovirus would grow satisfactorily on monkey kidney cell cultures.

Processing of Primary Cell Cultures

Primary cell cultures are prepared from fresh tissue, which is usually minced with a sharp sterile razor and dissociated with the aid of proteolytic enzymes (such as trypsin) into a cell suspension. The cells are washed with a physiological buffer (to remove the proteolytic enzymes used) and then suspended in a special growth medium containing a balanced salt solution, a buffer, necessary nutrients (vitamins, coenzymes, amino acids, glucose), and serum. Antibiotics may be added to inhibit bacterial growth. The cell suspension in the growth medium is placed in a tissue-culture vessel and incubated. The cells settle on the surface of the vessel and grow into a monolayer.

Continuous cell lines appear to be capable of an infinite number of doublings. Such cell lines may arise with the mutation of a cell strain, or more commonly from the establishment of cell cultures from malignant tissue. The karyotype of these cells is aneuploid (a variable multiple of the haploid chromosome number) and not diploid. These cells are also different morphologically from the cells of origin. They are usually less fastidious in their nutritional requirements. They do not attach as strongly as other cell cultures to the surface of the culture vessel, so under certain circumstances they can grow in suspension. They also have a tendency to grow on top of each other in multilayers on culture-vessel surfaces.

Even though cells from continuous cell lines are very different from normal cells in both genotype and phenotype, they are very useful in studies where large numbers of cells are required. Furthermore, they are easy to propagate serially. But because of their derivation from malignant tissue or their possession of malignant characteristics, such cells obviously are not used in virus production for human vaccines. Nevertheless, continuous cell lines have been extremely useful in cultivating many viruses previously difficult or impossible to grow.

Growth of viruses in tissue culture is shown in Fig. 21-19. It can be seen that the tissue structure deteriorates as the virus multiplies. This deterioration is called the cytopathic effect (CPE).

Animals

Some viruses cannot be cultivated in cell culture or in embryonated chicken eggs and must be propagated in living animals. Mice, guinea pigs, rabbits, and primates are used for this purpose. Animal inoculation is also a good diagnostic tool because the animal can show typical disease symptoms and histological (tissue) sections of infected tissue can be examined microscopically.

The Origin of HeLa Cells

It was in the winter of 1951 when Henrietta Lacks, a young black woman of 31, went to the medical clinic of Johns Hopkins University in Baltimore to seek medical treatment. The examining physician found a malignant tumor within her cervix. Some of this cancerous tissue was taken to a laboratory for cultivation.

In spite of intensive radiation treatment, the tumor continued to grow. Eight months after her first visit to the clinic, the cancer had spread throughout her whole body and she died. But the tumor cells taken from Henrietta Lacks thrived; they divided and doubled their number every 24 hours. Cells taken previously from the tumors of dozens of other patients had not grown at all, or grew only poorly and then died off.

The cancer cells of Henrietta Lacks continued to flourish in culture in Petri dishes. These cells, now code-named HeLa cells, became one of the best-known continuous tissue-culture cell lines. HeLa cells are widely used in research because they are so readily available, so versatile, and so easy to propagate serially. They have been dubbed the "cells that would not die." Thus Henrietta Lacks left behind her the first widely available model of human tissue in vitro for scientific investigation. Perhaps her legacy will help to conquer the disease that vanquished her in 1952.

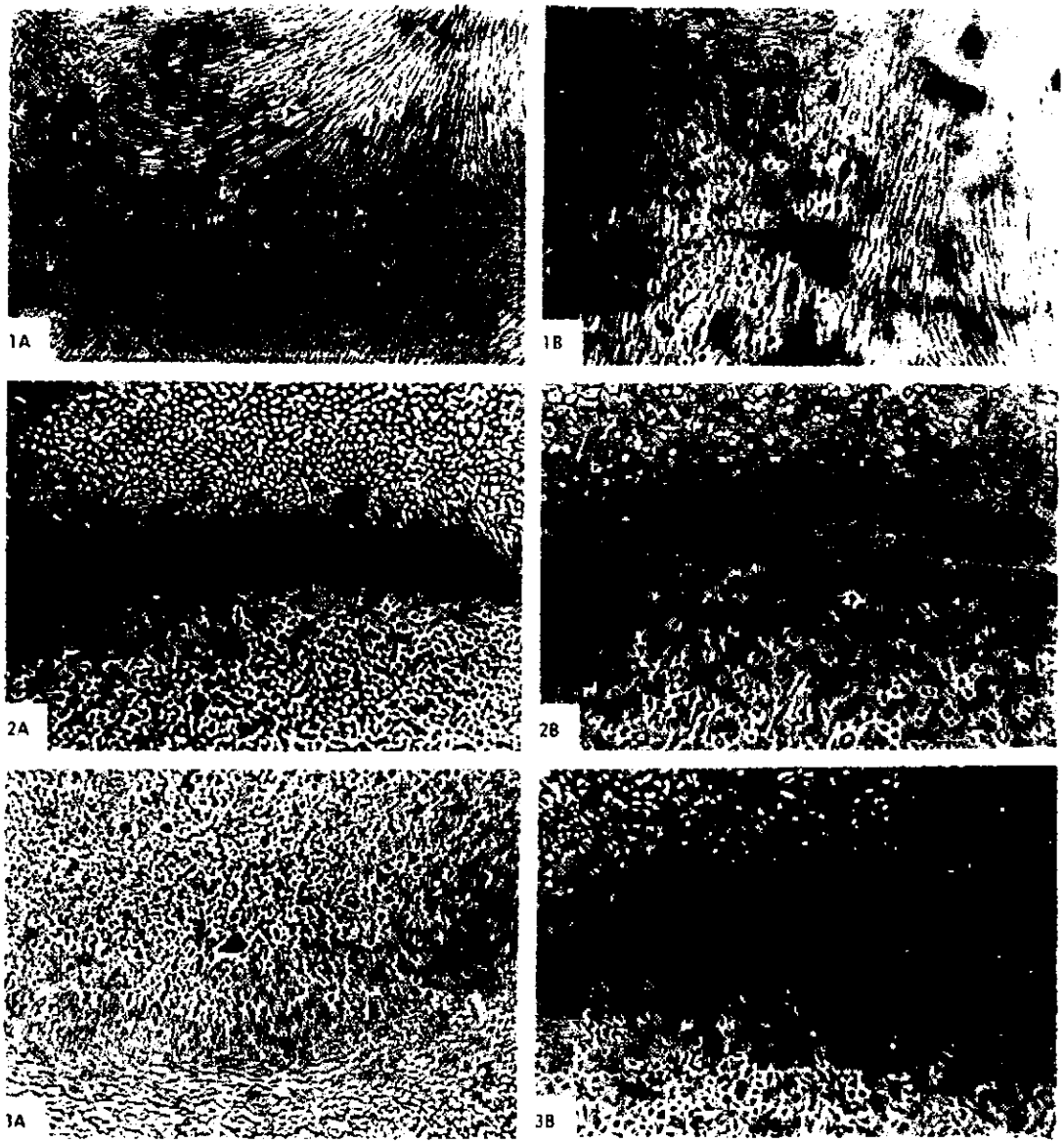


Figure 21-19. Light-microscope view of tissue cultures used for the cultivation of viruses. (1A) Normal human lung fibroblast cell culture. (1B) Human lung fibroblast cell culture infected with varicella (chicken pox) virus. Characteristic cytopathic effect (CPE) seen: rounded and enlarged cells with splitting of cell sheet (layer) and stranding. (2A) Normal monkey kidney cell, line GL V3A. (2B) Monkey kidney cell line GL V3A, infected with poliovirus. CPE seen: cell shrinkage with retracted margins exhibiting angular shapes. (3A) Normal rabbit cell line RK 13. (3B) Rabbit cell line RK 13, infected with rubella (German measles) virus. CPE seen: discrete foci or centers of aggregated cells. Magnification approx (X850). (*Courtesy of A. F. Doss, McGill University.*)

CULTIVATION OF PLANT VIRUSES

Plant viruses can be cultivated by direct mechanical inoculation of virus suspensions by rubbing on leaves of living plants. Rubbing is accomplished with the aid of an abrasive such as carborundum. This can lead to formation of local lesions as well as general infection. Some plant viruses can replicate to large numbers in infected plants. For example, a single hair cell of an infected tobacco plant may contain over 10^7 tobacco mosaic virions. Indeed, as much as 10 percent of the dry weight of infected leaves may be tobacco mosaic virus. Transfer of infection from cell to cell occurs mainly by direct transfer of virions via **plasmodesmata** (intercellular bridges). Also, in most plant diseases, infected cells can continue to manufacture virus without either lysing or dying.

In the last decade some progress has been made in the preparation of plant cell protoplasts for the cultivation of plant viruses. For instance, isolated protoplasts from mesophyll cells of tobacco can be infected directly with tobacco mosaic virus. Progress also has been made in the development of monolayer cultures of susceptible cells from the insects that are vectors of some plant viral diseases. For example, rhabdovirus has been cultivated in leafhopper cell culture giving a yield of over 10,000 virions per cell.

EFFECTS OF VIRUS INFECTION ON CELLS

Cell Death

Disease symptoms in the host due to virus infection vary from none to massive destruction of infected cells leading to cell death. In cell tissue culture, groups of killed cells (**plaques**) have been used in the enumeration of viruses because the number of plaques is proportional to the number of infectious virus particles present. Each virion gives rise to a single plaque, just as a bacterium gives rise to a single colony (Fig. 21-20).

Other effects of cell infection by viruses include formation of giant cells (**polykaryotes**), creation of genetic changes such as chromosomal breakage, induction of **interferon** production (see Chap. 32) by the infected cell that prevents infection of healthy cells, appearance of **pocks** or necrotic lesions on the chorioallantoic membrane of embryonated eggs, and formation of **inclusion bodies**.

Inclusion Bodies

Before it was possible to study the morphology of viruses at the high magnifications provided by the electron microscope, investigators using light microscopy had observed intracellular structures, or inclusion bodies, associated with virus diseases (Fig. 21-21). In 1887 J. B. Buist noted small particles in the

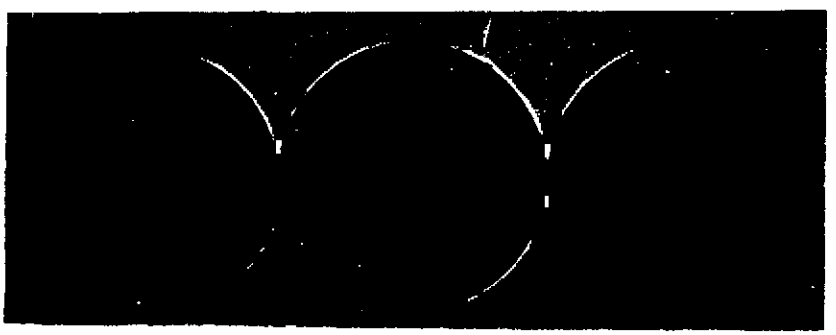


Figure 21-20. Virus particles in a suspension may be enumerated by means of a plaque assay. Shown are plaques of reovirus on a monolayer of L-929 mouse fibroblast cells in tissue-culture dishes. Note that increasing dilution of the virus suspension results in decreasing plaque numbers in the dishes. (*Courtesy of Collette Oblin, McGill University.*)

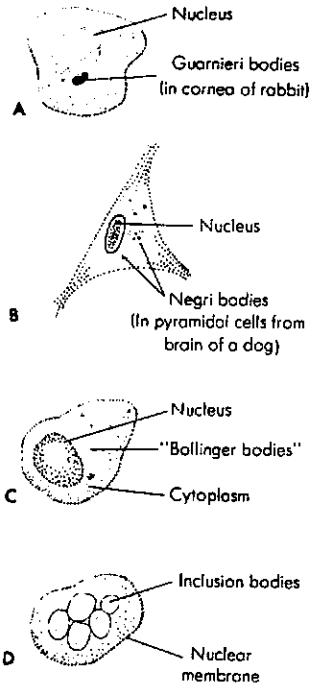


Figure 21-21. Inclusion bodies produced by viruses in certain host tissues. (A) Guarnieri bodies of variola (smallpox) virus in the cytoplasm of rabbit corneal cells. (B) Negri bodies in the cytoplasm of Purkinje cells (nerve cells of the brain) infected with rabies virus. (C) Bollinger bodies in the cytoplasm of cells infected with fowl pox virus. (D) Intranuclear inclusions in epithelial cells of rabbit cornea inoculated with herpesvirus.

cytoplasm of cells surrounding the lesions of smallpox. These he called elementary bodies. E. Paschen made the same observation independently in 1906. It is now known that these Paschen bodies are aggregates, or colonies, of virions growing in the cytoplasm of the host cell. In 1892, G. Guarnieri reported having seen small round particles in the cytoplasm of similar cells. These Guarnieri bodies are also thought to consist of aggregates of unassembled virus subunits and intact virions.

Characteristic inclusion bodies are found in the cytoplasm of the Purkinje cells of the cerebellum and in certain other nerve cells in cases of rabies infection. Finding these typical inclusions (called Negri bodies after their discoverer) is diagnostic of the disease.

Inclusion bodies have been found in connection with many other virus diseases. They occur in the cytoplasm in most pox diseases (smallpox, sheep pox, fowl pox), rabies, molluscum contagiosum, and others. Intranuclear inclusions are found in chicken pox, herpes, and the polyhedral diseases of insects. Intranuclear and intracytoplasmic inclusions may be found in the same cell in instances of multiple infection. Some inclusions are useful in establishing diagnosis while the significance of others is not yet known.

Inclusion bodies are for the most part characteristic of the virus causing the infection and even suggest definite pathological changes in the cell. It is generally true, however, that inclusion bodies are aggregates of unassembled virus subunits and intact virions in infected cells. It is experimentally possible to remove inclusion bodies from the cell and use them as inoculum to infect other cells.

PROGRESSIVE AND FATAL DISEASES ASSOCIATED WITH VIRUSES IN ANIMALS

Some progressive or gradually extending diseases which usually terminate in death are poorly understood and require much research. Some of these are or may be caused by viruses, such as classic slow virus diseases and cancer. (The conventional viral diseases are discussed in Chap. 37.)

Classic Slow Virus Diseases

These diseases have a *slow* progressive course, usually with a fatal outcome. (Incubation periods are measured in years!) They are caused by transmissible agents whose properties and behavior (for example, unusual resistance to ultraviolet radiation and heat) suggest an unconventional or atypical virus. Indeed, these agents have been called prions (proteinaceous infectious particles) because they appear to have no nucleic acids at all, protein being their only detectable component. If prions in fact do not contain nucleic acids, their ability to replicate would seem to pose a challenge to the central dogma of molecular biology.

There are four classic slow virus diseases. Each may be described as a neu-

rological disease. They are **kuru** and **Creutzfeldt-Jakob disease** of humans and **scrapie** and **transmissible mink encephalopathy** (TME) of animals. (The term *encephalopathy* describes these diseases well because each involves widespread destructive cerebral changes due to degeneration without inflammation.)

Scrapie is so named because the diseased animal tends to scrape against fixed objects. It is a chronic infection of the central nervous system of sheep. TME, a disease found in mink farms in the United States, may have arisen from mink fed on the meat of sheep contaminated with scrapie virus. It is also possible that skunks and raccoons harbor this virus naturally.

Kuru and Creutzfeldt-Jakob disease are similar degenerative diseases of the human central nervous system. Symptoms include tremors, progressive uncoordinated movements, and mental deterioration. Fortunately, both are rare, and kuru is restricted to the Foré people of New Guinea. Kuru was spread among these people by ingestion of infectious human brain tissue during ritual cannibalism of the dead as a mourning ceremony. Now that ritual cannibalism has been eliminated, the disease has been declining in prevalence and should soon disappear. (Dr. D. Carleton Gajdusek of the National Institute of Neurological Diseases and Strokes studied this disease extensively and was awarded the 1976 Nobel prize in medicine for his pioneering work.) Unlike kuru, which is restricted geographically, Creutzfeldt-Jakob disease has a worldwide distribution. The onset generally occurs between the ages of 35 and 65.

Cancer and Viruses

More than 100 clinically distinct types of cancer are recognized, each having a unique set of symptoms and requiring a specific course of therapy. However, most of them can be grouped into four major categories:

1 ***Leukemias.*** Abnormal numbers of white cells (leukocytes) are produced by the bone marrow.
2 ***Lymphomas.*** Abnormal numbers of lymphocytes (a type of leukocyte) are produced by the spleen and lymph nodes.
3 ***Sarcomas.*** Solid tumors grow from derivatives of embryonal mesoderm, such as connective tissues, cartilage, bone, muscle, and fat.
4 ***Carcinomas.*** Solid tumors grow from epithelial tissues, the most common form of cancer; epithelial tissues are the internal and external body surface coverings and their derivatives and thus include skin, glands, nerves, breasts, and the linings of the respiratory, gastrointestinal, urinary, and genital systems.

Cancer has three major characteristics: **hyperplasia**, **anaplasia**, and **metastasis**. Hyperplasia is the uncontrolled proliferation of cells. Anaplasia is structural abnormality of cells (these cells also have a loss of or reduction in their functions). Metastasis is the ability of a malignant cell to detach itself from a tumor and establish a new tumor at another site within the host.

For a long time microbiologists entertained the idea that cancer might be caused by viruses. This was because some early experiments associated viruses with some cancers. In the early 1900s, both leukemias and sarcomas in chickens were shown to be caused by viruses. But for many years these discoveries were not considered relevant to the cause of human cancer. Furthermore, human cancer did not appear to be infectious, and there were no confirmed isolations of an **etiologic** (causative) virus; the idea that viruses are the cause of human cancer became less attractive.

However, in recent years significant evidence has accumulated to show that

some viruses do in fact cause cancer in animals. These findings revived the idea that human cancers might also be caused by viruses.

Both RNA and DNA viruses have been found capable of inducing cancer in animals; in these animals the affected cells are transformed, resulting in the formation of tumors. (A transformed cell acquires phenotypic, biochemical, and other properties distinctly different from uninfected cells or infected cells in which tumors are not produced. Such changes arise from modification of a cell's genome induced by its incorporation of DNA from other cells or from viruses.) Such tumor-inducing viruses are called oncogenic viruses.

Among the viruses whose virions contain RNA, only some members of the family *Retroviridae* (more commonly known as the RNA tumor viruses) cause cancer in animals. These members (of the subfamily *Oncovirinae*) replicate through a DNA intermediate. None of the other RNA viruses, all of which replicate through an RNA intermediate, are known to cause cancer. In contrast, among the DNA viruses, members of at least three families (*Herpesviridae*, *Adenoviridae*, and *Papovaviridae*) can cause cancer in animals.

All the viruses that can cause cancer in animals induce synthesis of DNA that is present in the nucleus and that codes for proteins which are not virion structural proteins. A common characteristic of all oncogenic viruses is that the viral genome in some way becomes either integrated or intimately associated with the host DNA. The host cell does not lyse—a situation similar to the model of lysogeny in bacteria infected with temperate phages. As mentioned earlier in this chapter, if the viral genome is RNA, it serves as a template for the synthesis of a DNA molecule complementary to it; the enzyme reverse transcriptase is responsible for this synthesis giving rise to a DNA-RNA hybrid. A conventional DNA polymerase enzyme is then used to synthesize a strand of DNA complementary to the first strand. This results in a double-stranded DNA molecule synthesized from the viral RNA and called a **provirus**, which now can be integrated into the host DNA. In this way transformation and tumors are induced in host cells.

There are differences in the ability of oncogenic viruses to cause cancer. Some RNA tumor viruses, some herpesviruses, and one papovavirus cause cancer in nonlaboratory situations. In contrast, some other RNA tumor viruses, adenoviruses, and most papovaviruses are found to cause cancer only in laboratory animals. The DNA tumor viruses primarily induce cancers only in foreign hosts, that is, hosts the viruses do not infect outside the laboratory.

Furthermore, the transforming viruses differ from each other in the probability that only one virus particle can cause transformation and in the fraction of cells that are transformed in a genetically susceptible population. This means that, in the laboratory, transforming viruses differ in the efficiency with which they cause cancer. They also differ as to how dependent the transformation is on a specific type of differentiated cell.

Oncogenic DNA Viruses

DNA tumor viruses, such as adenovirus 12, polyoma virus, and simian virus 40 (SV 40), can transform susceptible cells rapidly. But usually only a small percentage of cells can be transformed.

Polyoma virus is endemic in wild and laboratory mouse populations. SV 40 cannot induce tumors in the monkey (natural host) but can do so in rodents in the laboratory. The Epstein-Barr virus (EBV), a herpesvirus, has been consis-

tently associated with certain human neoplasias, or tumors. It was discovered by Epstein and Barr in 1964 in cultured Burkitt's lymphoma cells. Burkitt's lymphoma is a cancer of the lymphoid system. EBV has also been implicated in infectious mononucleosis (regarded by some authorities as a self-limiting leukemia), in Hodgkin's disease (a form of lymphoma), and in nasopharyngeal carcinoma. Other herpesviruses, such as herpes virus (HSV) types 1 and 2, have also been implicated in certain human cancers: cancers of the lip of mouth have been associated with HSV1, and cervical cancer has been associated with HSV2.

Much of the mechanism of transformation has been learned from the SV40 virus infection of mouse cells. Infection of certain cells leads to integration of the viral DNA with cell DNA under conditions that do not allow full virus expression, which would normally lead to virus production and cell death. But sometimes this nonproductive infection (no progeny virus and consequently no cell death) allows formation of an early gene product essential for virus replication. Transformation is the accidental result of the continued presence of this early viral product.

Oncogenic RNA Viruses

The RNA sarcoma viruses are the most strongly transforming viruses. They transform many different types of cells, e.g., fibroblasts, myoblasts, iris epithelial cells, etc.; they can also transform all of the cells in a culture.

The genetic basis for transformation has been elucidated with Rous sarcoma virus infection of chicken fibroblast cells. The RNA viral genome contains a gene that is responsible for transformation and is not essential for virus replication. Upon infection of susceptible cells, the transformation gene, along with all the other viral genes, is synthesized as DNA, using the viral genome as template, and is integrated at a unique site in the cellular DNA. The viral gene for transformation, together with all other viral genes, is transcribed, giving rise to a product that causes cell transformation. In addition, progeny virus is produced (a situation different from that of the DNA oncogenic viruses). The existence of a specific gene for transformation and its integration to become a stable component of the cell genome make Rous sarcoma virus a strongly transforming virus capable of transforming many types of cells. This explains why Rous sarcoma virus has a greater probability of transforming susceptible cells than do oncogenic DNA viruses.

Until recently RNA viruses were not isolated from human cancer cells, although some circumstantial evidence implicated them in this disease. For example, molecular components were found in human leukemic cells which are related to similar components in RNA tumor viruses. However, recently Robert Gallo and coworkers of the National Cancer Institute in Bethesda, Maryland, and Yorio Hinuma and colleagues at Kyoto University separately isolated the first identified human cancer virus associated with a rare form of human leukemia. As of 1982, the virus had been discovered in four cutaneous T-cell leukemia or lymphoma patients in the United States. The virus is a new RNA tumor virus (retrovirus).

VIROIDS

Viroids are nucleic acid entities of relatively low molecular weight (1.1 to 1.3 $\times$ 10^5) and unique structure that cause several important diseases of cultivated

Oncogenes, Retroviruses, and Cancer

Oncogenes (from *oncos*, the Greek word for "mass" or "tumor") were originally defined as the genetic elements (genomic sequences) of retroviruses responsible for the malignant transformation of host cells. These oncogenes have also been found in spontaneous tumors. Furthermore, DNA sequences homologous to the transforming genes of certain retroviruses have been found in normal, untransformed cells. Thus these oncogenes are not themselves of viral origin but are cellular genes that the viruses picked up by recombination events during the course of infection. This means that most of the oncogenes found in viruses and cancer cells have counterparts, either identical or closely similar, among the normal genes of the human and animal body. In fact, oncogenes are found in all the cells of the mammalian body except the red blood cells, which do not possess nuclei; but the majority of cells never become cancerous. Oncogenes have the potential of causing cancerous transformation of cells when appropriately activated. Some 20 oncogenes have been identified, most of which were originally discovered in viruses that cause cancers in laboratory animals.

The structures of the cellular counterparts of the viral oncogenes (termed cellular oncogenes) have been conserved throughout evolutionary history. This suggests that they have essential roles to play in cellular physiology, probably in cell differentiation or regulation of cell division. At the present time, we still do not know what makes these genes go awry and induce uncontrolled cell division and abnormal cell differentiation patterns. It is speculated that an oncogene can be induced to act in the cancer process in many different ways: (1) by undergoing mutation, (2) by being abnormally activated when thrown into association with some other genes, (3) by being removed from natural repressors that regulate its activities, (4) by producing oncogene products that may be growth factors that stimulate cell division and contribute to the uncontrollable growth of cancer cells, and (5) by overexpression due to the accidental integration of a retrovirus next to it. The evidence implicating cellular oncogenes in the development of human cancers is still largely circumstantial. Nevertheless, these oncogenes are currently the subject of many exciting and promising studies.

plants. Such diseases include potato spindle tuber, citrus exocortis, chrysanthemum stunt, and cucumber pale fruit. Viroids are the smallest known agents of infectious disease. Unlike viruses, viroids do not possess a protein coat and exist only as short, infectious molecules of RNA. In spite of their small size, viroids replicate autonomously in cells of susceptible plant species. Some known viroids are single-stranded, covalently closed circular RNA molecules, and others are single-stranded linear RNA molecules.

It is not known how viroids cause disease. It is thought that their location in the nucleus (since most infectivity of viroids is associated with cell chromatin material) as well as their apparent inability to act as mRNA suggest that they cause host symptoms by interference with gene regulation in the infected host cells. Present evidence also suggests that viroidlike nucleic acids may exist in organisms other than higher plants. They may be responsible for certain diseases of humans and animals (although how they escape the destructive action of nucleases in animal cells is unexplained at the present time).

QUESTIONS

1 What association did the following people have with virology?

Jenner	Loeffler and Frosch
Waterhouse	Stanley
Pasteur	Theiler
Chamberland	Enders
Ivanowski	Baltimore
Beijerinck	Lacks

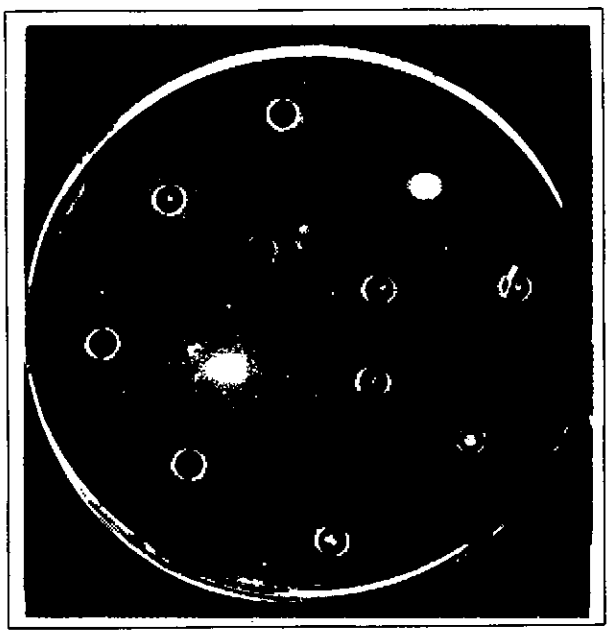
2 Cite three types of viral vaccines prepared from tissue cultures.
3 Discuss the characteristic symmetry of spherical and of rod-shaped viruses.
4 What is the difference between a naked and an enveloped virion?
5 Draw a simple virion, identifying the following structures: capsid, capsomere, nucleic acid core, and envelope.
6 How is the genome of a virus different from that of a cell of a higher organism?
7 What components of a virion are made of protein?
8 Explain why some single-stranded RNA viruses have plus strands while others have minus strands.
9 Compare the enzymatic activity of RNA polymerase with that of RNA-dependent DNA polymerase.
10 Compare the mode of multiplication of viruses with that of some representative bacteria.
11 Explain how the mechanism of adsorption of virus to animal cells is different from that of adsorption to plant cells.
12 Describe the mechanisms of penetration of animal viruses into host cells.
13 Compare the functions of early mRNA and late mRNA in the process of replication of viral components.
14 Describe the process by which virions are released from host cells.
15 How does the yield of virus particles per cell compare between a phage-infected bacterium and a virus-infected animal or plant cell?
16 Describe the temporal events in the replication of herpesvirus.
17 Discuss the primary characteristics used in the classification of viruses.
18 How do the plant virologists differ from the animal and bacterial virologists in their approach to viral classification?
19 Prepare a separate stylized drawing of a virion to represent each of the following: (*a*) *Reoviridae*, (*b*) *Poxviridae*, (*c*) *Orthomyxoviridae*, (*d*) tobamovirus, (*e*) closterovirus, and (*f*) *Rhabdoviridae*.
20 Describe three techniques for cultivating viruses in the laboratory. How do these methods differ from methods for culturing bacteria?
21 What are the advantages and disadvantages of primary cell cultures and continuous cell lines for the cultivation of viruses?
22 What type of cell culture is generally used for viral vaccine production?
23 In what ways have plant viruses been cultivated in vitro?
24 What are inclusion bodies? Where are they found? What is their significance?
25 What new concepts have we learned about virology from an understanding of slow virus diseases?
26 Which are the RNA-containing viruses that can cause cancer in animals?
27 Describe some unique characteristics of the viruses that cause cancer in animals.

28 What are some of the differences among oncogenic viruses in their ability to cause cancer?
29 Compare the known mechanism of transformation by an oncogenic DNA virus with that by an oncogenic RNA virus. Which virus is more efficient in transforming susceptible cells?
30 Has a human cancer virus been isolated and identified? Explain.
31 What is a viroid? Why is it unique?
32 What are oncogenes and where are they found?
33 Describe some ways by which oncogenes are activated to cause cancer.

REFERENCES

The references cited for Chap. 20 are also applicable to this chapter.

PART SIX
CONTROL OF MICROORGANISMS



Penicillin—Its Discovery and Development

Penicillin was discovered in 1927 by Alexander Fleming, a microbiologist working at St. Mary's Hospital in London.

Fleming was conducting experiments in search of new antibacterial agents, particularly ones that would be effective against wound infections. In the course of these experiments, he observed a plate culture of *Staphylococcus aureus* that had been contaminated by a mold. The area around the edges of the mold colony was clear—no bacterial colonies. Apparently, the staphlyococcus cells near the mold growth were inhibited or killed by the mold. Further studies of this phenomenon revealed that it was a mold of the genus *Penicillium* that produced a substance which was very potent against staphylococci and hence very attractive as a potential chemotherapeutic agent. Fleming named the substance *penicillin*.

Penicillin was effective against bacteria in laboratory cultures, but was it effective in the human body? This question needed to be answered. Unfortunately, the amount of penicillin produced in the mold cultures was extremely small. In addition, serious problems were encountered in attempts to isolate and purify penicillin.

The first clinical trial with a crude penicillin preparation was conducted on February 12, 1941. The patient, an Oxford policeman, was dying from a staphylococcus infection. The administration of penicillin resulted in an initial dramatic improvement, but five days later, when the supply of penicillin was exhausted, the staphylococci reemerged, the infection spread, and the patient died. This was a tragic end to a trial that did not succeed only because there was not enough penicillin available to treat the patient. The major problem continued to be the failure of large-scale production and recovery of penicillin.

Britain, at this point (1940–41), was engaged in a grim war. There was little likelihood that a major share of her national resources could be diverted to an intensive program for development of penicillin.

Fortunately, the British reports of penicillin attracted the attention of Americans. As a result, the Rockefeller Foundation invited Harold W. Florey, a professor of pathology at Oxford University, who had investigated the development of penicillin as a chemotherapeutic agent, and N. G. Heatley, his colleague, to the United States to explore means of large-scale production of penicillin. They arrived in the United States on July 2, 1941. Meetings were arranged with members of the National Research Council, Charles Thom, a world class mycologist with the U.S. Department of Agriculture, and others. Work on penicillin production began immediately at the U.S. Department of Agriculture's Northern Regional Research Laboratory in Peoria, Illinois, which had a record of achievements in microbial fermentations.

The U.S. Office of Scientific Research and Development, aware of the tremendous potential of penicillin for treatment of casualties of war, gave this project top priority. Major pharmaceutical companies and universities were called in to cooperate in the research and development of penicillin production. The results proved dramatic. Fleming's original mold cultures produced 2 units/ml; within a matter of months improvements in technology increased the yield to 900 units/ml; today the yield is approximately 50,000 units/ml!

In the autumn of 1941 there was little penicillin available in the United States for treatment of patients. One year later, as a result of the collaborative efforts of governments, universities, and industry, appreciable quantities were available.

Few other discoveries have contributed as much as that of penicillin (and the antibiotics that followed) to the health and welfare of people worldwide. The story of penicillin provides an elegant example of the benefits gained from collaboration of scientists from government agencies, industry, and universities.

Preceding page. Inhibition of bacteria by chemotherapeutic chemicals. The clear zone around the disks is evidence that the chemical agent in the disk, which diffuses into the inoculated agar medium, inhibits growth of the bacteria. (*Courtesy of BBL Microbiology Systems.*)

Chapter 22 The Control of Microorganisms by Physical Agents

OUTLINE

The term **control** as used here refers to the reduction in numbers and/or activity of the total microbial flora. The principal reasons for controlling microorganisms are: to prevent transmission of disease and infection, to prevent contamination by or growth of undesirable microorganisms, and to prevent deterioration and spoilage of materials by microorganisms.

Microorganisms can be removed, inhibited, or killed by various **physical agents, physical processes,** or **chemical agents.** A variety of techniques and agents are available; they act in many different ways, and each has its own limits of application.

In the first part of this chapter we will consider the fundamentals of control which are applicable when either physical or chemical methods are used. In the second part, we will describe the physical agents and physical methods for reducing or eliminating the viable microbial population.

Before considering the physical means or chemical agents by which microorganisms are controlled, it is important to understand some of the characteristics of a microbial population when exposed to a destructive agent. We shall use bacteria as an example.

FUNDAMENTALS OF CONTROL

The term death, as used in microbiology, is defined as the irreversible loss of the ability to reproduce. Viable microorganisms are capable of multiplying; dead microorganisms do not multiply (grow). The determination of death requires laboratory techniques that indicate whether growth occurs when the sample is inoculated into a suitable medium. The failure of a microorganism to grow when inoculated into an appropriate medium indicates that the organism is no longer able to reproduce, and the failure to reproduce is the criterion of death. A complicating factor in this definition is that the response of the organism may not be the same in all media. For example, a suspension of *Escherichia coli*

exposed to a heat treatment may yield a greater number of survivors if a plating medium of trypticase soy agar is used rather than a medium containing bile salts such as deoxycholate agar.

The Rate of Death of Bacteria

When one drops a suspension of bacteria into a bottle of hot acid or an incinerator, the bacteria may all be killed so fast that it is not possible to measure the death rate. However, less drastic treatment may result in the cells being killed over a longer period of time, at a constant *exponential* rate that is essentially the inverse of their exponential growth pattern.

Exponential death can be understood easily in terms of a simple model. Imagine that each cell is a *target* and that a large number of *bullets* (i.e., units of a physical or chemical agent) are being sprayed at them in a random manner, as with a machine gun; that is, no one is aiming the gun directly at a target. Common sense dictates some rules about the way bacteria die under these conditions. To begin, we assume that a single hit kills a bacterium.

The probability of hitting a target is proportional to the number of targets, i.e., the number of bacteria, *present.* Intuition tells us that if we shoot randomly at many targets, we have a good chance of hitting one, but as time goes on, the number of targets not yet hit decreases steadily and it becomes harder and harder to hit the remaining ones. (Hitting a target again and again does not count; a bacterium can be killed only once.) Let us take a simple numerical example. Assume that we have an initial population of 1 million targets. We shower them with bullets for 1 min and manage to hit 90 percent, so that there are now 100,000 survivors left. Then we shower them with bullets 1 min more, but since we only have one-tenth as many targets as in the first round, we hit only one-tenth as many. In other words, this time we hit 90,000 of the targets and have 10,000 survivors. We shower these with bullets another minute, and since we have only one-tenth as many as in the last minute, we again hit only one-tenth as many, or 9,000. This pattern repeats itself until there are no targets left, as shown in Table 22-1. But notice that it is just as hard to kill the last nine bacteria as it was to kill the first 900,000. In fact, we can never be sure that we have killed the last one; all that we can do is give the targets enough overkill for there to be a good chance that the last has been hit.

The pattern of death among *Bacillus anthracis* spores exposed to 5% phenol is shown in Fig. 22-1. The number of survivors is plotted against time; however, curve A is an arithmetic plot and curve B is a logarithmic plot. Both curves show that some portion of the population dies during any given unit of time,

Table 22-1. A Theoretical Case of the Order of Death of Bacteria When Exposed to a Lethal Agent

Time	Survivors	Deaths per Unit Time	Total Deaths
0	1,000,000	0	0
1	100,000	900,000 = 90%	900,000
2	10,000	90,000 = 90%	990,000
3	1,000	9,000 = 90%	999,000
4	100	900 = 90%	999,900
5	10	90 = 90%	999,990
6	1	9 = 90%	999,999

SOURCE: O. Rahn, *Physiology of Bacteria*, McGraw-Hill, New York, 1932.

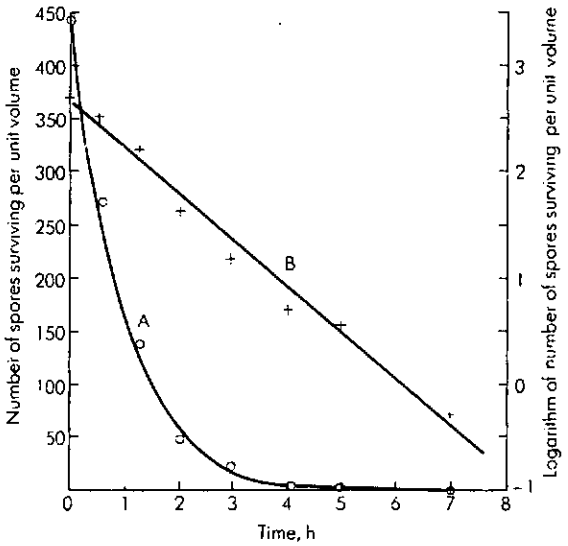


Figure 22-1. The death curve of *Bacillus anthracis* spores exposed to 5% phenol. Curve A: Number of survivors expressed arithmetically per unit volume plotted against time. Curve B: Logarithm of number of surviving bacteria plotted against time.

but what the logarithmic plot also reveals is that *the death rate is constant*. The points fall on a straight line, and the slope of the curve is the measure of the death rate.

Results such as those shown in Fig. 22-1 are obtainable only when all conditions are kept strictly uniform, including the age and the physiological condition of all the microorganisms in the population. If the cells in the microbial population vary in age or physiological stage of growth, they will exhibit differences in susceptibility to the agent. As a consequence the logarithmic plot of survivors will not fall in a straight line. Both the slope of the death curve and its form are affected by the species of microorganism and the homogeneity of the cells in the population.

The probability of hitting a target is also *proportional to the number of bullets shot*, i.e., concentration of the chemical or intensity of the physical agent. Intuition again tells us that the more bullets we shoot in a given time, the faster the targets will be hit. If the targets are bacteria and the bullets are x-rays or ultraviolet light, it stands to reason that the cells will be killed faster as the *intensity* of the radiation increases. If the bullets are molecules of some chemical agent, the cells will be killed more rapidly as the *concentration* of the agent increases (up to a certain limit, of course).

The longer we shoot, the more targets we hit, but the more targets we have, the longer it takes to hit them all. This is an obvious restatement of the exponential death pattern. It simply means that it takes time to kill the population, and if we have many cells, we must treat them for a longer time to be reasonably sure that all of the bacteria are dead.

Conditions Influencing Antimicrobial Action

Microorganisms are not simple physical targets. Many biological characteristics influence the rate at which microorganisms are killed or inactivated by various agents. Many factors must be considered in the application of any physical or

chemical agent used to inhibit or destroy microbial populations. It is not possible to prescribe one agent that will be effective for the control of microorganisms for all materials and all circumstances. Hence it is necessary to evaluate each situation separately in order to select a process that research and experience have shown will accomplish the desired result. Some of the biological characteristics of the cells, as well as environmental conditions which influence the efficacy of antimicrobial agents (physical and chemical), are outlined below. More specific information is presented where the application of a particular antimicrobial agent is described.

Environment

The physical or chemical properties of the medium or substance carrying the organisms, i.e., the environment, has a profound influence on the rate as well as the efficacy of microbial destruction. For example, the effectiveness of heat is much greater in acid than in alkaline material. The consistency of the material (aqueous or viscous) will markedly influence the penetration of the agent, and high concentrations of carbohydrates generally increase the thermal resistance of organisms.

The presence of extraneous organic matter can significantly reduce the efficacy of an antimicrobial agent by inactivating it or protecting the microorganism from it.

An increase in temperature, when used with another agent such as a chemical, hastens the destruction of microorganisms. This phenomenon is illustrated in Fig. 22-2.

Kinds of Microorganisms

Species of microorganisms differ in their susceptibility to physical and chemical agents. In sporeforming species, the growing vegetative cells are much more susceptible than the spore forms; bacterial spores are extremely resistant. In fact, bacterial spores are the most resistant of all living organisms in their capacity to survive under adverse physical and chemical conditions. The relative resistance of bacterial spores in comparison with other microorganisms is shown in Table 22-2.

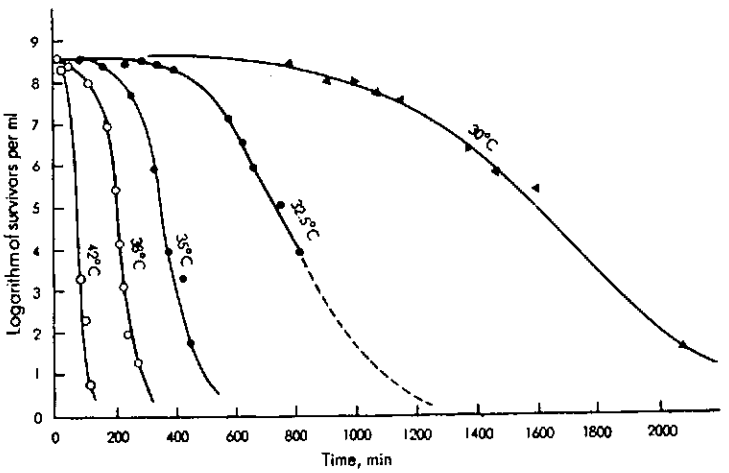


Figure 22-2. Increasing temperature also decreases bacterial survival when the concentration of the disinfectant remains constant. In this experiment *Escherichia coli* was exposed to phenol at a concentration of 4.62 g/liter at temperatures between 30 and 42°C. The number of survivors, expressed logarithmically, is plotted against time. (*From R. C. Jordan and S. E. Jacobs, J Hyg, 44:210, 1945. Courtesy of Cambridge University Press.*)

Table 22-2. Resistances of Bacterial and Mold Spores and of Viruses, Relative to the Resistance of *Escherichia coli* as Unity

Sterilizing Agent	*Escherichia coli*	Bacterial Spores	Mold Spores	Viruses and Bacteriophages
Phenol	1	100,000,000	1–2	30
Formaldehyde	1	250		2
Dry heat	1	1,000	2–10	±1
Moist heat	1	3,000,000	2–10	1–5
Ultraviolet	1	2–5	5–100	5–10

SOURCE: O. Rahn, *Bacteriol Rev*, 9:1, 1945.

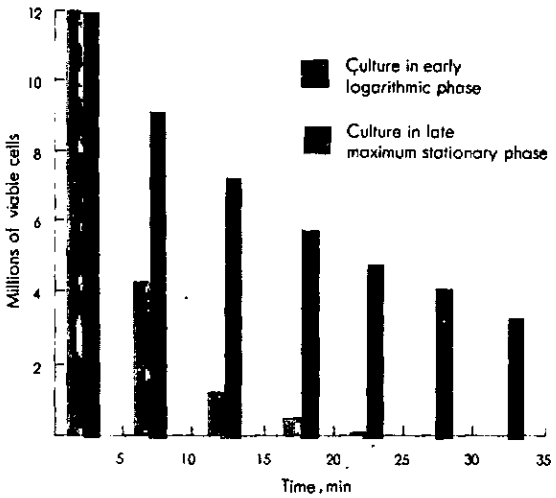


Figure 22-3. Comparative susceptibility of young and old cells to a lethal agent. The young cells are all killed within 25 min, but a considerable part of the more resistant, older cells still survives. (*Courtesy of Martin Frobisher et al., Fundamentals of Microbiology, Saunders, Philadelphia, 1974.*)

Physiological State of Cells

The physiological state of cells may influence susceptibility to an antimicrobial agent. Young, actively metabolizing cells are apt to be more easily destroyed than old, dormant cells in the case of an agent that causes damage through the interference with metabolism; nongrowing cells would not be affected. A comparison of the susceptibility of young and old cells to a lethal agent is shown in Fig. 22-3.

Mode of Action of Antimicrobial Agents

The many processes and substances used as antimicrobial agents manifest their activity in one of several ways. For both academic and practical reasons, it is important to know how microorganisms are inhibited or killed. Knowledge of the mode of action of a particular agent may make it possible to predict the conditions under which it will function most effectively as well as the kinds of microorganisms it will be most effective against. A great deal of research has been performed to determine the specific site of action of various agents. Such investigations are complicated by the fact that when a cell is exposed to a lethal agent, many changes can be observed. A domino effect occurs once the initial inhibitory or lethal process is inflicted. The real problem is to establish the *primary site of damage* responsible for inhibition or death.

In a general way, one may view the possible sites of action of an antimicrobial agent by recalling certain features of the microbial cell. A normal living cell

contains the multitude of enzymes responsible for metabolic processes. A semipermeable membrane (cytoplasmic membrane) maintains the integrity of the cellular contents; the membrane selectively controls the passage of substances between the cell and its external environment and is also the site of some enzyme reactions. The cell wall proper provides a protective covering to the cell in addition to participating in certain physiological processes. Damage at any of these areas may initiate a number of subsequent changes leading to the death of the cell. It is necessary to bear in mind that there are many sites of damage to the cell and that the damage may be caused by one or more of a variety of agents.

The manner in which antimicrobial agents inhibit or kill can be attributed to the following kinds of actions:

Damage to the cell wall or inhibition of cell-wall synthesis
Alteration of the permeability of the cytoplasmic membrane
Alteration of the physical or chemical state of proteins and nucleic acids
Inhibition of enzyme action
Inhibition of protein or nucleic acid synthesis

Examples of these modes of action will be described as we discuss specific physical agents in this chapter and chemical agents in Chaps. 23 and 24.

PHYSICAL AGENTS

The major physical agents or processes used for the control of microorganisms are temperature (high and low), desiccation, osmotic pressure, radiation, and filtration.

High Temperatures

Microorganisms can grow over a wide range of temperatures, from very low temperatures characteristic of psychrophiles to the very high growth temperatures characteristic of thermophiles. Every type has an optimum, minimum, and maximum growth temperature. Temperatures above the maximum generally kill, while those below the minimum usually produce stasis (inhibition of metabolism) and may even be considered preservative. The amount of water present in the environment at any temperature has a significant effect upon microorganisms in terms of their survival.

High temperatures combined with high moisture is one of the most effective methods of killing microorganisms. It is important to distinguish between dry heat and moist heat in any procedure for microbial control. Moist heat kills microorganisms by coagulating their proteins and is much more rapid and effective than dry heat, which destroys microorganisms by oxidizing their chemical constituents. Two examples will illustrate the difference. Spores of *Clostridium botulinum* are killed in 4 to 20 min by moist heat at 120°C, whereas a 2-h exposure to dry heat at the same temperature is required. Spores of *B. anthracis* are destroyed in 2 to 15 min by moist heat at 100°C, but with dry heat 1 to 2 h at 150°C is required to achieve the same result (Tables 22-3 and 22-4).

Vegetative cells are much more sensitive to heat than are spores; the higher level of "water activity" in the vegetative cells accounts for this. Cells of most bacteria are killed in 5 to 10 min at 60 to 70°C (moist heat). Vegetative cells of yeasts and other fungi are usually killed in 5 to 10 min by moist heat at 50 to

Table 22-3. Some Quoted Destruction Times of Bacterial Spores by Moist Heat

Organism	Destruction times, min							
	At 100°C	At 105°C	At 110°C	At 115°C	At 120°C	At 125°C	At 130°C	At 134°C
Bacillus anthracis	2–15	5–10						
B. subtilis	Many hours			40				
A putrefactive anaerobe	780	170	41	15	5.6			
Clostridium tetani	5–90	5–25						
Cl. welchii	5–45	5–27	10–15	4	1			
Cl. botulinum	300–530	40–120	32–90	10–40	4–20			
Soil bacteria	Many hours	420	120	15	6–30	4		1.5–10
Thermophilic bacteria		400	100–300	40–110	11–35	3.9–8.0	3.5	1
Cl. sporogenes	150	45	12					

SOURCE: G. Sykes, *Distinfection and Sterilization*, 2d ed., Lippincott, Philadelphia, 1965.

Table 22-4. Some Quoted Killing Times of Bacterial Spores by Dry Heat

Organism	Destruction times, min						
	At 120°C	At 130°C	At 140°C	At 150°C	At 160°C	At 170°C	At 180°C
Bacillus anthracis			Up to 180	60–120	9–90		3
Clostridium botulinum	120	60	15–60	25	20–25	10–15	5–10
Cl. welchii	50	15–35	5				
Cl. tetani		20–40	5–15	30	12	5	1
Soil spores				180	30–90	15–60	15

SOURCE: G. Sykes, *Disinfection and Sterilization*, 2d ed., Lippincott, Philadelphia, 1965.

60°C; their spores are killed in the same time but at temperatures of 70 to 80°C. Bacterial spores are much more resistant to high temperatures as shown in Tables 22-3 and 22-4. The susceptibility of viruses to heat is generally similar to that of mesophilic vegetative bacterial cells.

Thermal Death Time and Decimal Reduction Time

Thermal death time refers to the shortest period of time to kill a suspension of bacteria (or spores) at a prescribed temperature and under specific conditions. Another unit of measurement of the destruction of microorganisms by heat is the **decimal reduction time.** This is the time in minutes to reduce the population by 90 percent, or stated differently, it is the time in minutes for the thermal-death-time curve to pass through one log cycle (see Fig. 22-4). Figure 22-5 illustrates a thermal-death-time curve for spores of bacterial species responsible for a type of canned-food spoilage, e.g., flat sour spoilage.

From the definition of these terms, it is clear that they express a time-temperature relationship to killing. In thermal death time, the temperature is selected as the fixed point and the time varied. Decimal reduction time is a modification of thermal death time which measures a 90 percent rather than 100 percent kill rate. In the experimental determination of these values, it is an absolute requirement that the conditions be rigidly controlled. Attention must

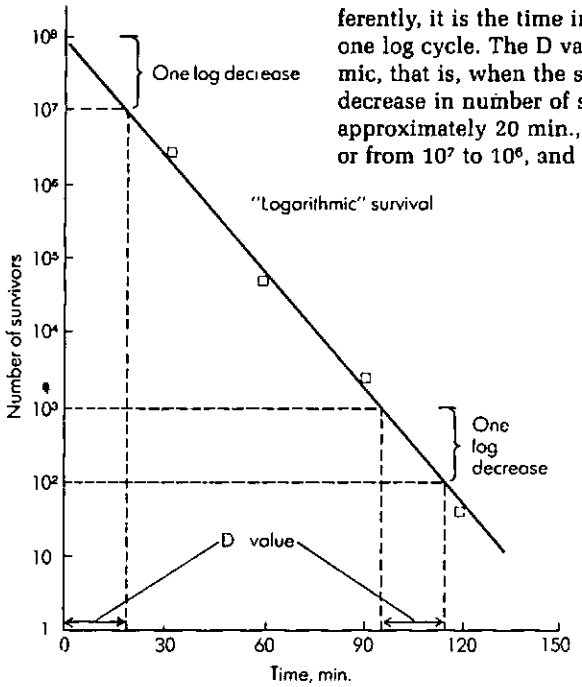


Figure 22-4. Graph illustrating the concept of decimal reduction time (D value), the time in minutes to reduce the microbial population by 90 percent. Stated differently, it is the time in minutes for the thermal-death-time curve to pass through one log cycle. The D value is independent of time when the response is logarithmic, that is, when the same length of time is required to accomplish any given log decrease in number of survivors. For example, the D value in this illustration is approximately 20 min., the time required to reduce the survivors from 10^8 to 10^7, or from 10^7 to 10^6, and so on.

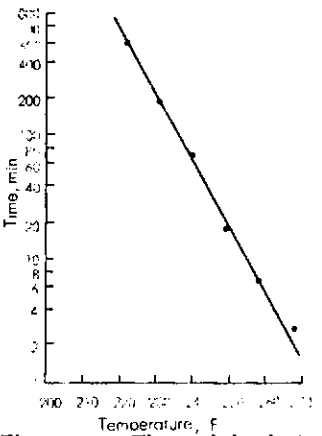


Figure 22-5. Thermal-death-time curve for spores of a type of bacterium encountered in food spoilage. (*Courtesy of W. C. Frazier, Food Microbiology, McGraw-Hill, New York, 1958.*)

be given to the nature of the medium, pH, and the number of organisms, since all these factors have a bearing on the susceptibility of the microorganisms to heat.

Thermal-death-time data and decimal-reduction-time data are extremely important in many applications of microbiology. The canning industry, for example, carries out extensive studies on this subject to establish satisfactory processing temperatures for the preservation of canned foods.

Application of High Temperatures for Destruction of Microorganisms

The killing action of heat is, as we have seen, a time-temperature relationship affected by numerous conditions that must be taken into consideration in selecting the time and temperature required to reduce the microbial population to the desired level. Practical procedures by which heat is employed are conveniently divided into two categories: moist heat and dry heat.

Moist Heat. The application of moist heat for inhibiting or destroying microorganisms is discussed by the method used to obtain the desired result.

Steam Under Pressure. Heat in the form of saturated steam under pressure is the most practical and dependable agent for sterilization. Steam under pressure provides temperatures above those obtainable by boiling, as shown in Table 22-5. In addition, it has the advantages of rapid heating, penetration, and moisture in abundance, which facilitates the coagulation of proteins.

The laboratory apparatus designed to use steam under regulated pressure is

Table 22-5. Temperature of Steam under Pressure

Steam Pressure, lb/in²	Temperature, °C
0	100.0
5	109.0
10	115.0
15	121.5
20	126.5

SOURCE: J. J. Perkins, *Principles and Methods of Sterilization*, Charles C. Thomas, Springfield, Ill., 1956.

called an autoclave (see Fig. 22-6). It is essentially a double-jacketed steam chamber equipped with devices which permit the chamber to be filled with saturated steam and maintained at a designated temperature and pressure for any period of time. In the operation of an autoclave it is absolutely essential that the air in the chamber be completely replaced by saturated steam. If air is present, it will reduce the temperature obtained within the chamber substantially below that which would be realized if pure saturated steam were under the same pressure. It is not the pressure that kills the organisms but the temperature of the steam.

The autoclave is an essential unit of equipment in every microbiology laboratory. Many media, solutions, discarded cultures, and contaminated materials are routinely sterilized with this apparatus. Generally, but not always, the autoclave is operated at a pressure of approximately 15 lb/in² (at 121°C). The time of operation to achieve sterility depends on the nature of the material being sterilized, the type of the container, and the volume. For example, 1000 test tubes containing 10 ml each of a liquid medium can be sterilized in 10 to 15 min at 121°C; 10 liters of the same medium contained in a single container would require 1 h or more at the same temperature to ensure sterilization.

Fractional Sterilization. Some microbiological media, solutions of chemicals, and biological materials cannot be heated above 100°C without being damaged. If, however, they can withstand the temperature of free-flowing steam (100°C), it is possible to sterilize them by fractional sterilization (tyndallization). This method involves heating the material at 100°C on three successive days with incubation periods in between. Resistant spores germinate during the incubation

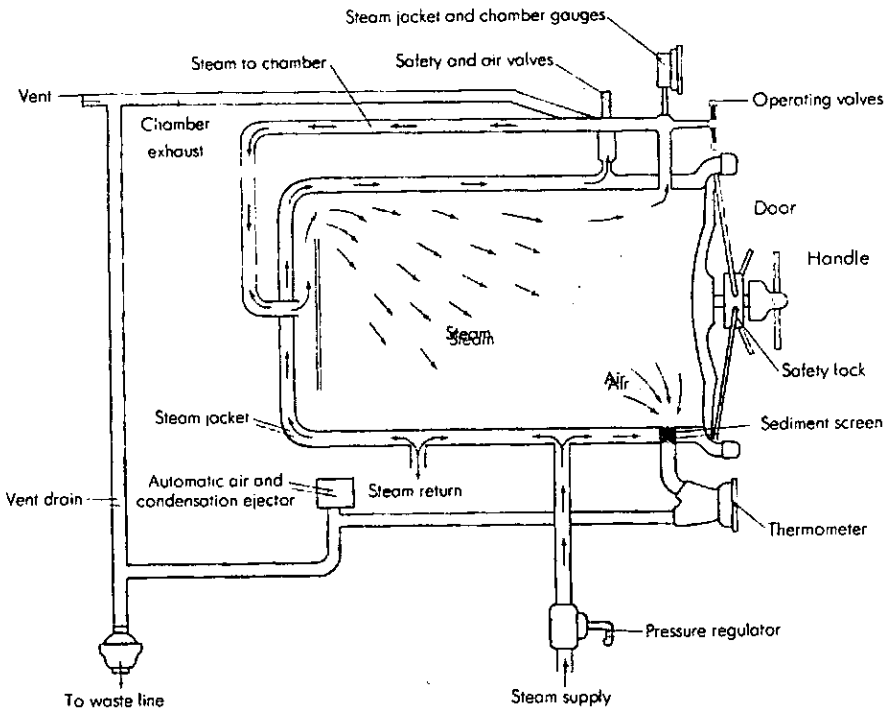


Figure 22-6. Pressure steam sterilizer (autoclave), cross-sectional view illustrating operational parts and path of steam flow. (*Courtesy of Wilmont Castle Company.*)

periods; on subsequent exposure to heat, the vegetative cells will be destroyed. If spores are present and do not germinate during the incubation periods, the material will not be sterilized. An apparatus known as the **Steam Arnold** is used for this technique; however, it is also possible to operate an autoclave with free-flowing steam for this purpose.

Boiling Water. Contaminated materials or objects exposed to boiling water cannot be sterilized with certainty. It is true that all vegetative cells will be destroyed within minutes by exposure to boiling water, but some bacterial spores can withstand this condition for many hours. The practice of exposing instruments for short periods of time in boiling water is more likely to bring about disinfection (destruction of vegetative cells of disease-producing microorganisms) rather than sterilization. Boiling water cannot be (and is not) used in the laboratory as a method of sterilization.

Pasteurization. Milk, cream, and certain alcoholic beverages (beer and wine) are subjected to a controlled heat treatment (called **pasteurization**) which kills microorganisms of certain types but does not destroy all organisms. Pasteurized milk is not sterile milk. The pasteurization of milk is discussed in Chap. 28.

Dry-Heat: *Hot-Air Sterilization.* Dry-heat, or hot-air, sterilization is recommended where it is either undesirable or unlikely that steam under pressure will make direct and complete contact with the materials to be sterilized. This is true of certain items of laboratory glassware, such as Petri dishes and pipettes, as well as oils, powders, and similar substances. The apparatus employed for this type of sterilization may be a special electric or gas oven or even the kitchen stove oven. For laboratory glassware, a 2-h exposure to a temperature of 160°C is sufficient for sterilization.

Incineration. Destruction of microorganisms by burning is practiced routinely in the laboratory when the transfer needle is introduced into the flame of the Bunsen burner. A note of caution should be added here. When the transfer needle is sterilized, care should be exercised to prevent *spattering*, since the droplets which fly off are likely to carry viable organisms. The danger from spattering can be greatly reduced or eliminated by using a Bunsen burner or an electric heat coil equipped with a tube into which the transfer needle can be inserted (see Fig. 22-7).

Incineration is used for the destruction of carcasses, infected laboratory animals, and other infected materials to be disposed of. Special precautions need to be taken to ensure that the exhaust fumes do not carry particulate matter containing viable microorganisms into the atmosphere.

Low Temperatures

Temperatures below the optimum for growth depress the rate of metabolism, and if the temperature is sufficiently low, growth and metabolism cease. Low temperatures are useful for preservation of cultures, since microorganisms have a unique capacity for surviving extreme cold. Agar-slant cultures of some bacteria, yeasts, and molds are customarily stored for long periods of time at refrigeration temperatures of about 4 to 7°C. Many bacteria and viruses can be maintained in a deep-freeze unit at temperatures from −20 to −70°C. Liquid nitrogen, at a temperature of −196°C, is used for preserving cultures of many viruses and microorganisms, as well as stocks of mammalian tissue cells used

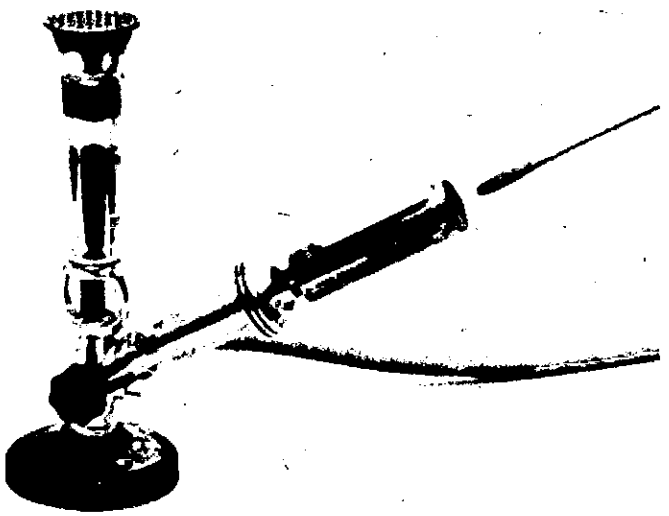


Figure 22-7. When a transfer needle is placed in a flame, spattering may occur with resultant spread of living organisms. To prevent this, one can use a Bunsen burner which is modified so that the transfer needle is exposed to a flame within a tubular space, as shown here.

in animal virology and many other types of research. In all these procedures, the initial freezing kills a fraction of the population, but the survivors may remain viable for long periods. (See Chap. 8.)

From these facts it is immediately apparent that low temperatures, however extreme, cannot be depended upon for disinfection or sterilization. Microorganisms maintained at freezing or subfreezing temperatures may be considered dormant; they perform no detectable metabolic activity. This static condition is the basis of successful application of low temperatures for the preservation of foods. Thus from a practical standpoint, high temperatures may be considered as microbicidal and low temperatures (freezing or lower) as microbistatic.

Desiccation

Desiccation of the microbial cell causes a cessation of metabolic activity, followed by a decline in the total viable population. In general, the time of survival of microorganisms after desiccation varies, depending on the following factors:

1 The kind of microorganism
2 The material in or on which the organisms are dried
3 The completeness of the drying process
4 The physical conditions to which the dried organisms are exposed, e.g., light, temperature, and humidity

Species of Gram-negative cocci such as gonococci and meningococci are very sensitive to desiccation; they die in a matter of hours. Streptococci are much more resistant; some survive weeks after being dried. The tubercle bacillus (*Mycobacterium tuberculosis*) dried in sputum remains viable for even longer periods of time. Dried spores of microorganisms are known to remain viable indefinitely.

In the process of lyophilization, organisms are subjected to extreme dehydra-

tion in the frozen state and then sealed in a vacuum. In this condition, desiccated (lyophilized) cultures of microorganisms remain viable for many years.

Osmotic Pressure

When two solutions with differing concentrations of solute are separated by a semipermeable membrane, there will occur a passage of water, through the membrane, in the direction of the higher concentration. The trend is toward equalizing the concentration of solute on both sides of a membrane. The solute concentration within microbial cells is approximately 0.95 percent. Thus if cells are exposed to solutions with higher solute concentration, water will be drawn out of the cell. The process is called **plasmolysis**. The reverse process, that is, passage of water from a low solute concentration into the cell, is termed **plasmoptysis**. The pressure built up within the cell as a result of this water intake is termed **osmotic pressure**. These phenomena can be observed more conveniently with animal cells since they do not have rigid cell walls. Plasmolysis results in dehydration of the cell, and as a consequence metabolic processes are retarded partially or completely. The antimicrobial effect is similar to that caused by desiccation. Because of the great rigidity of microbial cell walls (except for protozoa), the cell-wall structure does not exhibit distortions as a result of plasmolysis or plasmoptysis. However, changes in the cytoplasmic membrane, and particularly shrinkage of the protoplast from the cell wall, can be observed during plasmolysis.

Radiation

Energy transmitted through space in a variety of forms is generally called **radiation**. For our purposes, the most significant type of radiation is probably **electromagnetic radiation**, of which light and x-rays are examples. Electromagnetic radiation has the dual properties of a *continuous* wave phenomenon and a *discontinuous* particle phenomenon; the particles are packets, or **quanta**, of energy, sometimes called **photons**, which vibrate at different frequencies. Radiation of a given frequency can also be described by its wavelength, λ; it is measured in angstroms, where 10,000 Å = 1 μm, and the energy of the radiation in electron volts (ev) is given by 12,350/λ. The various parts of the electromagnetic spectrum, distinguished by their wavelengths, are shown in Fig. 22-8.

Electromagnetic radiation can interact with matter in one of two general ways.

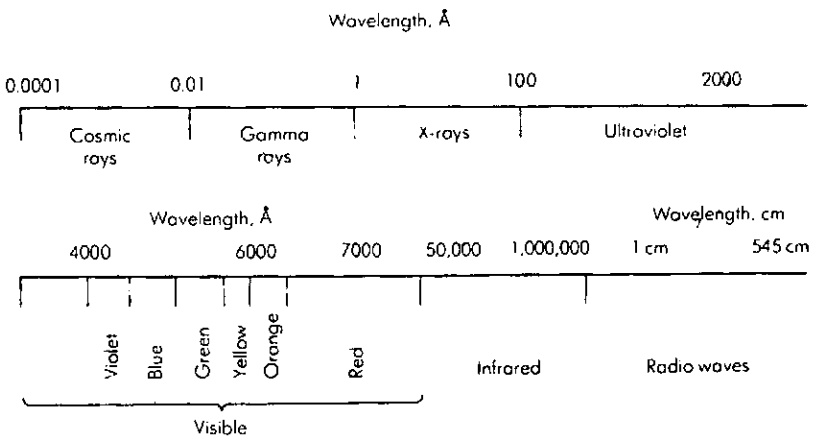


Figure 22-8. Spectrum of radiant energy. (1 Å = 0.1 nm.)

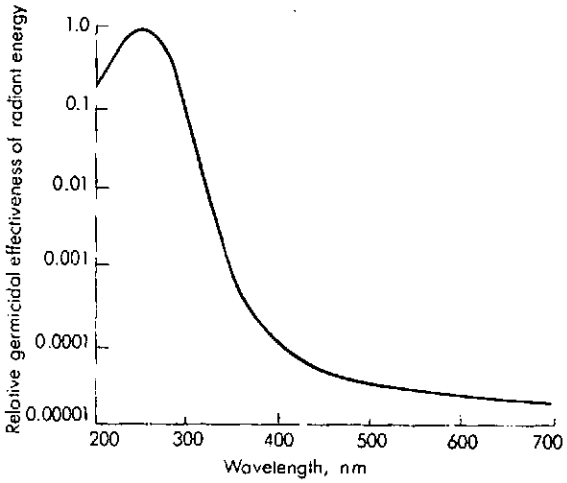


Figure 22-9. Relative germicidal effectiveness of radiant energy between 2000 and 7000 Å. *(Courtesy of General Electric Company, Lamp Division, Publication LD-11.)*

Gamma rays and x-rays, which have energies of more than about 10 eV, are called **ionizing radiations** because they have enough energy to knock electrons away from molecules and ionize them. When such radiations pass through cells, they create free hydrogen radicals, hydroxyl radicals, and some peroxides, which in turn can cause different kinds of intracellular damage. Moreover, since this damage is produced in a variety of materials, ionizing radiations are rather nonspecific in their effects. Less energetic radiation, particularly ultraviolet light, does not ionize; it is **absorbed** quite specifically by different compounds because it excites electrons and raises them to higher energy levels, thus creating different chemical species that can engage in a variety of chemical reactions not possible for unexcited molecules.

In addition to electromagnetic radiation, organisms may be subjected to acoustic radiation (sound waves) and to subatomic particles, such as those released in radioactive decay. The atomic era has alerted us to the damaging potential of radiation. Consequently, a tremendous expenditure of research effort is being directed toward determining the minimum dosage which affects cells, how radiations damage cells, and how the damage can be prevented. Microorganisms have been used for the major part of this research for the same reasons they are used in so many other areas of basic biological research; they are easy to grow and lend themselves to rapid, efficient experimentation.

Besides the fundamental research in radiation microbiology, there have been many developments in the application of ionizing radiation to sterilize biological materials. This method is called **cold sterilization** because ionizing radiations produce relatively little heat in the material being irradiated. Thus it is possible to sterilize heat-sensitive substances, and such techniques are being developed in the food and pharmaceutical industries.

Ultraviolet Light

The ultraviolet portion of the spectrum (Fig. 22-8) includes all radiations from 150 to 3900 Å. Wavelengths around 2650 Å have the highest bactericidal efficiency (see Fig. 22-9). Although the radiant energy of sunlight is partly com-

posed of ultraviolet light, most of the shorter wavelengths of this type are filtered out by the earth's atmosphere (ozone, clouds, and smoke). Consequently, the ultraviolet radiation at the surface of the earth is restricted to the span from about 2670 to 3900 Å. From this we may conclude that sunlight, under certain conditions, has microbicidal capacity, but to a limited degree.

Many lamps are available which emit a high concentration of ultraviolet light in the most effective region, 2600 to 2700 Å. Germicidal lamps, which emit ultraviolet radiations, are widely used to reduce microbial populations. For example, they are used extensively in hospital operating rooms, in aseptic filling rooms, in the pharmaceutical industry, where sterile products are being dispensed into vials or ampules, and in the food and dairy industries for treatment of contaminated surfaces.

An important practical consideration in using this means of destroying microorganisms is that ultraviolet light has very little ability to penetrate matter. Even a thin layer of glass filters off a large percentage of the light. Thus, only the microorganisms on the surface of an object where they are exposed directly to the ultraviolet light are susceptible to destruction.

Mode of Action. Ultraviolet light is absorbed by many cellular materials but most significantly by the nucleic acids, where it does the most damage. The absorption and subsequent reactions are predominantly in the pyrimidines of the nucleic acid. One important alteration is the formation of a *pyrimidine dimer* in which two adjacent pyrimidines become bonded. Unless dimers are removed by specific intracellular enzymes, DNA replication can be inhibited and mutations can result. (See Chap. 12.)

X-Rays (Roentgen Rays)

X-rays are lethal to microorganisms and higher forms of life (see Table 22-6). Unlike ultraviolet radiations, they have considerable energy and penetration ability. However, they are impractical for purposes of controlling microbial populations because (1) they are very expensive to produce in quantity and (2) they are difficult to utilize efficiently, since radiations are given off in all directions from their point of origin. However, x-rays have been widely employed experimentally to produce microbial mutants, as mentioned in Chap. 12.

Gamma Rays

Gamma radiations are high-energy radiations emitted from certain radioactive isotopes such as ^{60}Co. As a result of the major research programs with atomic energy, large quantities of radioisotopes have become available as by-products of atomic fission. These isotopes are potential sources of gamma radiations. Gamma rays are similar to x-rays but are of shorter wavelength and higher energy. They are capable of great penetration into matter, and they are lethal to all life, including microorganisms.

Because of their great penetrating power and their microbicidal effect, gamma rays are attractive for use in commercial sterilization of materials of considerable thickness or volume, e.g., packaged foods and medical devices. However, certain technical problems must be resolved for practical applications, e.g., development of radiation sources for large-scale use and the design of equipment to eliminate any possible hazards to the operators.

Results of quantitative studies on the effect of ionizing radiations on the cells

Table 22-6. Median Lethal Dose of X-Rays for Various Species of Organisms

Organism		Median Lethal Dose, rd*
Viruses:	Tobacco mosaic	200,000
	Rabbit papilloma	100,000
Bacteria:	*Escherichia coli*	5,000
	Bacillus mesentericus	130,000
Algae:	*Mesotenium*	8,500
	Pandorina	4,000
Protozoa:	*Colpidium*	330,000
	Paramecium	300,000
Vertebrates:	Goldfish	750
	Mouse	450
	Rabbit	800
	Rat	600
	Monkey	450
	Humans (?)	400

* A rad (radiation absorbed dose), abbreviated rd, is the dose which delivers 100 ergs/g of irradiated material; it is equal to 6×10^{13} eV.

SOURCE: Modified from E. Paterson, in R. Paterson (ed.), *The Treatment of Malignant Disease by Radium and X Rays*, E. Arnold, London, 1948; *McGraw-Hill Encyclopedia of Science and Technology*, vol. 11, p. 244, McGraw-Hill, New York, 1971.

have resulted in the establishment of the "target" theory of action. This implies that the radiant-energy particle makes a "direct hit" on some essential substance such as DNA within the bacterial cell, causing ionization which results in the death of the cell.

Cathode Rays (Electron-Beam Radiation)

When a high-voltage potential is established between a cathode and an anode in an evacuated tube, the cathode emits beams of electrons, called cathode rays or electron beams. Special types of equipment have been designed which produce electrons of very high intensities (millions of volts), and these electrons are accelerated to extremely high velocities. These intense beams of accelerated electrons are microbicidal as well as having other effects on biological and nonbiological materials.

The electron accelerator, a type of equipment which produces the high-voltage electron beam, is used today for the sterilization of surgical supplies, drugs, and other materials. One of the unique features of the process is that the material can be sterilized after it has been packaged (the radiations penetrate the wrappings) and at room temperature. Electron-beam radiation has limited power of penetration; but within its limits of penetration, sterilization is accomplished on very brief exposure.

The susceptibility of microorganisms to doses of different radiations is shown in Table 22-7.

Surface Tension and Interfacial Tension

The interface, or boundary, between a liquid and a gas is characterized by unbalanced forces of attraction between the molecules in the surface of the liquid and in the interior. A molecule at the surface of the liquid-air interface is pulled strongly toward the interior of the liquid beneath it, whereas

Table 22-7. Lethal Doses of Different Radiations

Type of Organism	Lethal Doses, mrd			
	Cathode Rays from van de Graaff Accelerators*	From Capacitron Pulsed Beam†	Gamma Rays from ^{60}Co	X-rays from 3-MeV Source
Vegetative:				
Nonpathogenic	0.1–0.25			
Pathogenic	0.45–0.55	0.1–0.25	0.15–0.25	0.03–0.5
Bacterial spores	0.5–2.1	0.2–0.4	1.5‡	0.5–2.0
Molds	0.25–1.15	0.35–0.4	0.2–0.3	0.25–1.0
Yeasts	0.5–1.0		0.3	0.25–1.5

* Various authors.
† Huber and colleagues, quoted by Hannan, 1955.
‡ Approximate.
SOURCE: G. Sykes, "Methods and Equipment for Sterilization of Laboratory Apparatus and Media," in J. R. Norris and D. W. Ribbons (eds.), *Methods in Microbiology*, vol. 1, Academic, New York, 1969.

molecules in the interior of the liquid are attracted uniformly in all directions. This behavior of molecular forces at the liquid-air interface imparts a distinctive characteristic to the surface of a liquid, known assurface tension. Surface forces also exist between two immiscible liquids and at the interface between a solid and a liquid. Here they are referred to asinterfacial tension. Changes in surface tension may alter the permeability charcteristics of the cytoplasmic membrane, causing leakage of cellular substances, which results in damage to the cell.

Filtration

Bacteriological Filters

For many years a variety of filters have been available to the microbiologist which can remove microorganisms from liquids or gases. These filters are made of different materials—an asbestos pad in the Seitz filter, diatomaceous earth in the Berkefeld filter, porcelain in the Chamberland-Pasteur filter, and sintered glass disks in other filters.

The mean pore diameter in these bacteriological filters ranges from approximately one to several micrometers; most filters are available in several grades, based on the average size of the pores. However, it should be understood that these filters do not act as mere mechanical sieves; porosity alone is not the only factor preventing the passage of organisms. Other factors, such as the electric charge of the filter, the electric charge carried by the organisms, and the nature of the fluid being filtered, can influence the efficiency of filtration.

In recent years a new type of filter termed the **membrane** or **molecular filter** has been developed whose pores are of a uniform and specific predetermined size. Membrane or molecular filters are composed of biologically inert cellulose esters. They are prepared as circular membranes of about 150-μm thickness and contain millions of microscopic pores of very uniform diameter (see Fig. 22-10). Filters of this type can be produced with known porosities ranging from approximately 0.01 to 10 μm. Membrane filters are used extensively in the laboratory and in industry to sterilize fluid materials. They have been adapted to microbiological procedures for the identification and enumeration of microorganisms from water samples and other materials (see Chap. 26.)

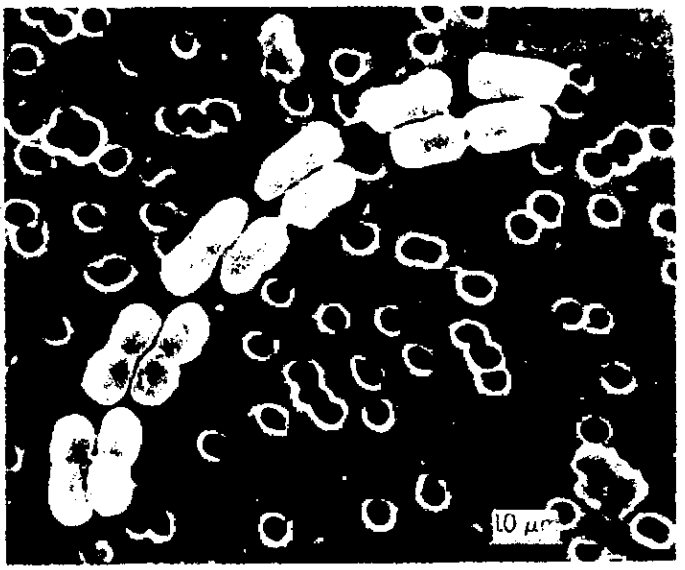


Figure 22-10. Bacteria from a marine water sample are retained by this membrane filter. (*Courtesy of Pall Corporation.*)

It is customary to force the fluid through the filter by applying a negative pressure to the filter flask by use of a vacuum or water pump or to impose a positive pressure above the fluid in the filter chamber, thus forcing it through. Upon completion of filtration, precautions must be taken to prevent contamination of the filtered material when it is transferred to other containers.

The development of **high-efficiency particulate air (HEPA) filters** has made it possible to deliver clean air to an enclosure such as a cubicle or a room. This type of air filtration together with a system of **laminar airflow** is now used extensively to produce dust- and bacteria-free air. (See. Fig. 22-11.)

A summary of the application of physical agents for the control of microorganisms is provided in Table 22-8.

Table 22-8. Application of Physical Agents for Controlling Microorganisms

Method	Recommended Uses	Limitations
Moist heat		
Autoclave	Sterilizing instruments, linens, utensils, and treatment trays, media and other liquids	Ineffective against organisms in materials impervious to steam; cannot be used for heat-sensitive articles
Free-flowing steam or boiling water	Destruction of nonsporeforming pathogens; sanitizes bedding, clothing, and dishes	Cannot be guaranteed to produce sterilization on one exposure
Dry heat		
Hot-air oven	Sterilizing materials impermeable to or damaged by moisture, e.g., oils, glass, sharp instruments, metals	Destructive to materials which cannot withstand high temperatures for long periods

Table 22.8. *(continued)*

Method	Recommended Uses	Limitations
Incineration	Disposal of contaminated objects that cannot be reused.	Size of incinerator must be adequate to burn largest load promptly and completely; potential of air pollution
Radiation		
Ultraviolet light	Control of airborne infection; disinfection of surfaces	Must be absorbed to be effective (does not pass through transparent glass or opaque objects); irritating to eyes and skin; low penetration
X-ray, gamma, and cathode radiation	Sterilization of heat-sensitive surgical materials and other medical devices	Expensive and requires special facilities for use
Filtration		
Membrane filters	Sterilization of heat-sensitive biological fluids	Fluid must be relatiely free of suspended particulate matter
Fiberglass filters (HEPA)	Air disinfection	Expensive
Physical cleaning		
Ultrasonics	Effective in decontaminating delicate cleaning instruments	Not effective alone, but as adjunct procedure enhances effectiveness of other methods
Washing	Hands, skin, objects	Sanitizes; reduces microbial flora

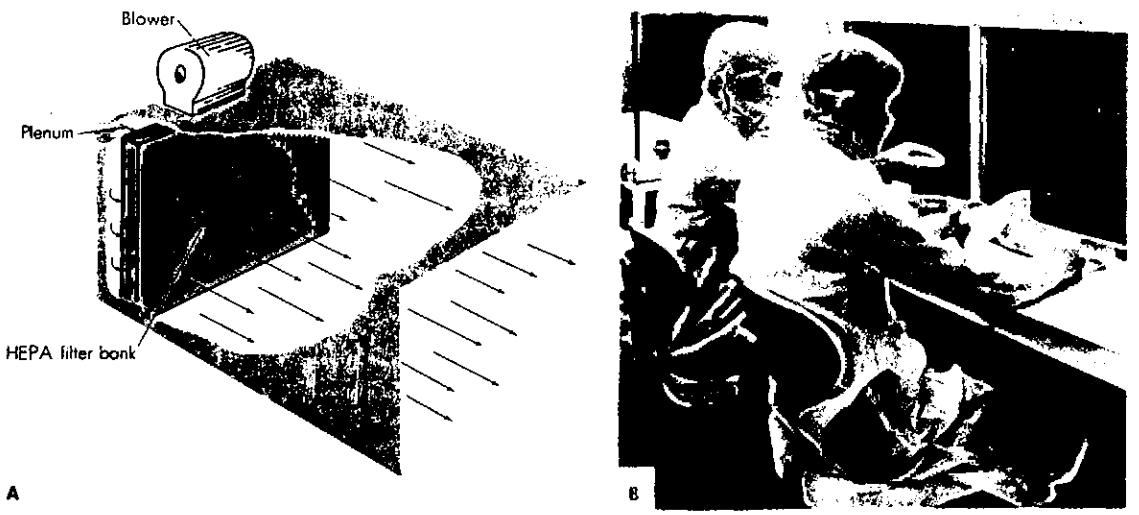


Figure 22-11. Laminar-airflow system. (A) Schematic drawing of horizontal laminar-flow tunnel. Arrows in tunnel denote parallel flow of air through a room. (*Redrawn from M. S. Favero, "Industrial Applications of Laminar Air-flow," in Developments in Industrial Microbiology, American Institute of Biological Sciences, Washington, 1970, vol. 11.*) (B) Laboratory personnel performing sterility test in laminar-airflow unit. (*Courtesy of B. Phillips, Becton, Dickinson & Company.*)

QUESTIONS

1 Describe the death-rate pattern of bacteria when exposed to a lethal agent.
2 Enumerate the conditions which affect the death of microorganisms when they are exposed to an antimicrobial agent.
3 What are the terms by which one can express, quantitatively, the resistance of microorganisms to high temperatures? Distinguish between the meaning of each of these terms.
4 Compare vegetative cells of bacteria with bacterial spores in terms of resistance to heat. What is thought to account for the difference?
5 Describe the process of fractional sterilization, or tyndallization.
6 List several physical agents (or processes) that produce a microbistatic condition.
7 How are microorganisms affected by subzero temperatures?
8 The mechanism of antimicrobial action caused by desiccation is similar to that caused by plasmolysis. Explain why.
9 List several different kinds of radiations that are destructive to microorganisms. Comment on the practical application of each.
10 What is a membrane or molecular filter? How does it differ from older types of bacteriological filters in terms of how it removes microorganisms?
11 What method of sterilization would be appropriate for each of the following?
   (a) Petri dishes for laboratory use.
   (b) Water
   (c) Usual laboratory media, e.g., nutrient agar
   (d) A dry powder product
   (e) A heat-labile solution of vitamins
   (f) A heat-labile antibiotic solution
   (g) Contaminated hospital linens

REFERENCES

See Chap. 23.

Chapter 23 Control by Chemical Agents

OUTLINE

A large number of chemical compounds have the ability to inhibit the growth and metabolism of microorganisms or to kill them. Commercial products which incorporate these compounds are available for controlling microbial populations in many different circumstances. For example, solutions of some chemical compounds are used to reduce the microbial flora of the oral cavity; other chemical compounds are recommended for reducing the microbial population in the dust of hospital floors. No single chemical agent is best for any and all purposes. This is not surprising in view of the variety of conditions under which they are used, their differences in mode of action, and the differences of resistance among microbial species. Experience and research have shown that certain kinds of chemicals are more appropriate and effective for certain situations. As a result, several classes of chemical substances have been identified and new compounds developed within these classes which have destructive properties in terms of their suitability for practical application.

In this chapter we will identify and characterize the major classes of chemical compounds used for the practical control of microorganisms. It is important to bear in mind that the efficacy of an antimicrobial agent is influenced by many factors, as described in Chap. 22.

CHARACTERISTICS OF AN IDEAL ANTIMICROBIAL CHEMICAL AGENT

As already stated, there is not a single chemical agent which is best for the control of microorganisms for any and all purposes. If there were an ideal general-purpose chemical antimicrobial agent, it would have to possess an

extremely elaborate array of characteristics. It is unlikely that such a compound will be found. Nevertheless, the specifications for such an ideal compound, as they are described below, can be aimed for in the preparation of new compounds. They should also be taken into consideration in the evaluation of new chemical agents proposed for practical use.

1 ***Antimicrobial activity.*** The capacity of the substance to kill or inhibit microorganisms is the first requirement. The chemical, at a low concentration, should have a broad spectrum of antimicrobial activity.
2 ***Solubility.*** The substance must be soluble in water or other solvents to the extent necessary for effective use.
3 ***Stability.*** Changes in the substance upon standing should be minimal and should not result in significant loss of germicidal action.
4 ***Nontoxicity to humans and other animals.*** Ideally, the compound should be lethal to microorganisms and noninjurious to humans and other animals.
5 ***Homogeneity.*** The preparation must be uniform in composition so that active ingredients are present in each application. Pure chemicals are uniform, but mixtures of materials may lack homogeneity.
6 ***Noncombination with extraneous organic material.*** Many disinfectants have an affinity for proteins or other organic material. When such disinfectants are used in situations where there is considerable organic material besides that of the microbial cells, little, if any, of the disinfectant will be available for action against the microorganisms.
7 ***Toxicity to microorganisms at room or body temperatures.*** In using the compound, it should not be necessary to raise the temperature beyond that normally found in the environment where it is to be used.
8 ***Capacity to penetrate.*** Unless the substance can penetrate through surfaces, its germicidal action is limited solely to the site of application. Sometimes, of course, surface action is all that is required.
9 ***Noncorroding and nonstaining.*** It should not rust or otherwise disfigure metals nor stain or damage fabrics.
10 ***Deodorizing ability.*** Deodorizing while disinfecting is a desirable attribute. Ideally the disinfectant itself should either be odorless or have a pleasant smell.
11 ***Detergent capacities.*** A disinfectant which is also a detergent (cleaning agent) accomplishes two objectives, and the cleansing action improves the effectiveness of the disinfectant.
12 ***Availability.*** The compound must be available in large quantities at a reasonable price.

DEFINITION OF TERMS

The following terms are used to describe the processes and chemical agents employed in controlling microorganisms.

Sterilization. The process of destroying all forms of microbial life. A *sterile* object, in the microbiological sense, is free of living microorganisms. The terms *sterile*, *sterilize*, and *sterilization* therefore refer to the complete absence or destruction of all microorganisms and should not be used in a relative sense. An object or substance is sterile or nonsterile; it can never be semisterile or almost sterile.

Disinfectant. An agent, usually a chemical, that kills the growing forms but not necessarily the resistant spore forms of disease-producing microorganisms. The term is commonly applied to substances used on inanimate objects. Disinfection is the process of destroying infectious agents.

Antiseptic. A substance that opposes sepsis, i.e., prevents the growth or action of microorganisms either by destroying microorganisms or by inhibiting their growth and metabolism. Usually associated with substances applied to the body.

Sanitizer. An agent that reduces the microbial population to safe levels as judged by public health requirements. Usually it is a chemical agent that kills 99.9 percent of the growing bacteria. Sanitizers are commonly applied to inanimate objects and are generally employed in the daily care of equipment and utensils in dairies and food plants and for glasses, dishes, and utensils in restaurants. The process of disinfection would produce sanitization; however, in the strict sense, sanitization implies a sanitary condition which disinfection does not necessarily imply.

Germicide (Microbicide). An agent that kills the growing forms but not necessarily the resistant spore forms of germs; in practice a germicide is almost the same thing as a disinfectant, but germicides are commonly used for all kinds of germs (microbes) for any application.

Bactericide. An agent that kills bacteria (adjective, bactericidal). Similarly, the terms **fungicide**, **virucide**, and **sporicide** refer to agents that kill fungi, viruses, and spores, respectively.

Bacteriostasis. A condition in which the growth of bacteria is prevented (adjective, bacteriostatic). Similarly, **fungistatic** describes an agent that stops the growth of fungi. Agents that have in common the ability to inhibit growth of microorganisms are collectively designated **microbistatic** agents.

Antimicrobial Agent. One that interferes with the growth and metabolism of microbes. In common usage the term denotes inhibition of growth, and with reference to specific groups of organisms such terms as **antibacterial** or **antifungal** are frequently employed. Some antimicrobial agents are used to treat infections, and they are called **chemotherapeutic** agents.

SELECTION OF A CHEMICAL AGENT FOR PRACTICAL APPLICATIONS

The major factors that need to be assessed in the process of selecting the most appropriate chemical agent for a specific practical application are:

1 ***Nature of the material to be treated.*** To cite an extreme example, a chemical agent used to disinfect contaminated utensils might be quite unsatisfactory for application to the skin; i.e., it might do serious injury to the tissue cells. Consequently, the substance selected must be compatible with the material to which it is applied.

2 ***Types of microorganisms.*** Chemical agents are not all equally effective against

bacteria, fungi, viruses, and other microorganisms. Spores are more resistant than vegetative cells. Differences exist between Gram-positive and Gram-negative bacteria; *Escherichia coli* is much more resistant to cationic disinfectants than *Staphylococcus aureus*. Differences in action also exist between strains of the same species. Therefore, the agent selected must be known to be effective against the type of organism to be destroyed.

3 ***Environmental conditions.*** The factors discussed in Chap. 22, e.g., temperature, pH, time, concentration, and presence of extraneous organic material, may all have a bearing on the rate and efficiency of antimicrobial action. The successful use of an antimicrobial agent requires an understanding of the influence of these conditions on the particular agent, so it can be employed under the most favorable circumstances.

MAJOR GROUPS OF CHEMICAL ANTIMICROBIAL AGENTS

The major antimicrobial agents can be grouped as shown below. We will discuss representative examples from each of these groups.

1 Phenol and phenolic compounds
2 Alcohols
3 Halogens
4 Heavy metals and their compounds
5 Dyes
6 Detergents
7 Quaternary ammonium compounds
8 Aldehydes
9 Gaseous agents

Phenol and Phenolic Compounds

Phenol has the distinction of being used successfully in the 1880s by Joseph Lister, a surgeon, to reduce infection of surgical incisions and surgical wounds. Lister became aware of Pasteur's studies which incriminated germs as the cause of infection. Accordingly, he instituted the practice of applying a solution of phenol (carbolic acid) to surgical incisions and wounds. The reduction in infections was striking. Later he developed the practice of spraying phenol into the operating room area to control infection.

Phenol has the additional distinction of being the standard against which other disinfectants of a similar chemical structure are compared to determine their antimicrobial activity. The procedure used is called the **phenol-coefficient technique.** This technique is described later in this chapter.

Phenol and phenolic compounds are very effective disinfectants. A 5% aqueous solution of phenol rapidly kills the vegetative cells of microorganisms; spores are much more resistant. Many derivatives of phenol have been prepared and evaluated for their antimicrobial activity. The chemical structures of phenol and a few phenol derivatives are shown in Fig. 23-1. Antimicrobial activity is enhanced by the addition of chemical substitutions in the phenol ring structure as shown in Table 23-1.

Hexylresorcinol, a derivative of phenol, is marketed in a solution of glycerin and water. It is a strong surface-tension reductant, which may account in part for its high bactericidal activity. A commercial product containing hexylresorcinol, S.T. 37, is so named because of its surface-tension value. Hexylresorcinol preparations are employed as general antiseptics.

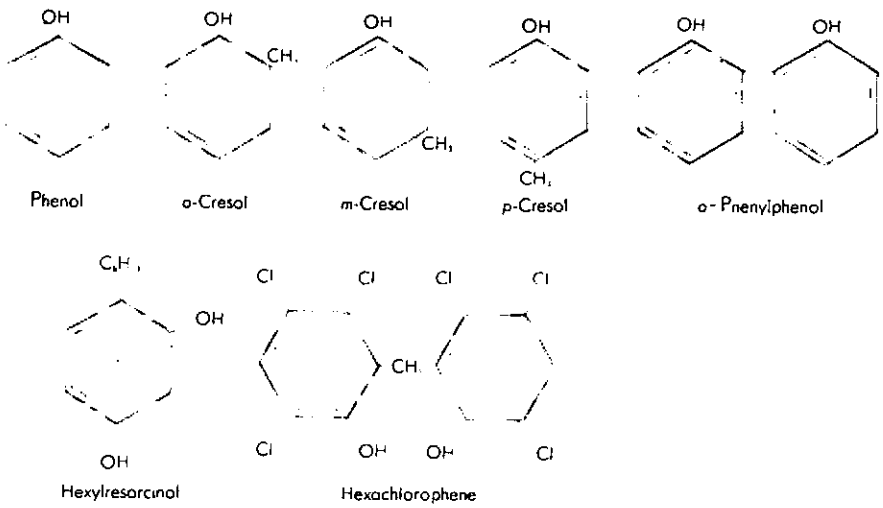


Figure 23-1. Phenol and some phenolic compounds which are used as disinfectants.

Table 23-1. Microbicidal Action of Phenol Derivatives (Phenol Coefficients at 37°C)*

Name	*Salmonella typhi*	*Staphylococcus aureus*	*Mycobacterium tuberculosis*	*Candida albicans*
Phenol	1.0	1.0	1.0	1.0
o-Cresol	2.3	2.3	2.0	2.0
m-Cresol	2.3	2.3	2.0	2.0
p-Cresol	2.3	2.3	2.0	2.0
4-Ethylphenol	6.3	6.3	6.7	7.8
2,4-Dimethylphenol	5.0	4.4	4.0	5.0
2,5-Dimethylphenol	5.0	4.4	4.0	4.0
3,4-Dimethylphenol	5.0	3.8	4.0	4.0
2,6-Dimethylphenol	3.8	4.4	4.0	3.5
4-*n*-Propylphenol	18.3	16.3	17.8	17.8
4-*n*-Butylphenol	46.7	43.7	44.4	44.4
4-*n*-Amylphenol	53.3	125.0	133.0	156.0
4-*tert*-Amylphenol	30.0	93.8	111.1	100.0
4-*n*-Hexylphenol	33.3	313.0	389.0	333.0
4-*n*-Heptylphenol	16.7†	625.0	667.0	556.0

* The higher the value the greater the microbicidal activity.
† Approximate.
SOURCE: From R. F. Prindle and E. S. Wright, "Phenolic Compounds," in C. A. Lawrence and S. S. Black, *Disinfection, Sterilization, and Preservation*, Lea & Febiger, Philadelphia, 1968.

Practical Applications. Phenolic substances may be either bactericidal or bacteriostatic, depending upon the concentration used. Bacterial spores and viruses are more resistant than are vegetative cells. Some phenolics are highly fungicidal. The antimicrobial activity of phenolics is reduced at an alkaline pH and by organic material. Low temperatures and the presence of soap also reduce antimicrobial activity.

Pure crystalline phenol is colorless. Aqueous solutions of from 2 to 5% can be employed to disinfect such materials as sputum, urine, feces, and contami-

nated instruments or utensils. Solutions of pure phenol have limited application. However, derivatives of phenol diluted in detergents or some other carrier find use in many commercial antiseptic and disinfectant preparations. One of the widely used phenolic derivatives is *o*-phenylphenol. Combination of compounds of this class with detergents results in products with good disinfectant as well as detergent properties.

Mode of Action. Exposure of microbial cells to phenolic compounds produces a variety of effects. Depending upon the concentration of the phenolic compound to which microbial cells were exposed, researchers have described results such as disruption of cells, precipitation of cell protein, inactivation of enzymes, and leakage of amino acids from the cells. Although the specific mode of action is not clear, there is a consensus that the lethal effect is associated with physical damage to the membrane structures in the cell surface, which initiates further deterioration.

Alcohols

Ethyl alcohol, CH_3CH_2OH, in concentrations between 50 and 90%, is effective against vegetative or nonsporeforming cells. For practical application a 70% concentration of alcohol is generally used.

Ethyl alcohol cannot be relied upon to produce a sterile condition. Concentrations which are effective against vegetative cells are practically inert against bacterial spores. In his book *Disinfection and Sterilization*, Sykes notes that "There is one record of survival of anthrax spores in alcohol for 20 years and another one of the *Bacillus subtilis* for 9 years."

Methyl alcohol is less bactericidal than ethyl alcohol; furthermore, it is highly poisonous. Even the fumes of this compound may produce permanent injury to the eyes, and is not generally employed for the destruction of microorganisms. The higher alcohols—propyl, butyl, amyl, and others—are more germicidal than ethyl alcohol. In fact, there is a progressive increase in germicidal power as the molecular weight of alcohols increases (as shown in Table 23-2). Since alcohols of molecular weight higher than that of propyl alcohol are not miscible in all proportions with water, they are not commonly used in disinfectants. Propyl and isopropyl alcohols in concentrations ranging from 40 to 80% are bactericidal for vegetative cells.

Table 23-2. Phenol Coefficients of Alcohols

	Phenol Coefficient	
Alcohol	Against *Salmonella typhi*	Against *Staphylococcus aureus*
Methyl, CH_3OH	0.026	0.03
Ethyl, CH_3CH_2OH	0.04	0.039
n-Propyl, $CH_3CH_2CH_2OH$	0.102	0.082
Isopropyl, $(CH_3)_2CHOH$	0.064	0.054
n-Butyl, $CH_3(CH_2)_2CH_2OH$	0.273	0.22
n-Amyl, $CH_3(CH_2)_3CH_2OH$	0.78	0.63
n-Hexyl, $CH_3(CH_2)_4CH_2OH$	2.3	
n-Heptyl, $CH_3(CH_2)_5CH_2OH$	6.8	
n-Octyl, $CH_3(CH_2)_6CH_2OH$	21.0	0.63

SOURCE: G. Sykes, *Disinfection and Sterilization*, 2d ed., Lippincott, Philadelphia, 1965.

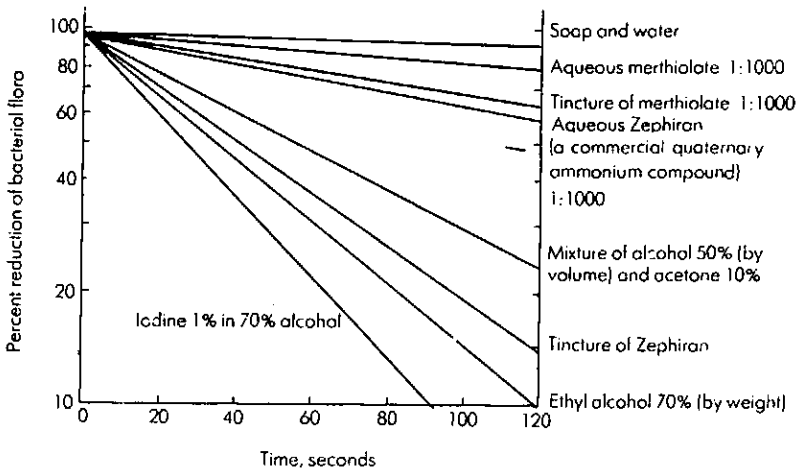


Figure 23-2. Comparative effectiveness of washing with various antiseptic solutions. This chart summarizes a large number of tests. In each test, the calculated bacterial flora immediately before the antiseptic was applied was considered as 100 percent; the residual flora immediately after use of the antiseptic is shown as a proportion of the original one. The steeper the curve, the greater the effect. (Note: 1:1000 means 1 part in 1000.) {*Courtesy of P. B. Price, "Skin Antisepsis," in J. H. Brewer (ed.), Lectures on Sterilization, Duke, Durham, N.C., 1957.*}

Practical Applications. Alcohol is effective in reducing the microbial flora of skin and for the disinfection of clinical oral thermometers. The comparative effectiveness of alcohol and other disinfectants applied to skin is shown in Fig. 23-2. Alcohol concentrations above 60% are effective against viruses; however, the effectiveness is influenced considerably by the amount of extraneous protein material in the mixture. The extraneous protein reacts with the alcohol and thus protects the virus.

Mode of Action. Alcohols are protein denaturants, and this property may, to a large extent, account for their antimicrobial activity. Alcohols are also solvents for lipids, and hence they may damage lipid complexes in the cell membrane. They are also dehydrating agents. This may account for the relative ineffectiveness of absolute alcohol on "dry" cells; it is possible that very high concentrations remove so much water from the cell that the alcohol is unable to penetrate. The severe dehydration occurring under these conditions would result in a bacteriostatic condition. Some of the effectiveness of alcohol for surface disinfection can be attributed to its cleansing or detergent action which results in mechanical removal of microorganisms.

Halogens

Iodine

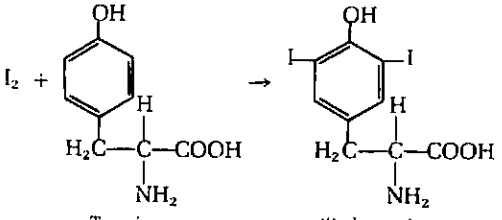
Iodine is one of the oldest and most effective germicidal agents. It has been in use for more than a century, having been recognized by the U.S. Pharmacopoeia in 1830. Pure iodine is a bluish-black crystalline element having a metallic luster. It is only slightly soluble in water but readily soluble in alcohol and aqueous solutions of potassium or sodium iodide. The element is traditionally used as a germicidal agent in a form referred to as **tincture of iodine.** There are several preparations available, such as 2% iodine plus 2% sodium iodide diluted in alcohol, 7% iodine plus 5% potassium iodide in 83% alcohol, and 5% iodine and 10% potassium iodide in aqueous solution. Iodine is also used in the form of substances known as **iodophors.** Iodophors are mixtures of iodine with surface-active agents which act as carriers and solubilizers for the iodine. One of these agents is polyvinylpyrrolidone (PVP); the complex can be expressed as

PVP-I. Iodine is released slowly from this complex. Iodophors possess the germicidal characteristics of iodine and have the additional advantages of non-staining and low irritant properties.

Practical Applications. Iodine is a highly effective bactericidal agent and is unique in that it is effective against all kinds of bacteria. Iodine also possesses sporicidal activity; however, the rate at which the spores are killed is markedly influenced by the conditions under which they are exposed, e.g., amount of organic material and extent of dehydration. In addition, it is highly fungicidal and is to some extent virucidal.

Iodine solutions are chiefly used for the disinfection of skin, and for this purpose they rank among the best disinfectants. The effectiveness of iodine preparations for the reduction of the microbial flora of skin is well recognized (see Fig. 23-2). Iodine preparations are effective for other purposes, such as disinfection of water, disinfection of air (iodine vapors), and sanitization of food utensils.

Mode of Action. The mechanism by which iodine exerts its antimicrobial activity is not specifically understood. Iodine is an oxidizing agent, and this fact may account for its antimicrobial action. Oxidizing agents can irreversibly oxidize and thus inactivate essential metabolic compounds such as proteins with sulfhydryl groups. It has also been suggested that the action may involve the halogenation of tyrosine units of enzymes and other cellular proteins requiring tyrosine for activity.



Tyrosine Diiodotyrosine

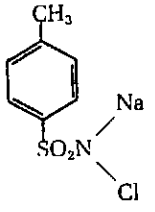
Chlorine and Chlorine Compounds

Chlorine, either in the form of gas or in certain chemical combinations, represents one of the most widely used disinfectants. The compressed gas in liquid form is almost universally employed for the purification of municipal water supplies. Chlorine gas is difficult to handle unless special equipment is available to dispense it. Hence, its usefulness in the gaseous state is limited to large-scale operations such as water-purification plants, where it is feasible for installing suitable equipment for safe handling.

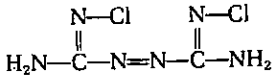
There are available many compounds of chlorine which can be handled more conveniently than free chlorine and which, under proper conditions of use, are equally effective as disinfectants. One class of compounds in this category is the **hypochlorites.** Calcium hypochlorite, $Ca(OCl)_2$ (also known as chlorinated lime), and sodium hypochlorite, NaOCl, are popular compounds.

The **chloramines** represent another category of chlorine compounds used as disinfectants, sanitizing agents, or antiseptics. Chemically they are characterized by the fact that one or more of the hydrogen atoms in an amino group of a

compound are replaced with chlorine. The simplest of these is monochloramine, NH_2Cl. Chloramine-T and azochloramide, two of several germicidal compounds in this category, have more complex chemical structures.



Chloramine-T



Azochloramide

One of the advantages of the chloramines is stability; they are more stable than the hypochlorites in terms of prolonged release of chlorine.

Practical Applications. Semmelweis is credited with having used hypochlorites in 1846 to 1848 in an attempt to reduce the incidence of childbed fever. Medical students were required to wash their hands and soak them in a hypochlorite solution before examining patients.

Chlorine compounds are very widely used to control microorganisms. Major applications are in water treatment, in the food industry, for domestic uses, and in medicine.

Products containing calcium hypochlorite are used for sanitizing dairy equipment and eating utensils in restaurants. Solutions of sodium hypochlorite of a 1% concentration are used for personal hygiene and as a household disinfectant; higher concentrations of 5 to 12% are also employed as household bleaches and disinfectants and for use as sanitizing agents in dairy and food-processing establishments. The amount of hypochlorite added should provide a residual concentration of approximately 1 mg per liter of free chlorine.

Chlorine compounds have been used to disinfect open wounds, to treat athlete's foot, to treat other infections, and as a general disinfectant.

Mode of Action. The antimicrobial action of chlorine and its compounds comes through the hypochlorous acid formed when free chlorine is added to water:

$$Cl_2 + H_2O \rightarrow HCl + HClO \text{ (hypochlorous acid)}$$

Similarly, hypochlorites and chloramines undergo hydrolysis, with the formation of hypochlorous acid. The hypochlorous acid formed in each instance is further decomposed:

$$HClO \rightarrow HCl + \underset{\substack{\text{Formed from chlorine, hypochlorites, chloramines}}}{O}$$

The oxygen released in this reaction (nascent oxygen) is a strong oxidizing agent, and through its action on cellular constituents, microorganisms are destroyed. The killing of microorganisms by chlorine and its compounds is also

Table 23-3. Some Compounds of Heavy Metals That Have Antimicrobial Activity

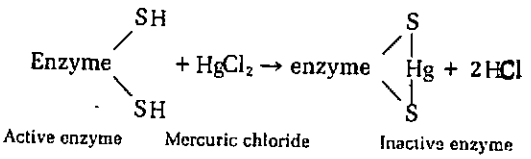
Heavy Metal	Examples of Compounds	Applications
Mercury	Inorganic compounds:	Bactericidal in dilutions of 1:1,000; limited use because of corrosive action, high toxicity to animals, and reduction of effectiveness in presence of organic material; insoluble compounds, used in ointments as antiseptics
	Mercuric chloride (bichloride of mercury)	
	Mercurous chloride	
	Mercuric oxide	
	Ammoniated mercury	
	Organic compounds:	Less irritating and less toxic than the inorganic mercury compounds; employed as antiseptics on cutaneous and mucosal surfaces; may be bactericidal or bacteriostatic
	Mercurochrome	
	Metaphen	
	Merthiolate	
	Mercresin	
Silver	Colloidal silver compounds:	Consist of protein in combination with metallic silver or silver oxide (colloidal solution); bacteriostatic or bactericidal effect is a function of the free silver ions released from the combination; used as antiseptics, silver nitrate is the most widely used of these compounds, all of which are germicidal and employed as antiseptics in specific conditions; silver nitrate is bactericidal for most organisms at a dilution of 1:1,000; many states require that the eyes of newborns be treated with a few drops of 1% silver nitrate solution to prevent ophthalmia neonatorum, a gonococcal infection of eyes
	Silver nitrate	
	Silver lactate	
	Silver picrate	
Copper	Copper sulfate	Much more effective against algae and molds than bacteria; 2 ppm in water sufficient to prevent algal growth; used in swimming pools and open water reservoirs; used in the form of Bordeaux mixture as a fungicide for prevention of certain plant diseases.

due in part to the direct combination of chlorine with proteins of the cell membranes and enzymes.

Heavy Metals and Their Compounds

Most of the heavy metals, either alone or in certain compounds, exert a detrimental effect upon microorganisms. The most effective are mercury, silver, and copper. Examples of these are summarized in Table 23-3.

Mode of Action. Heavy metals and their compounds act antimicrobially by combining with cellular proteins and inactivating them. For example, in the case of mercuric chloride the inhibition is directed at enzymes which contain the *sulfhydryl* grouping. Note below that the effect is upon the sulfhydryl group.



High concentrations of salts of heavy metals like mercury, copper, and silver coagulate cytoplasmic proteins, resulting in damage or death to the cell. Salts of heavy metals are also precipitants, and in high concentrations such salts could cause the death of a cell.

Dyes

Two classes of dye compounds which have antimicrobial properties are of special interest to microbiologists. These are triphenylmethane and acridine dyes.

Triphenylmethane Dyes

Included in this category are malachite green, brilliant green, and crystal violet. As a rule Gram-positive organisms are more susceptible to lower concentrations of these compounds than are Gram-negative ones. Crystal violet will inhibit Gram-positive cocci at a dilution of 1:200,000 to 1:300,000; 10 times this concentration is required to inhibit *Escherichia coli*. *Staphylococcus aureus* is inhibited by malachite green at a concentration of 1:1,000,000; a concentration of about 1:30,000 is required to inhibit *E. coli*. This general relationship between Gram reaction and susceptibility to triphenylmethane dyes has a number of practical applications.

Practical Applications. Certain media can be made selective by the incorporation of low concentrations (about 1:100,000) of the dyes crystal violet, brilliant green, or malachite green. Gram-positive bacteria will be inhibited. Media of this kind are used extensively in public health microbiology, where detection of *E. coli* is important. Susceptibility to various dyes can also be used for identification of bacteria. Three species of *Brucella* can be distinguished by their patterns of resistance to several dyes. Crystal violet has also been used as a fungicide. A concentration of 1:10,000 is lethal for *Monilia* and *Torula*, and a concentration of 1:1,000,000 is inhibitory.

Mode of Action. The mode of action of triphenylmethane dyes is uncertain, but there is speculation that they exert their inhibitory effect by interfering with cellular oxidation processes.

Acridine Dyes

Two examples of dyes derived from acridine are acriflavine and tryptoflavine. These compounds exhibit selective inhibition against bacteria, particularly staphylococci and gonococci. Gonococci are inhibited by tryptoflavine in dilutions of 1:10,000,000 to 1:50,000,000. They possess little, if any, antifungal activity. Presently, they have less application than before the advent of antibiotics and other chemotherapeutic agents. They are used to some extent for the treatment of burns and wounds and for ophthalmic application and bladder irrigation.

Synthetic Detergents

Surface-tension depressants, or wetting agents, employed primarily for cleansing surfaces are called **detergents**. Soap is an example. However, soap is a poor detergent in hard water. For this reason many new more efficient cleaning agents have been developed, called *surfactants* or **synthetic detergents**, many of which are superior to soap. They do not form precipitates in alkaline or acid water, nor do they produce deposits with minerals found in hard water. They are

extensively used in laundry and dishwashing powders, shampoos, and other washing preparations. Some are also highly bactericidal.

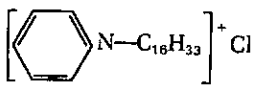
Chemically, detergents are classified as follows:

1 Those which ionize with the detergent property resident in the *anion* are referred to as anionic detergents. For example,

$[C_9H_{19}COO]^- Na^+$ — A soap

$[C_{12}H_{25}OSO_3]^- Na^+$ — Sodium lauryl sulfate

2 Those which ionize with the detergent property resident in the *cation* are referred to as cationic detergents. For example,



Cetylpyridinium chloride (Ceepryn)

3 A third category of detergents is nonionic; i.e., they do not ionize. However, these substances do not possess significant antimicrobial activity.

Insofar as reduction of the microbial flora from surfaces such as skin and clothing is concerned, the real value of ordinary soaps lies in the mechanical removal of microorganisms. Soaps reduce surface tension and thereby increase the wetting power of the water in which they are dissolved. Soapy water has the ability to emulsify and disperse oils and dirt. The microorganisms become enmeshed in the soap lather and are removed by the rinse water. Various chemicals have been incorporated into soaps to enhance their germicidal activity.

Cationic detergents are regarded as more germicidal than anionic compounds and will be discussed separately here as quaternary ammonium compounds.

Quaternary Ammonium Compounds

Most compounds of the germicidal cationic-detergent class are quaternary ammonium salts. Their characteristic structure with reference to a common inorganic ammonium salt such as ammonium chloride is shown in Fig. 23-3. The R_1, R_2, R_3, and R_4 groups are carbon groups linked to the nitrogen atom as shown in Fig. 23-3C. The R groups may be any one of a large number of different alkyl groups. Accordingly, a very large number of different quaternary ammonium compounds have been synthesized and evaluated for their antimicrobial activity. Several are available commercially as effective antimicrobial agents for a variety of uses (see Fig. 23-4).

The bactericidal power of the quaternaries is exceptionally high against Gram-positive bacteria, and they are also quite active against Gram-negative organisms. Bactericidal concentrations range from dilutions of one part in a few thousand to one part in several hundred thousand, as shown in Table 23-4. Another of their characteristics is the ability to manifest bacteriostatic action far beyond their bactericidal concentration. For example, the limit of bactericidal action for a given compound may be at a dilution of 1:30,000; yet it may be bacteriostatic in dilutions as high as 1:200,000. The action of these compounds demonstrates the need to distinguish between static and lethal activity in test procedures for the evaluation of disinfectants.

Table 23-4. Some Bactericidal Concentrations* of Three Quaternary Ammonium Compounds

Organism	Lethal Concentrations†		
	Cetrimide	Ceepryn	Zephirol
Staphylococcus	20,000‡	83,000	18,000
	35,000	218,000	20,000
	218,000		38,000
			50,000
			200,000
Streptococcus pyogenes	20,000	42,000	40,000
		127,000	
Escherichia coli	3,000	66,000	12,000
	27,500	67,000	27,000
	30,000		
Salmonella typhi	13,000	15,000	10,000
		48,000	20,000
		62,000	
Pseudomonas aeruginosa	3,500		2,500
	5,000		
Proteus vulgaris	7,500	34,000	1,300

* These figures have been collected from various published sources; they were therefore obtained with different testing techniques.

† Expressed as 1 part quaternary ammonium compound in stated volume of diluent; e.g., see footnote ‡.

‡ 1 part cetrimide in 20,000 parts of diluent.

SOURCE: G. Sykes, *Disinfection and Sterilization*, 2d ed., Lippincott, Philadelphia, 1965.

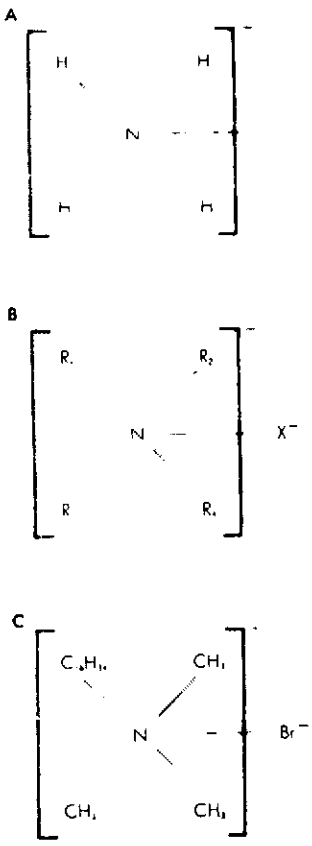


Figure 23-3. Chemical structure of quaternary ammonium compounds shown in relation to the structure of ammonium chloride. (A) Ammonium chloride. (B) The general structure of a quaternary ammonium compound. R_1, R_2, R_3, and R_4 are carbon-containing groups, and the X^- is a negatively charged ion such as Br^- or Cl^-. (C) The quaternary ammonium compound CTAB, or cetrimide.

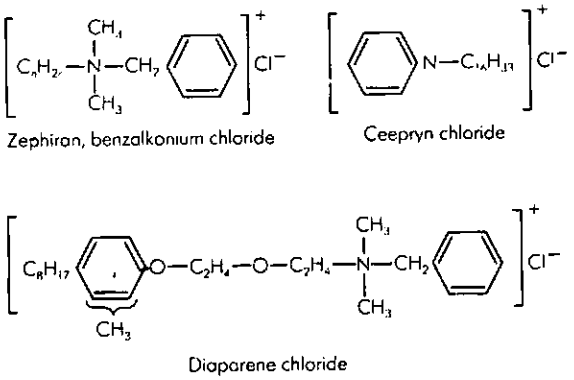


Figure 23-4. Some examples of quaternary disinfectants.

Quaternaries have been shown to be fungicidal as well as destructive to certain of the pathogenic protozoa. Viruses appear to be more resistant than bacteria and fungi.

Practical Applications. The combined properties of germicidal activity and detergent action, plus such other features as low toxicity, high solubility in water, stability in solution, and noncorrosiveness, have resulted in many applications of quaternaries as disinfectants and sanitizing agents. They are used as skin

disinfectants, as a preservative in ophthalmic solutions, and in cosmetic preparations. Quaternaries are widely used for control of microorganisms on floors, walls, and other surfaces in hospitals, nursing homes, and other public places. They are used to sanitize food and beverage utensils in restaurants as well as surfaces and certain equipment in food-processing plants. Other applications are to be found in the dairy, egg, and fishing industries to control microbial growth on surfaces of equipment and the environment in general.

Mode of Action. A variety of damaging effects of quaternaries upon microorganisms have been observed. These include denaturation of proteins, interference with glycolysis, and membrane damage. Experimental evidence suggests that the most likely site of the damage to the cell is the cytoplasmic membrane; the quaternaries alter the vital permeability features of this cell structure.

Aldehydes

Among the class of chemicals with the general formula RCHO (aldehydes), several of the low-molecular-weight compounds are antimicrobial. Two of the most effective are formaldehyde and glutaraldehyde. Both are highly microbicidal, and both have the ability to kill spores (sporicidal).

Formaldehyde

Formaldehyde (HCHO) is the simplest compound in the aldehyde series. It is a gas that is stable only in high concentrations and at elevated temperatures. At room temperature it polymerizes, forming a solid substance. The important polymer is paraformaldehyde, a colorless substance which rapidly yields formaldehyde upon heating. Formaldehyde is also marketed in aqueous solution as formalin, which contains 37 to 40% formaldehyde.

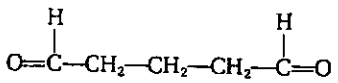
The fumes of formaldehyde are noxious; they are irritating to tissues and eyes.

Practical Applications. Formaldehyde in solution is useful for sterilization of certain instruments. Formaldehyde in gaseous form can be used for disinfection and sterilization of enclosed areas. Formalin and paraformaldehyde are two principal sources of formaldehyde when it is used for gaseous disinfection. Vaporization of formaldehyde from either of these sources into an enclosed area for an adequate time will cause sterilization, vegetative cells being killed more quickly than spores. Humidity and temperature have a pronounced effect on the microbicidal action of formaldehyde; in order to sterilize an enclosure the temperature must be about room temperature (22°C) and the relative humidity between 60 to 80 percent. One of the disadvantages of this process is the limited ability of the formaldehyde vapors to penetrate covered surfaces.

Mode of Action. Formaldehyde is an extremely reactive chemical. It combines readily with vital organic nitrogen compounds such as proteins and nucleic acids. It is likely that interaction of formaldehyde with these cellular substances accounts for its antimicrobial action.

Glutaraldehyde

Glutaraldehyde is a saturated dialdehyde with the formula



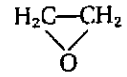
A 2% solution of this chemical agent exhibits a wide spectrum of antimicrobial activity. It is effective against vegetative bacteria, fungi, bacterial and fungal spores, and viruses. It is used in the medical field for sterilizing urological instruments, lensed instruments, respiratory therapy equipment, and other special equipment.

Gaseous Agents

Certain kinds of medical devices that need to be available in a sterile condition are made of materials that are damaged by heat. Examples are plastic syringes, blood transfusion apparatus, and catheterization equipment. The same is true for routinely used laboratory ware, such as plastic pipettes, Petri dishes, and other equipment, that is packaged and sterilized ready for use. On occasion there is need to disinfect or sterilize an enclosed area. Sterilization by means of gaseous agents is effective and practical for such situations. The main agents currently used for gaseous sterilization are ethylene oxide, β-propiolactone, and formaldehyde. We have discussed formaldehyde in the preceding section under aldehydes.

Ethylene Oxide

Ethylene oxide is a relatively simple organic compound having the formula



It is a liquid at temperatures below 10.8°C (51.4°F). Above this temperature it vaporizes rapidly. Vapors of this compound in air are highly flammable even in low concentrations. In this respect it is very much like diethyl ether. This objectionable feature was overcome by preparing mixtures of ethylene oxide in carbon dioxide or Freon, which are now available commercially. The carbon dioxide–ethylene oxide or Freon–ethylene oxide mixtures are nonflammable, and there is no alteration of the microbicidal activity of the ethylene oxide. The carbon dioxide and the Freon merely serve as inert diluents which prevent flammability.

Ethylene oxide is a unique and powerful sterilizing agent. Its use for sterilizing heat- or moisture-sensitive materials in hospitals, industry, and laboratories has become universal. Bacterial spores, which are many times more resistant than vegetative cells as measured by other antimicrobial agents, show little resistance to destruction by this agent. Figure 23-5 illustrates the sporicidal action of this gas. An outstanding and desirable feature of ethylene oxide is its power to

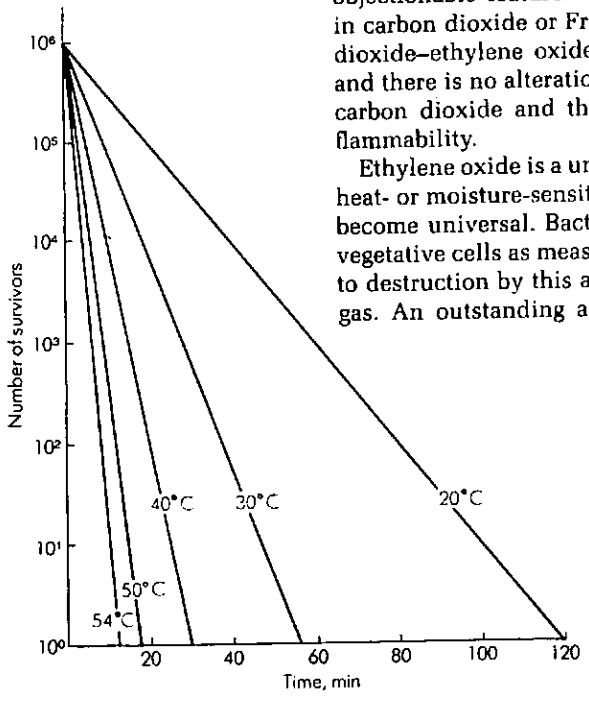


Figure 23-5. Decrease in numbers of *Bacillus subtilis* spores on paper strips surviving at various temperatures in gaseous ethylene oxide at 1200 mg/liter and 40 percent relative humidity. (*Courtesy of R. R. Ernst, "Ethylene Oxide Gaseous Sterilization for Industrial Applications," in G. B. Phillips and W. S. Miller (eds.) Industrial Sterilization, Duke, Durham, N.C., 1973.*)

penetrate. It will pass through and sterilize large packages of materials, bundles of cloth, and even certain plastics. It must be used with caution, although devices are available for its safe, routine laboratory use. The commercially available apparatus for this purpose is essentially an autoclave modified to allow the chamber to be filled with the gas under controlled conditions. The concentration of ethylene oxide, as well as the temperature and humidity, are critical factors which together determine the time required to achieve sterilization. Modern autoclaves are equipped with controls to maintain the desired concentration of ethylene oxide and the proper temperature and humidity.

An evaluation of the antimicrobial action of ethylene oxide and a comparison of its efficacy with other agents is shown in Table 23-5.

Practical Application. Ethylene oxide has been established as an effective sterilizing agent for heat- and moisture-sensitive materials. Effective usage requires careful control of three parameters: ethylene oxide concentration, temperature, and moisture. The varieties of materials on which it is used include spices, biological preparations, soil, plastics, certain medical preparations, and contaminated laboratory equipment. It has been used in the space program by both the Americans and the Russians for decontaminating spacecraft components. Among the advantages already mentioned for this agent as a sterilant is its remarkable penetration and its broad spectrum of activity against microorganisms, including spores. In addition, it is effective at relatively low temperatures,

Table 23-5. Evaluation of Selected Germicides

Class	Use Concentration	Activity Level*
Ethylene oxide (gas) (in autoclave-type equipment at 55 to 60°C)	450–800 mg/l	High
Glutaraldehyde, aq.	2%	High
Formaldehyde + alcohol	8% + 60–70%	High
Formaldehyde, aq.	3–8%	High to Intermediate
Iodine + alcohol	0.5–70%	Intermediate
Alcohols	70–95%	Intermediate
Chlorine compounds	4–5%	Intermediate
Phenolic compounds	0.5–3%	Intermediate to low
Iodophors	75–150 ppm	Intermediate to low
Quaternary ammonia compounds	1:750	Low
Mercurial compounds	1:500–1:1000	Low

Interpretation of Activity Level

	Test Organism					
	Bacteria			Fungi‡	Viruses	
	Vegetative†	Tubercle Bacillus	Spores		Lipid and Medium-Sized	Nonlipid and Small
High	+	+	+	+	+	+
Intermediate	+	+	−	+	+	+
Low	+	−	−	+	+	−

* + means that cidal effect can be expected.

† Common forms of bacterial cells, e.g., *Staphylococcus*.

‡ Includes usual asexual spores but not necessarily dried chlamydospores and sexual spores.

SOURCE: Courtesy of E. H. Spaulding, Temple University.

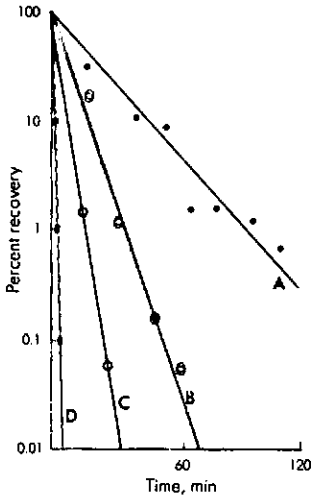


Figure 23-6. Effect of β-propiolactone concentration on death rate of spores of *Bacillus subtilis* var. *niger*. Relative humidity, 80 ± 5 percent; temperature, 27 ± 2°C. Concentrations: (A) 0.1, (B) 0.2, (C) 0.4, (D) 1.6 mg/liter. (*Courtesy of R. K. Hoffman and B. Warshowsky, Appl Microbiol, 6:358, 1958.*)

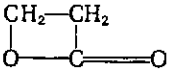
and it does not damage materials exposed to it. One of its disadvantages is its comparatively slow action upon microorganisms.

Mode of Action. The mode of action of ethylene oxide is believed to be alkylation reactions with organic compounds such as enzymes and other proteins. Alkylation consists in the replacement of an active hydrogen atom in an organic compound, e.g., the hydrogen atom in a free carboxyl, amino, or sulfhydryl group, with an alkyl group. In this reaction the ring in the ethylene oxide molecule splits and attaches itself where the hydrogen was originally. This reaction would inactivate an enzyme with a sulfhydryl group:

$$H_2C\text{—}CH_2 + \text{enzyme—SH} \rightarrow \underset{\text{Inactive}}{\text{enzyme—S—}CH_2CH_2OH}$$
$$\quad\quad\ \ O$$

β-Propiolactone

This compound is a colorless liquid at room temperature with a high boiling point (155°C) and has the formula



β-Propiolactone

It is not flammable like ethylene oxide but is a vesicant and lachrymator and consequently must be handled with care. It lacks the penetrating power of ethylene oxide but is considerably more active against microorganisms; it is sporicidal, fungicidal, and virucidal. The sporicidal activity of β-propiolactone is shown in Fig. 23-6. Whereas the usual concentration of ethylene oxide for sterilization purposes is 400 to 800 mg/liter, only 2 to 5 mg of β-propiolactone is required. β-propiolactone is very effective in destroying microorganisms on surfaces. However, the fact that it has a low power of penetration coupled with its alleged carcinogenic properties has restricted its use as a practical sterilizing agent.

EVALUATION OF ANTIMICROBIAL CHEMICAL AGENTS

Laboratory techniques for the evaluation of antimicrobial chemical agents are conducted by one of three general procedures. In each the chemical agent is tested against a specified microorganism referred to as the test organism.

Tube-Dilution and Agar-Plate Techniques

1 Liquid water-soluble substances appropriately diluted are dispensed into sterile test tubes, to which are added a measured amount of the test organism. At specified intervals, a transfer is made from this tube into tubes of sterile media that are then incubated and observed for the appearance of growth. It is necessary in this type of procedure to ascertain whether the inhibitory action is bactericidal and not bacteriostatic. This approach can also be used to determine the number of organisms killed per unit time by performing a plate count on samples taken at appropriate intervals.

2 The chemical agent is incorporated into an agar medium or broth, inoculated with the test organism, incubated, and then observed for (a) decrease in the amount of growth or (b) complete absence of growth.
3 A plate of agar medium is inoculated with the test organism, and the chemical agent is placed on this medium. Following incubation, the plate is observed for a zone of inhibition (no growth) around the chemical agent. This is particularly suitable for semisolid preparations. It may also be used for liquid solutions, in which case the solution is first impregnated in absorbent paper or confined by a hollow cylinder placed on the agar surface.

For evaluation of gaseous substances, paper strips impregnated with known numbers of bacterial spores are exposed to the gas under prescribed conditions, after which they are cultured for survivors. These general procedures are illustrated in Fig. 23-7.

Phenol-Coefficient Method

A specific official test method based on the principle outlined in the first procedure above is the AOAC phenol-coefficient method, sometimes called the FDA method. (These abbreviations refer to the Association of Official Agricultural Chemists and the Food and Drug Administration, respectively.) This procedure is suitable for testing disinfectants miscible with water and exerting their antimicrobial action in a manner similar to that of phenol. The test organism employed in this procedure is a specific strain of either *Salmonella typhi* or *Staphylococcus aureus*. The temperature at which the test is performed, the manner of making subcultures, the composition of the subculture medium, the size of the test tubes, and other details of the test are spelled out in the official procedure. Briefly, the test is performed as follows.

To a series of dilutions of the disinfectant being tested (5 ml per tube), 0.5 ml of 24-h broth culture of the test organism is added. At the same time, similar additions, in the same amounts, are made to a series of dilutions of phenol. All tubes (disinfectant + organisms and phenol + organisms) are placed in a 20°C water bath. At intervals of 5, 10, and 15 min, subcultures are made with a loop transfer needle into sterile tubes of medium. The inoculated subculture tubes are incubated and subsequently examined for growth. The greatest dilution of the disinfectant killing the test organism in 10 min but not in 5 min is divided by the greatest dilution of phenol showing the same result. The number obtained by this division is the phenol coefficient of the substance tested. An example of the type of result obtained in this test and the method of calculation of the phenol coefficient are shown in Table 23-6.

It should be emphasized that no single microbiological test method is suitable for the evaluation of all germicidal chemicals for all applications recommended. Therefore, one must exercise care in selecting a test method for a specific chemical agent, so the results obtained will be meaningful and reproducible and lend themselves to some degree of practical interpretation. The ultimate criterion for the effectiveness of a germicidal agent is its performance under practical conditions. However, the laboratory test should provide a reliable index of its practical value.

GENERAL OBSERVATIONS

From the foregoing description of antimicrobial chemical agents, it is apparent that a large variety of substances is available for a diversity of applications. This

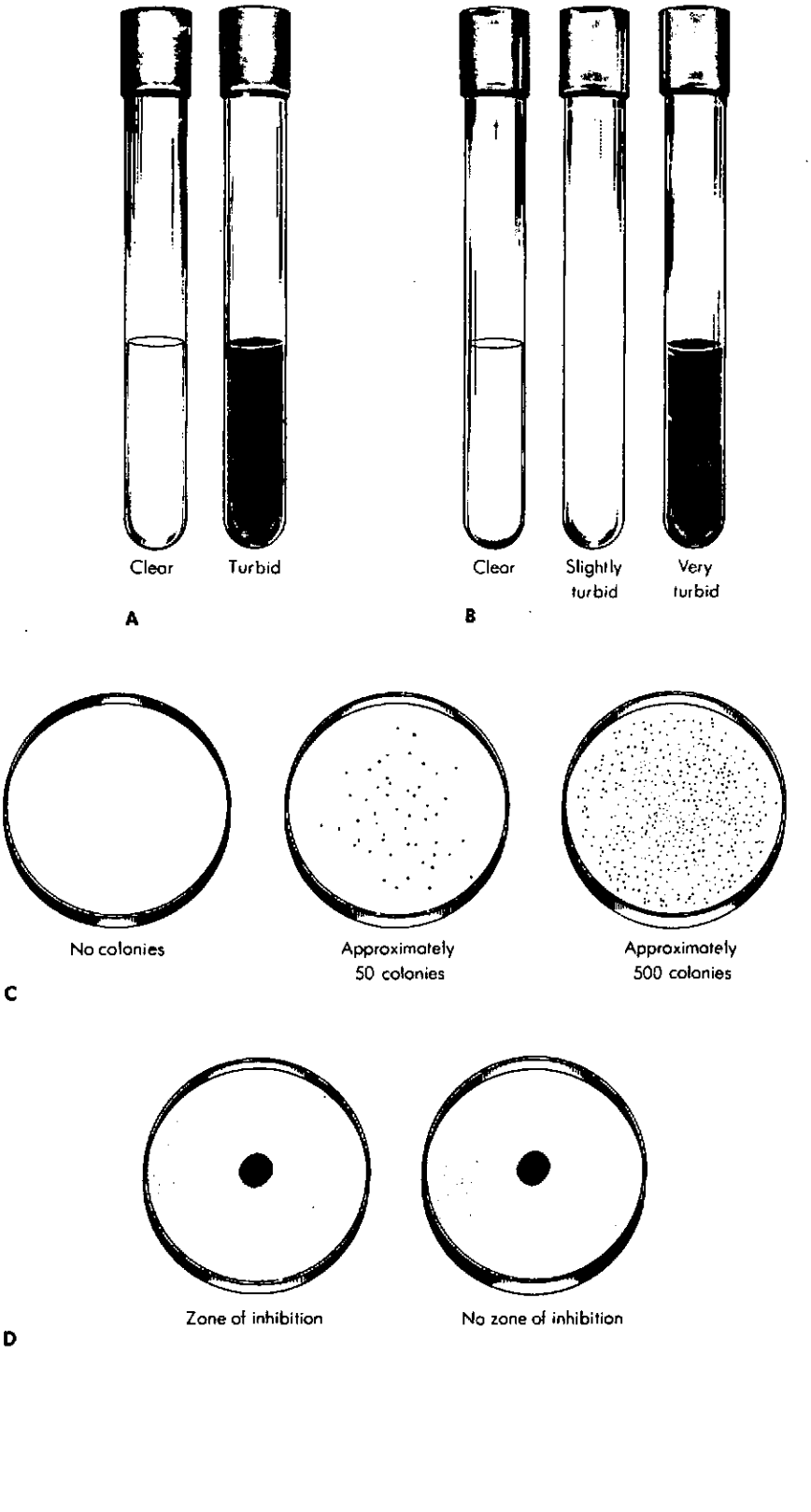


Figure 23-7. Laboratory evaluation of chemical antimicrobial agents: (A) No growth or growth in broth; (B) increased growth in broth as concentration of chemical agent is decreased; (C) increased growth in nutrient agar plates as concentration of chemical agent is decreased; (D) inhibition of growth by chemical agent applied to center of inoculated medium in Petri dish; zone of inhibition develops if compound is active. (*Courtesy of Procter and Gamble Company.*)

fact is compounded by the many proprietary products available to the public. Table 23-7 provides a summary of the various antimicrobial agents (or classes of agents) with a general statement of their applicability for practical usage. A summary of their modes of attack upon microbial cells is shown in Table 23-8.

Table 23-6. An Example of the Type of Result Obtained in the Phenol-Coefficient Method for Testing Disinfectants; Test Organism = *Salmonella typhi*

		Subculture Tubes*		
	Dilution	5 min	10 min	15 min
Disinfectant (X)	1:100	0	0	0
	1:125	+	0	0
	1:150	+	0	0
	1:175	+	+	0
	1:200	+	+	+
Phenol	1:90	+	0	0
	1:100	+	+	+

Phenol coefficient of (X) = $\frac{150}{90}$ = 1.6

NOTE: The phenol resistance of the test cultures must adhere to the following pattern:

	Phenol Dilution	5 min	10 min	15 min
Salmonella typhi	1:90	+ or 0	+ or 0	0
	1:100	+	+	+
Staphylococcus aureus	1:60	+	0	0
	1:70	+	+	

* 0 = no growth; + = growth.

Table 23-7. Application of Chemical Agents for Controlling Microorganisms

Chemical Agent	Recommended Use	Limitations
Phenol and phenolic compounds	General disinfectant	Microbial effectiveness limited; irritating and corrosive
Alcohols: ethyl and isopropyl	Skin and thermometer antiseptic	Antiseptic
Iodines	Disinfect skin	Irritating to mucous membranes
Chlorine	Water disinfection	Inactivated by organic material; pH dependent for effectiveness; objectionable taste and odor unless strictly controlled
Silver nitrate	Treating burns	Possible irritation
Mercurials	Skin disinfection	Slow-acting; toxic
Quaternaries	Skin disinfection	Not sporicidal
Formaldehyde	Sterilizing instruments; fumigation	Permeation poor; corrosive
Glutaraldehyde	Sterilizing instruments; fumigation	Stability limited
Ethylene oxide	Sterilizing heat-sensitive materials, instruments, and large equipment	Flammable; potentially explosive in pure form
β-propiolactone	Sterilizing instruments and heat-sensitive materials	Lacks penetrating power

Table 23-8. Sites of Action of Antimicrobial Chemical Agents Other Than Antibiotics

	Chemical Agents										
Sites of Action*	Acridine Dyes	Alcohols	Chlorine and Chlorine Compounds	Ethylene Oxide	Formaldehyde	Glutaraldehyde	Heavy-metal Salts	Iodine	Phenols	β-Propiolactone	Quaternary Compounds
Cell wall									+		
Cytoplasmic membrane		+							+		+
Proteins (denaturation)		+					+		+		+
Nucleic acids	+					+					
Enzymes with sulfhydryl (SH) groups			+	+		+	+	+		+	
Amino acids			+	+	+	+				+	

* In some instances the site of action is dependent upon the concentration of the chemical.

QUESTIONS

1 What is the distinction between the following terms?
   (a) Bactericidal and bacteriostatic
   (b) Sterile and disinfected
   (c) Virucidal and fungicidal
   (d) Germicidal and bactericidal
   (e) Sporicidal and bactericidal
2 List the major conditions influencing the effectiveness of antimicrobial chemical agents.
3 Compare the bactericidal property of phenol with that of other disinfectants of the phenolic type.
4 What relationship exists between various concentrations of ethyl alcohol and the higher alcohols?
5 List several halogens and compounds of halogens that are used to control microbial populations. Describe several practical applications for these agents. What is their mode of action upon microorganisms?
6 Give examples of the selective inhibition of dyes on microorganisms. Describe the use of such dyes in bacteriological media.
7 Explain the term cationic detergents. Describe their chemical structure; their practical use; and their mode of antimicrobial action.
8 What are the attractive features of sterilization by ethylene oxide? What kinds of materials are sterilized with ethylene oxide? Why?
9 A disinfectant is found to have a phenol coefficient of 3.0. What does this mean?
10 How would you demonstrate the antimicrobial capacity of an antiseptic ointment?

11 As a sterilizing agent, how does β-propiolactone compare with ethylene oxide?
12 Describe a laboratory experiment by which you could prove that the antimicrobial activity of a chemical in high dilutions was bacteriostatic.
13 Describe five different modes of antimicrobial activity exhibited by chemical antimicrobial agents. Identify the chemical agent involved in each example.
14 Why is it unlikely that a single "ideal" disinfectant agent will become available?

REFERENCES

Block, S. S. (ed.): *Disinfection, Sterilization, and Preservation*, 3d ed., Lea & Febiger, Philadelphia, 1983. *Fundamental principles and practical aspects of the control of microorganisms by chemical and physical methods to achieve disinfection, sterilization, and preservation. The mode of action of antimicrobial agents, bacterial resistance, and methods of testing are discussed. Each major category of chemical substances and physical agents is examined in terms of use and efficacy.*

Collins, C. H., M. C. Allwood, S. F. Bloomfield, and A. Fox (eds.): *Disinfectants: Their Use and Evaluation of Effectiveness*, Academic, New York, 1981. *This volume is one of a technical series (No. 16) published by the British Society for Applied Bacteriology. It provides brief descriptions of methods of testing disinfectants and describes the manner of using disinfectants in numerous environments such as the home, hospital, swimming pools, farm buildings, and milking areas.*

Favero, M. S.: *Sterilization, Disinfection, and Antisepsis in the Hospital*, in Lennette, E. H., et al. (eds.): *Manual of Clinical Microbiology*, 4th ed., Washington, American Society for Microbiology, 1985. *Practical information for control of microorganisms in hospitals.*

Hugo, W. B. (ed.): *Inhibition and Destruction of the Microbial Cell*, Academic, New York, 1971. *Covers inhibition and destruction of various groups of microorganisms (bacteria, fungi, viruses) by physical and chemical agents.*

Russell, A. D.: *The Destruction of Bacterial Spores*, Academic, New York, 1982. *A condensed, extensively referenced volume on the physical processes and chemical agents available for destruction of bacterial spores.*

Russell, A. D., W. B. Hugo, and G. A. J. Ayliffe (eds.): *Principles and Practice of Disinfection*, Blackwell Scientific Publications, Oxford, 1982. *A good general reference for methods used in disinfection.*

Sykes, G.: *Disinfection and Sterilization*, 2d ed., Lippincott, Philadelphia, 1965. *Contains practical information on the methods available for accomplishing sterilization and the major categories of chemical substances used for disinfectants.*

Chapter 24 Antibiotics and Other Chemotherapeutic Agents

OUTLINE

The treatment of a disease with a chemical substance is known as **chemotherapy**; the chemical substance is called a **chemotherapeutic agent**. Chemotherapy has been practiced for centuries, but it was only early in the present century (the mid-1930s) that this kind of therapy revolutionized the field of medicine. This turn in events is attributed to two discoveries. The first was the finding that sulfonamide compounds (sulfa drugs) could be used successfully for the treatment of certain bacterial diseases. The second was the discovery of a new and potent class of antibacterially active chemotherapeutic agents, namely, antibiotics. In this chapter we present a brief history of chemotherapy and then discuss

the modern chemotherapeutic substances. Emphasis is given to antibiotics and particularly the manner in which they inhibit or kill microbial cells.

CHEMOTHERAPEUTIC AGENTS AND CHEMOTHERAPY

Chemotherapeutic agents are chemical substances used for the treatment of infectious diseases or diseases caused by the proliferation of malignant cells. These substances are prepared in the chemical laboratory or obtained from microorganisms and some plants and animals. In general, naturally occurring substances are distinguished from synthetic compounds by the name antibiotics. Some antibiotics are prepared synthetically, but most of them are prepared commercially by microbial biosynthesis. Antitoxins and other substances produced by the bodies of infected animals are not considered to be chemotherapeutic agents; the compounds discussed in Chap. 23 used for killing or inhibiting microbial growth in vitro are not classified as chemotherapeutic agents but usually as disinfectants, antiseptics, or germicides.

To be useful as a chemotherapeutic agent a substance *must* have selective toxicity for the parasite, which means a low toxicity for host cells and high toxicity for the parasite. In other words, the substance must damage the parasite and cause little or no damage to the cells of the host. For this and other reasons antiseptics and germicides such as phenol, coal-tar derivatives, and many mercurial compounds are unsatisfactory as chemotherapeutic agents. Germicides are not selective in their action on cells, and they interfere with such natural defense mechanisms as phagocytosis: since they do not penetrate cells and tissues well, they do not come into contact with the parasites; because they are inactivated by protein, their effectiveness is destroyed by body fluids rich in protein; and, finally, the tissues killed by the germicide or antiseptic provide an excellent medium for microorganisms to grow.

Since the reasons enumerated above make some chemical compounds useless in treating microbial infections, it follows that a satisfactory chemotherapeutic agent must:

1 Destroy or prevent the activity of a parasite without injuring the cells of the host or with only minor injury to its cells
2 Be able to come in contact with the parasite by penetrating the cells and tissues of the host in effective concentrations
3 Leave unaltered the host's natural defense mechanisms, such as phagocytosis and the production of antibodies

HISTORICAL HIGHLIGHTS OF CHEMOTHERAPY

Quinine and Malaria

Europeans used natural quinine from the bark of the cinchona tree to treat malaria as early as 1630. It was used even earlier by South American Indians, who relieved symptoms of malarial fever by chewing the bark of the cinchona tree.

Ehrlich, Salvarsan, and Syphilis

Syphilis is the first known disease for which a chemotherapeutic agent was used. Mercury was used to treat syphilis as early as 1495, but it was not until about 1910, when an arsenical compound known as Salvarsan was synthesized by Paul Ehrlich (Fig. 24-1), that a specific drug capable of curing disease without

great danger to the patient was developed. Ehrlich's contributions were especially important because his was the first systematic and deliberate search for a compound that had potent microbicidal properties, low toxicity for humans and other animals, and good chemical stability. For this important discovery he was awarded a share, with Elie Metchnikoff, of the 1908 Nobel prize in physiology and medicine. Ehrlich's compound has now been replaced in syphilis therapy by arsphenamine, neoarsphenamine, and other arsenical compounds and antibiotics.

Domagk, Sulfonamides, and Bacterial Infections

Because of the effectiveness of Ehrlich's drug, it is rather surprising that no further significant development in the synthesis of chemotherapeutic agents occurred until 1935, when Domagk showed the therapeutic value of a group of compounds known as the **sulfonamides**. These substances are not specific for a special group of organisms, as arsphenamine is for *Treponema*, but are effective against a large variety of pathogenic organisms. Sulfanilamide, the first compound in this group to be synthesized, was made by Gelmo in 1908, and in 1913 Eisenberg studied the bactericidal properties of azo dyes with a sulfonamide grouping. Possibly progress in this field was delayed because of the hesitancy of physicians to accept a type of therapy that Ehrlich referred to as "the chemical knife," since it really amounted to "cutting out" microbes from tissues. Ehrlich was justified in this designation, because the use of such drugs was attended by danger to the patient comparable to surgical risk. Fortunately, the new chemotherapeutic agents are not dangerous drugs since they are carefully studied in the laboratory followed by extensive clinical trials. A more realistic explanation for nonacceptance of Ehrlich's concept of chemotherapy is that it was then generally believed that the struggle between host and parasite was too complex to permit such a direct attack. It would be better, his opponents reasoned, to stimulate the host's defenses.

Figure 24-1. Paul Ehrlich is generally regarded as having established chemotherapy as a science. His research in the early 1900s resulted in the synthesis of an arsenical compound (Salvarsan) for the treatment of syphilis. His research represented a major contribution to the systematic search for new drugs.

After Domagk's reports in 1935 in Germany and confirmatory work by investigators in other countries—notably England, France, and later the United States—interest in chemotherapy reached an all-time high. The compound on which Domagk reported was known as Prontosil. French chemists at the Pasteur Institute who studied its action on bacteria and attempted to improve it discovered that its antibacterial activity is due to the sulfanilamide moiety, previously synthesized and reported by Gelmo in 1908. This observation lighted the fuse for an explosive search for related compounds having therapeutic value. By 1945 it was estimated that several thousand derivatives of sulfanilamide had been made.

The important result of the search for new varieties of sulfonamides has been the development of drugs with increased antibacterial activities and fewer unfavorable reactions in the host animal. Some have been especially useful in certain types of infections, but sulfadiazine and sulfamerazine are extensively used because of their antibacterial effectiveness in a wide range of bacterial infections and because they are least likely to produce toxic reactions in the patient.

The sulfonamides are especially useful in the treatment of infections caused by meningococci and *Shigella*, respiratory infections caused by streptococci and staphylococci, and urinary infections due to Gram-negative organisms. They are

useful in the prevention of rheumatic fever, bacterial endocarditis, wound infections, and urinary-tract infections following surgery or catheterization.

Antibiotics, Fleming, and Penicillin

Antibiotics are a special kind of chemotherapeutic agent usually obtained from living organisms. The word **antibiotic** has come to refer to a metabolic product of one microorganism that in very small amounts is detrimental or inhibitory to other microorganisms It has been known for many years that antagonisms can exist between microorganisms growing in a common environment. The term **antibiosis** was first defined by Vuillemin in 1889 as a condition in which "one creature destroys the life of another in order to sustain his own, the first being entirely active and the second entirely passive; one is in unrestricted opposition to the life of the other." However, it can be seen that this definition is not entirely compatible with the present-day use of the term *antibiotics* proposed by Waksman in 1945 as applying to *those chemical substances of microbial origin which in small amounts exert antimicrobial activity.*

Antibiotics were known by their activities long before they were given the name by which we know them. Many years ago the Chinese used moldy soybean curd for the treatment of boils and controlled foot infections by wearing sandals furry with mold. In 1881, Tyndall reported that culture media cloudy with bacterial growth became clear when mold grew on the surface. Pasteur and Joubert found that pure cultures of anthrax bacilli grew well in urine but that when certain other organisms were present, the anthrax bacilli disappeared. This observation was related to that of Emmerich and Low, who demonstrated in 1901 that when liquid cultures of *Pseudomonas aeruginosa* were injected into rabbits, the animals were protected against anthrax. They called this material *pyocyanase* because they thought its activity was due to enzymes from *Bacillus pyocyaneus*, as *Ps. aeruginosa* was then called.

An early clinical application of bacterial antagonism was the use of lactobacilli in the treatment of dysentery, as recommended by Metchnikoff in 1899. This was an example of replacement therapy; i.e., a harmless microbe was able to eliminate and replace one that could cause disease. Modern antibiosis is based not on replacement but on utilization of an active inhibitory principle obtained from the antibiotic-producing microbes.

The first systematic search for, and study of, antibiotics, made by Gratia and Dath about 1924, resulted in the discovery of *actinomycetin* in strains of actinomycetes, soil organisms that are representative of the group that has given us a number of antibiotics since 1940. Actinomycetin was never used for the treatment of patients but was used to lyse cultures of bacteria for the production of vaccines.

In 1929 Alexander Fleming (Fig. 24-2A) noticed that an agar plate inoculated with *Staphylococcus aureus* had become contaminated with a mold and that the mold colony was surrounded by a clear zone, indicating inhibition of bacterial growth, or lysis of the bacteria (Fig. 24-2B). He was inspired to isolate and identify the mold and study its activities, but not until there was an urgent need for a better means of preventing death from infection of war wounds was the importance of Fleming's observation realized. With the aid of many investigators in England and the United States, and at a great deal of expense, the inhibitory substance from Fleming's "contaminant mold" became a "miracle drug." Be-

A

B

Figure 24-2. (A) Sir Alexander Fleming discovered the bacterial inhibitory properties of a metabolic product of *Penicillium notatum*. He called the substance penicillin. This discovery, in 1929, opened the era of antibiotics. For his contributions Fleming was knighted and shared the Nobel prize in physiology and medicine for 1945 with Ernst B. Chain, a chemist, and Sir Howard W. Florey, a physician. (B) Fleming's original plate demonstrated the inhibition of *Staphylococcus* (colonies at bottom) by a colony of *Penicillium notatum* (large white circle at top). This led to the discovery of penicillin. (*Courtesy of Robert Cruickshank.*)

cause the mold was identified as a *Penicillium* sp., Fleming called the antibiotic *penicillin*.

In 1939, René Dubos (Fig. 24-3) isolated from New Jersey soil a culture of *Bacillus brevis* which produced a substance that killed many Gram-positive bacteria. The cell-free extract produced from *B. brevis* by Dubos was found to contain two active principles now known as *gramicidin* and *tyrocidine*. These successes were followed closely by the discovery of *streptomycin* by Selman Waksman and associates.

Several thousand antibiotic substances have been isolated and identified since 1940. Many of them are of no practical importance as yet, but a few have changed the entire concept of chemotherapy. The popularity of antibiotics is due to their ability to destroy many kinds of pathogens and to their relatively nontoxic

Figure 24-3. In 1939, René Dubos isolated two antibiotics, gramicidin and tryocidine, from a soil bacterium, *Bacillus brevis*. (*Courtesy of National Library of Medicine.*)

properties to the host when given systemically. Few developments in the field of medicine have had as dramatic an effect as have antibiotics in the treatment of microbial infections.

CHARACTERISTICS OF ANTIBIOTICS THAT QUALIFY THEM AS CHEMOTHERAPEUTIC AGENTS

To be useful as chemotherapeutic agents antibiotics must have the following qualities:

1 They should have the ability to destroy or inhibit many different species of pathogenic microorganisms. This is what is meant by a "broad-spectrum" antibiotic.
2 They should prevent the ready development of resistant forms of the parasites.
3 They should not produce undesirable side effects in the host, such as sensitivity or allergic reactions, nerve damage, or irritation of the kidneys and gastrointestinal tract.
4 They should not eliminate the normal microbial flora of the host, because doing so may upset the "balance of nature" and permit normally nonpathogenic microbes, or particularly pathogenic forms normally restrained by the usual flora, to establish a new infection. The broad-spectrum antibiotics, for example, may eliminate the normal bacterial flora but not *Monilia* from the intestinal tract. Under these conditions the *Monilia* may establish an infection that is not controlled by antibiotic therapy.

ANTIBIOTICS AND THEIR MODE OF ACTION

Antibiotics can be classified in several ways. For example, some are bactericidal and others are bacteriostatic. They may be grouped on the basis of chemical structure. A third way of classifying antibiotics is on the basis of their mode of action, that is the manner in which they manifest their damage upon microbial cells. Our discussion in this section will be organized on the basis of this latter manner of grouping antibiotics, i.e., their mode of action.

The major points of attack of antibiotics on microorganisms include:

Inhibition of cell-wall synthesis
Damage to the cytoplasmic membrane
Inhibition of nucleic acid and protein synthesis
Inhibition of specific enzyme systems

In the discussion which follows we will characterize only a few of the many antibiotics in each of these categories.

Inhibition of Cell-Wall Synthesis

Among the antibiotics whose antimicrobial activity is expressed by inhibition of the biosynthesis of the peptidoglycan cell-wall structure are the penicillins, cephalosporins, cycloserine, vancomycin, and bacitracin.

As you may recall from Chap. 11, the substance that gives rigidity to the cell wall is the peptidoglycan (see Figs. 11-6 and 11-7). The structure of this compound is essentially that of a series of strands (polymers with repeating units of *N*-acetylglucosamine and *N*-acetylmuramic acid) that are cross-linked with small peptides, with a frequency and in a manner that imparts considerable rigidity to the cell wall. It is a protective covering for the bacterial cell.

As you will note from the description of the biosynthesis of peptidoglycan in

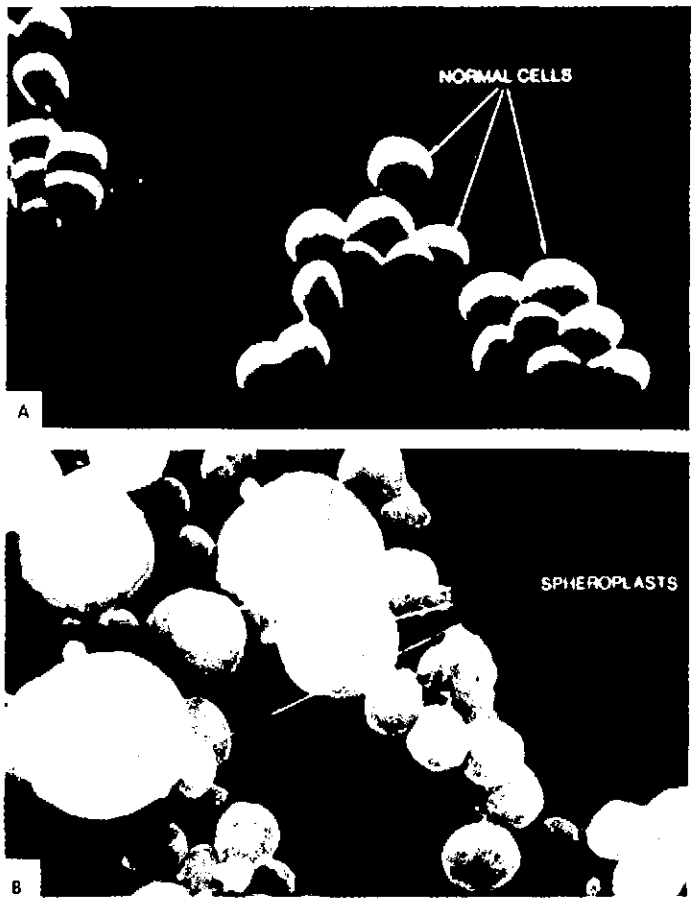


Figure 24-4. The morphologic changes, which occur in *Escherichia coli* as a result of exposure to penicillin, shown in this illustration, provide visual evidence that this antibiotic acts upon the cell wall. (A) Normal cells of *E. coli*. (B) *E. coli* cells after exposure to penicillin. Enlarged (bulged) defect indicates alteration of cell wall. (*Courtesy of Lilly Research Laboratories, Division of Eli Lilly and Company.*)

Chap. 11, the process involves numerous steps. Interference with any step in the sequence may inhibit cell-wall synthesis and result in the inability of the bacterium to survive because of the absence of a protective covering (cell wall).

Early experimental evidence suggested that some antibiotics exert their antimicrobial effect by inhibiting biosynthesis of the peptidoglycan polymer, resulting in the inhibition of cell-wall formation. Subsequent research identified the sequence of reactions in the biosynthetic pathway of the peptidoglycan and demonstrated that antibiotics like penicillin inhibited its formation. Some experimental observations that led to this conclusion can be summarized as follows:

1 Bacterial cells susceptible to penicillin can be protected from destruction if the medium in which they are exposed is of high osmotic pressure. The high osmotic pressure prevents the cells from bursting. Rod-shaped cells become spherical because they lack the cell structure which imparts shape. These cells without cell walls are called **spheroplasts**. (See Fig. 24-4).

2 Some species of bacteria such as the mycoplasmas lack the peptidoglycan structure and are not inhibited by penicillin.
3 Concentrations of penicillin below that which kill susceptible bacteria results in the accumulation of compounds that are precursors to peptidoglycan formation.

The Penicillins

The first of the modern antibiotics, and still one of the most useful, penicillin is produced by *Penicillium notatum* (Fig. 24-5), *Penicillium chrysogenum*, and by other species of molds. As previously noted, the first of these was isolated by Fleming in 1929, when he found it as a contaminant on a culture plate. Florey and his associates at Oxford University isolated the active ingredient and used the crude material clinically in 1940. Penicillin is selective for Gram-positive bacteria, some spirochetes, and the Gram-negative diplococci (*Neisseria*). Although it is rarely toxic in human patients, it may give rise to sensitivity reactions which vary from a mild skin reaction to severe anaphylaxis.

Penicillins are a class of β-lactam antibiotics of related structure with slightly different properties and activities. All penicillins have a common basic nucleus, a fused β-lactam-thiazolidine ring with different side chains which give each its unique properties (Fig. 24-6). Several chemically different penicillins are produced by biosynthesis in a single fermentation.

Natural penicillins can be prepared as salts of sodium, potassium, procaine, and other bases. The crystalline sodium or potassium salts are freely soluble in water, ethyl alcohol, ether, esters, and dioxane but only slightly soluble in chloroform and benzene. In pure crystalline form penicillins are colorless. The natural penicillins are inactivated by heat, cysteine, sodium hydroxide, penicillinase, and hydrochloric acid. They are not affected by the action of saliva or bile. Penicillin V exhibits greater stability than others in acids. Some of

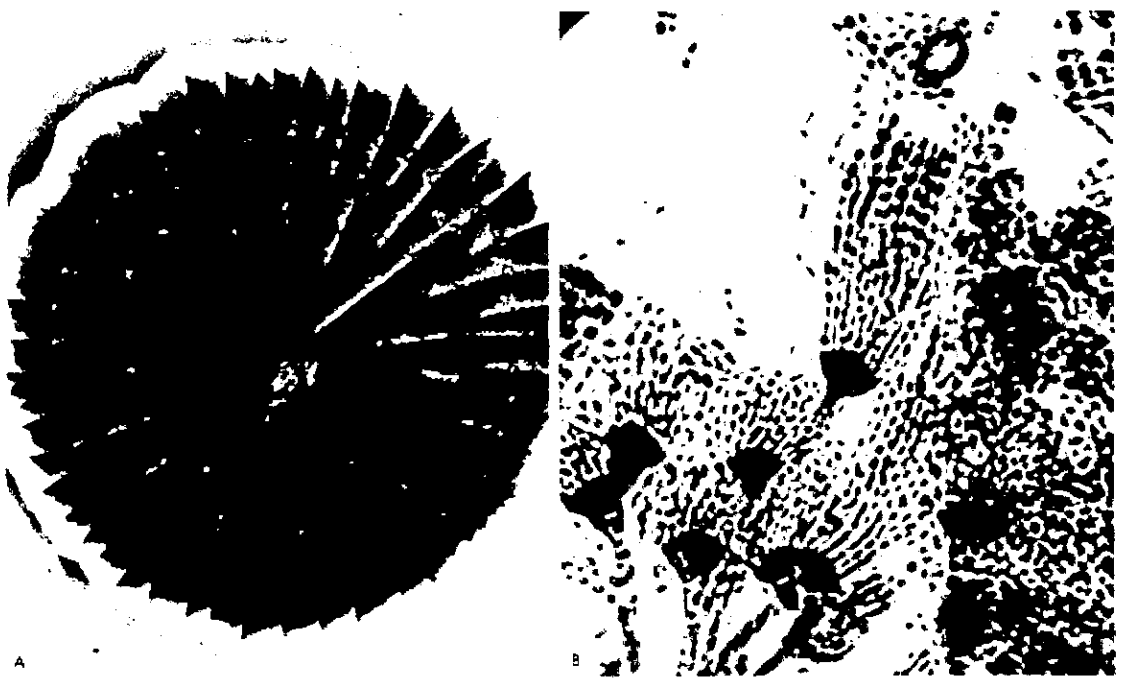


Figure 24-5. *Penicillium notatum*. (A) A colony on agar-plate culture; magnification X3. (B) A microscopic view showing spores and mycelia; magnification X400.

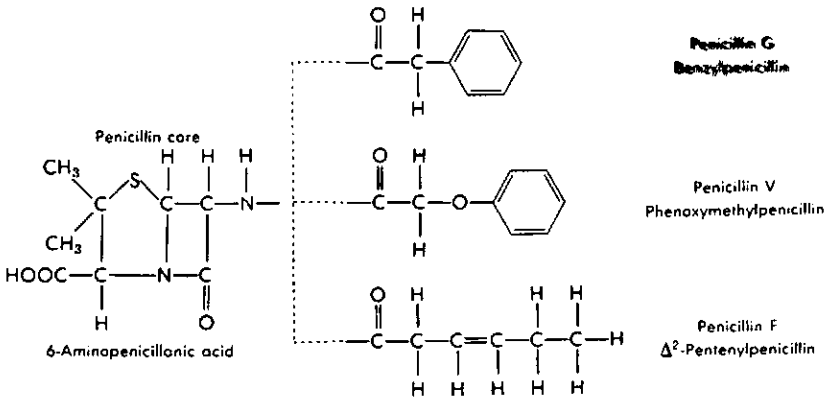


Figure 24-6. Some "natural" penicillins showing the basic core of 6-aminopenicillanic acid with side chains which differ, thereby conferring special properties.

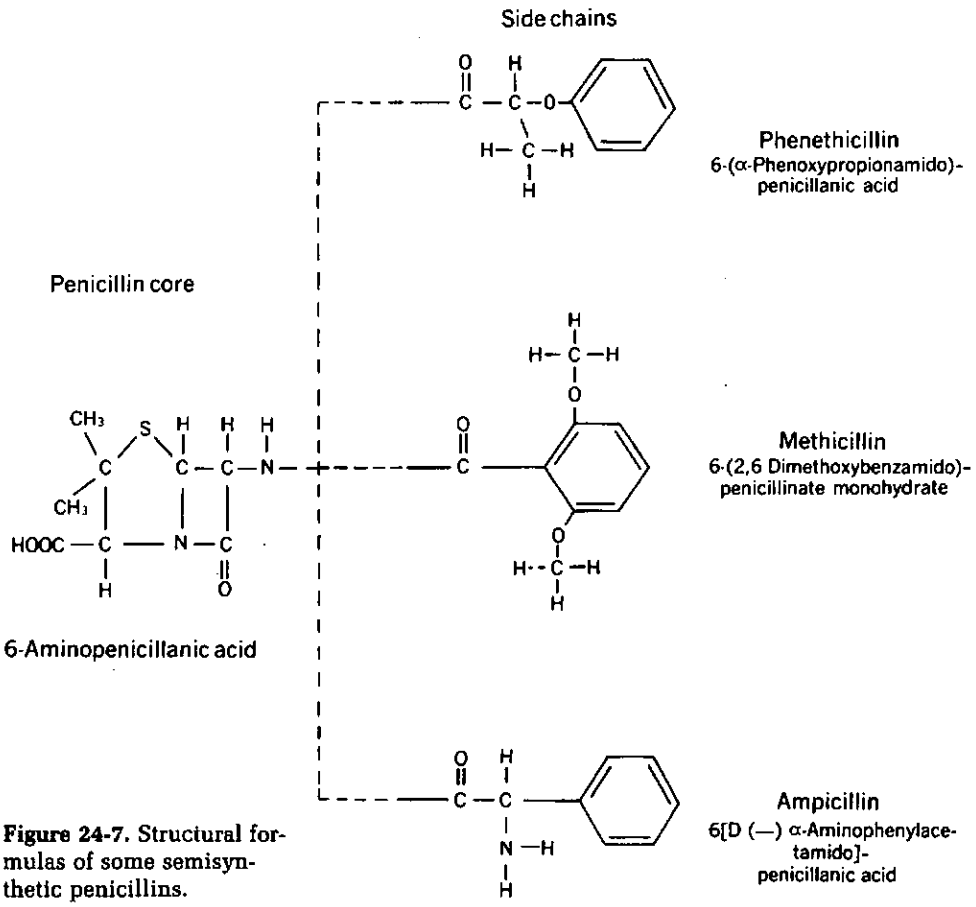


Figure 24-7. Structural formulas of some semisynthetic penicillins.

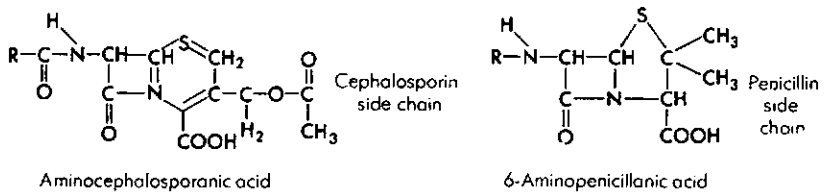


Figure 24-8. A comparison of the nucleus of cephalosporin (aminocephalosporanic acid) with the nucleus of penicillin (6-aminopenicillanic acid).

the new semisynthetic penicillins may be much more stable than those produced by biosynthesis, the "natural penicillins."

The first break in the production of the new semisynthetic penicillins was the discovery that the basic nucleus of the molecule, common to all penicillins, is 6-aminopenicillanic acid. The next step was to obtain 6-aminopenicillanic acid in quantity so that suitable side chains could be attached to it. This was a very difficult task, but it was then discovered that under suitable conditions *P. chrysogenum* would produce the basic nucleus in abundance by "interrupted biosynthesis," and that the side chains could be removed from penicillin G, produced by biosynthesis, by amidase enzymes, leaving the 6-aminopenicillanic acid free for attaching new side chains as desired.

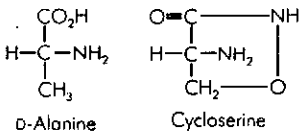


Figure 24-9. The chemical structures of D-alanine and the antibiotic cycloserine.

One of the first semisynthetic penicillins to be produced for clinical use was phenethicillin. It is more readily absorbed than penicillin V and just as effective as penicillin G. Another of the semisynthetic penicillins, methicillin, is more resistant to penicillinase and therefore is less likely to be inactivated.

Ampicillin

Ampicillin, another semisynthetic penicillin, acts against a broad spectrum of bacteria. It is strongly bactericidal and lacks toxicity, but it is not resistant to penicillinases. It is relatively stable to gastric acid and hence can be administered orally. The chemical structures of these three penicillins are shown in Fig. 24-7. Several additional semisynthetic penicillins have been developed for chemotherapeutic use.

Penicillins interfere with the final stages of peptidoglycan biosynthesis. The penicillins inhibit the transpeptidase reaction, namely, the cross-linking of the two linear polymers. The penicillins are bactericidal to growing cells.

Cephalosporins

Cephalosporins are a group of antibiotcs produced by a species of marine fungus, *Cephalosporium acremonium*, which bears considerable resemblance to *Penicillium* spp. They are effective against Gram-positive and Gram-negative bacteria. The cephalosporins have antibacterial properties similar to those of the semisynthetic penicillins. They are effective therapeutically and have a low toxicity. The nucleus of the cephalosporins (Fig. 24-8) resembles that of penicillin. As with penicillin, several semisynthetic cephalosporins have been manufactured commercially for therapeutic use.

As would be anticipated from the similarity in chemical structure of penicillin and cephalosporin, the mode of action of the cephalosporins is that of inhibition of the cross-linking transpeptidase. They are bactericidal to growing cells.

Cycloserine

Cycloserine, a relative simple compound, is related in structure to alanine (see Fig. 24-9). It was originally discovered as an antibiotic produced by streptomyces and is now manufactured through chemical synthesis. The main use of

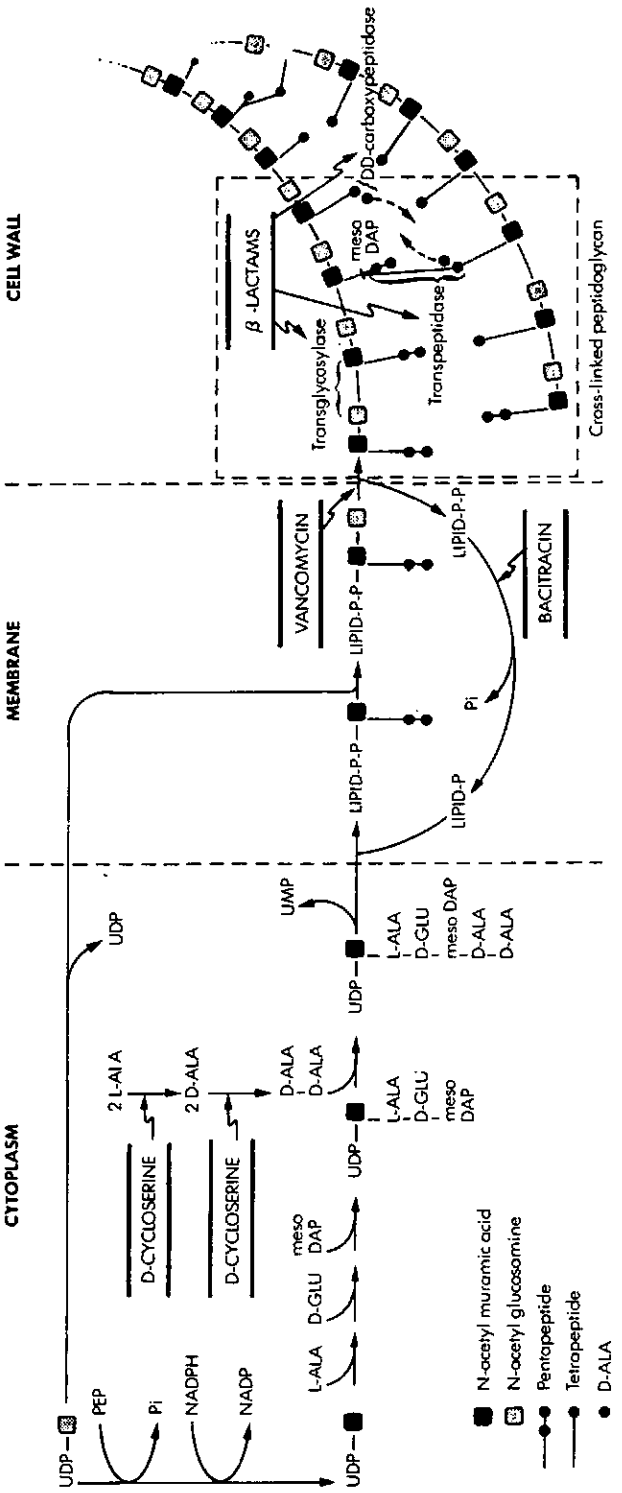


Figure 24-10. Schematic illustration of sites of attack of antibiotics on cell-wall synthesis (formation). (*Erwin F. Lessel, illustrator.*)

this antibiotic is in tuberculosis therapy. However, because of potential undesirable side effects, its utilization is limited.

Cycloserine manifests its inhibitory effect on peptidoglycan synthesis by interference with synthesis of the peptide moiety of the peptidoglycan. Specifically, it inhibits both alanine racemase and D-alanyl-D-alanine synthetase, the enzymes involved in the synthesis of the pentapeptide side chains.

Bacitracin

Bacitracin is a product of *Bacillus subtilis* and chemically is a polypeptide. Because of its toxicity to animal and human cells it cannot be used for systemic chemotherapy. It does have application for topical treatment of infections caused by Gram-positive bacteria.

Bacitracin interferes with regeneration of the monophosphate form of bactoprenol from the pyrophosphate form (lipid-P-P in Fig. 24-10).

Vancomycin

Vancomycin is an antibiotic produced by *Streptomyces orientalis*. It is a complex chemical entity consisting of amino acids and sugars.

Vancomycin inhibits peptidoglycan synthesis by binding the D-alanyl-D-alanine group on the peptide side chain of one of the membrane-bound intermediates.

A schematic summary of the modes of action of some antibiotics that exert their antibacterial effect through interference with cell-wall synthesis is shown in Fig. 24-10.

Damage to Cytoplasmic Membrane

Several polypeptide antibiotics produced by *Bacillus* spp. have the ability to damage cell-membrane structure. They adversely affect the normal permeability characteristics of the cell membrane. Included in this category are the **polymyxins, gramicidins,** and **tyrocidines** (see Fig. 24-11).

The polymyxins are particularly effective against Gram-negative organisms, while the tyrocidines and gramicidins are more effective against Gram-positive organisms.

These agents are bactericidal; they cause a leakage from the cytoplasmic content of the cell. Because of their toxicity to tissue they have limited application in chemotherapy.

Another category referred to as **polyene** antibiotics are large ring structures with many double bonds. Examples are *nystatin*, produced by *Streptomyces noursei*, and *amphotericin*, produced by *Streptomyces nodosus*. Polyene anti-

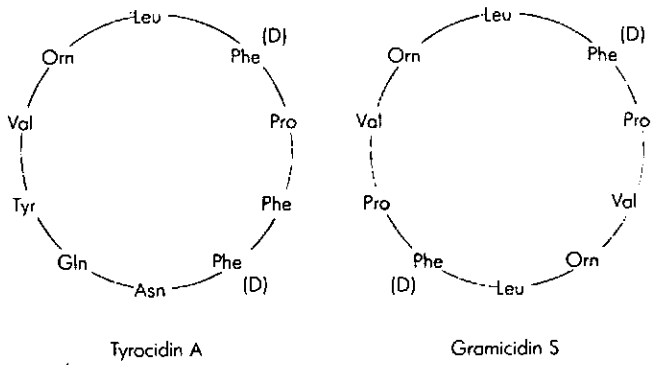


Figure 24-11. The structural formulas of tyrocidine A and gramicidin S, polypeptide antibiotics which exert their antibacterial action through binding with the cytoplasmic membrane. (Amino acid configuration is L except for those marked D.)

biotics act upon cells which have sterols in their cytoplasmic membrane. They act upon fungi (including yeasts) and animal cells but do not affect bacteria. Their antimicrobial action is attributed to their ability to increase cell permeability.

Inhibition of Nucleic Acid and Protein Synthesis

The process by which the cell synthesizes nucleic acids and proteins was described in Chap. 11. As you will recall, synthesis of these substances involves a number of intricate biochemical reactions. It is recommended that these reactions be reviewed to better comprehend the mode of action of those antibiotics that interfere with these metabolic processes.

Examples of the major categories of antibiotics affecting nucleic acid and protein synthesis are described below.

Streptomycin

Streptomycin is produced by *Streptomyces griseus* (Fig. 24-12), a soil organism isolated by Schatz, Bugie, and Waksman, who reported on its antibiotic activities in 1944. It is particularly important because it inhibits many organisms resistant to sulfonamides and penicillin. Its antibacterial spectrum includes many Gram-negative bacteria, including *Francisella tularensis* and some organisms in the

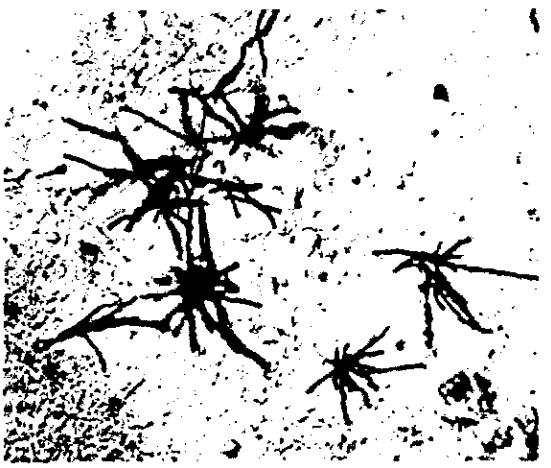


Figure 24-12. *Streptomyces griseus*, the organism that produces streptomycin. This antibiotic inhibits the growth of certain Gram-negative pathogens and *Mycobacterium tuberculosis*. (*Courtesy of the Institute of Microbiology, Rutgers University.*)

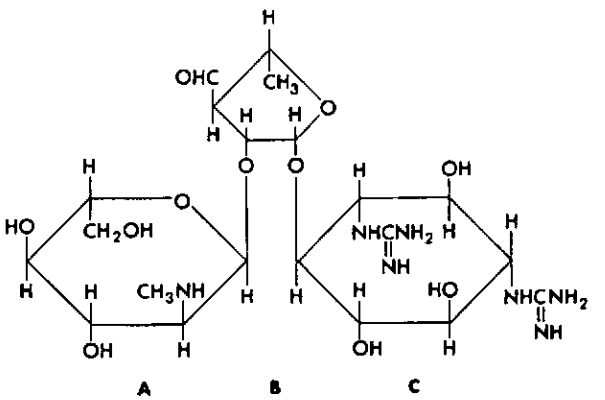
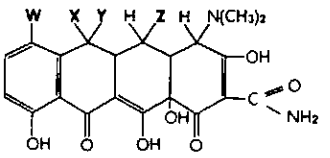


Figure 24-13. Streptomycin. This antibiotic consists of three components, linked glycosidically: (A) N-methyl-L-glycosamine, (B) streptose, and (C) streptidine.



Antibiotic	Position on molecule			
	W	X	Y	Z
Tetracycline	−H	−CH_3	−OH	−H
Oxytetracycline	−H	−CH_3	−OH	−OH
Chlortetracycline	−Cl	−CH_3	−OH	−H
Minocycline	−$N(CH_3)_2$	−H	−H	−H
Doxycycline	−H	−CH_3	−H	−OH

Figure 24-14. Tetracyclines, broad-spectrum antibiotics produced from *Streptomyces*, differ slightly in chemical structure as shown in the positions labeled "w," "x," "y," and "z" in the above molecule.

salmonella group. It is inhibitory for several species of *Mycobacterium*, including *Mycobacterium tuberculosis*. Highly purified streptomycin is nontoxic to humans and other animals when given in small doses, but it appears to have a cumulative detrimental effect on a specific region of the nervous system when given as a medication over long periods of time.

Streptomycin is characterized chemically as an **aminoglycoside** antibiotic; its structure is shown in Fig. 24-13. Other aminoglycoside antibiotics are kanamycin, produced by *Streptomyces kanamyceticus*, and neomycin, produced by *Streptomyces fradiae* and other species of streptomyces.

Streptomycin and other aminoglycoside antibiotics inhibit protein synthesis by combining irreversibly with the 30S subunit mRNA Thus the normal synthetic sequence is disrupted.

Tetracyclines

Chlortetracycline, *oxytetracycline*, *tetracycline*, *doxycycline*, and *minocycline* are generic names for five antibiotics having similar biological and chemical properties. As a group they are commonly called **tetracyclines.** Their structural formulas are shown in Fig. 24-14. Note that the antibiotic produced by *Streptomyces aureofaciens* is chlortetracycline, while *Streptomyces rimosus* produces oxytetracycline. They are broad-spectrum antibiotics with similar antimicrobial spectra, and cross resistance of bacteria to them is common.

Hydrochlorides and bases of the tetracyclines are extremely stable as dry powders. In solutions, tetracycline retains its activity for 3 weeks or more, whereas chlortetracycline and oxytetracycline are less stable. As shown in Fig. 24-14, tetracycline, oxytetracycline, chlortetracycline, minocycline, and doxycycline are chemically very similar. It is not surprising, therefore, that there are no great differences in their activity. The antimicrobial spectra are similar, and all are bacteriostatic in their action. Organisms that are resistant to one are likewise resistant to the others. Tetracycline has a low order of toxicity in laboratory animals. It is readily absorbed from the intestinal tract; hence it is effective when given orally.

The tetracyclines inhibit protein synthesis through interference with the binding of aminoacyl-tRNA to the 30S subunit ribosome.

Chloramphenicol

Chloramphenicol is a broad-spectrum antibiotic active against many Gram-positive and Gram-negative bacteria. Its antimicrobial spectrum is similar to

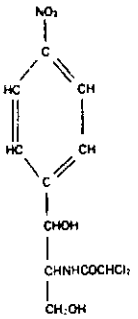


Figure 24-15. Structure of chloramphenicol, a broad-spectrum antibiotic from *Streptomyces venezuelae*.

that of tetracycline. It is also bacteriostatic. Chemically, it is a nitrobenzene ring with nonionic chlorine (Fig. 24-15). The possibility of serious side affects such as blood dyscrasias have limited the use of this antibiotic as a general antibacterial agent.

Chloramphenicol inhibits protein synthesis by combining with the 50S subunit ribosome. The transpeptidation and translocation functions associated with this site are blocked.

Erythromycin

Erythromycin is produced by a strain of *Streptomyces erythraeus* isolated from soil collected in the Philippines. Erythromycin is active against the Gram-positive bacteria, some Gram-negative bacteria, and pathogenic spirochetes. With regard to antimicrobial spectrum and clinical usefulness, it resembles penicillin, but it is also active against organisms that become resistant to penicillin and streptomycin. It is, therefore, often prescribed to those patients with allergies when penicillin is indicated.

Erythromycin belongs to the chemical class of antibiotics known as macrolides. Structurally it contains a large lactone ring linked with amino sugars through glycosidic bonds. Erythromycin inhibits protein synthesis as a result of binding on the 50S subunit ribosome; the steps of transpeptidation and translocation in protein synthesis are blocked.

The specific steps in protein synthesis which are interrupted by various antibiotics are summarized in Fig. 24-16.

Inhibition of Specific Enzyme Systems

The sulfonamides, which were discussed earlier in this chapter for their role in the development of chemotherapy, represent a category of compounds whose antibacterial attack is directed toward a specific essential enzyme. There are numerous sulfonamides as shown in Table 24-1. All of them have the same basic structure. This structure is related to the compound p-aminobenzoic acid. Many bacteria require p-aminobenzoic acid (PABA) as a precursor to their synthesis of the essential coenzyme tetrahydrofolic acid (THFA). PABA is a structural part of the THFA acid molecule. The selective action of sulfonamides is explained by the fact that the PABA molecule and a sulfonamide molecule are so very similar that the sulfonamide may enter the reaction in place of the PABA and block the synthesis of an essential cellular constituent, which in this case is THFA, as shown in Fig. 24-17. The cellular functions of the THFA coenzyme include amino acid synthesis, thymidine synthesis, etc. Lack of this

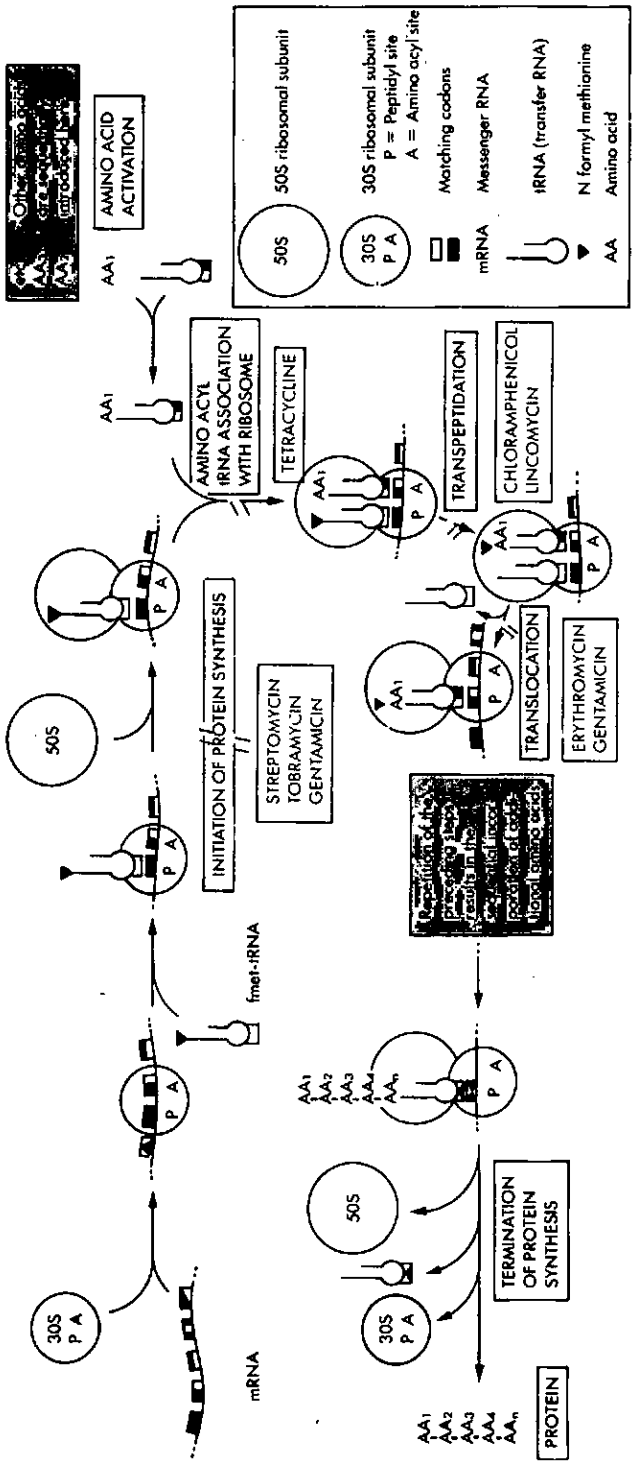
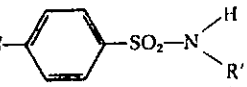
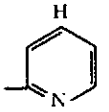
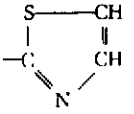
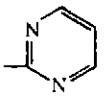
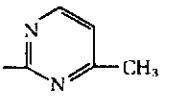
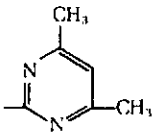
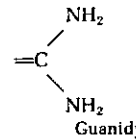


Figure 24-16. Schematic illustration of sites of action of antibiotics on the sequence of protein synthesis. (*Erwin F. Lessel, illustrator.*)

Table 24-1. Some Examples of Sulfonamides

The basic structure for the sulfonamides is  which is a p-aminobenzenesulfonamide. The sulfonamides differ primarily by virtue of the different substituents in the R′ position, as indicated.

Name*	R′
Sulfanilamide	H
Sulfapyridine (*N*′-2-Pyridylsulfanilamide)	 Pyridine
Sulfathiazole (*N*′-2-Thiazolysulfanilamide)	 Thiazole
Sulfadiazine (*N*′-2-Sulfanilamidopyrimidine)	 Pyrimidine
Sulfamerazine [*N*′-(4-Methyl-2-pyrimidyl)-sulfanilamide]	 4-Methylpyrimidine
Sulfamethazine [*N*′-(4,6-Dimethyl-2-pyrimidyl)-sulfanilamide]	 4,6-Dimethylpyrimidine
Sulfaguanidine (*N*′-Guanylsulfanilamide)	 Guanidyl

* The common name is followed by the systematic name.

coenzyme will quite obviously disrupt normal cellular activity. Sulfonamides will inhibit growth of those cells which synthesize their THFA from PABA and will not interfere with the growth of those cells (including mammalian host cells) which require the vitamin folic acid and reduce it directly to THFA. This accounts for the selective antibacterial action of sulfonamides and makes them useful in the treatment of many infectious diseases.

This mode of action is an example of competitive inhibition between an essential metabolite (PABA) and a metabolic analog (a sulfonamide). After the antimicrobial activity of sulfonamides was discovered, D. D. Woods, an English bacteriologist observed that its effect could be reversed by PABA. Although, in

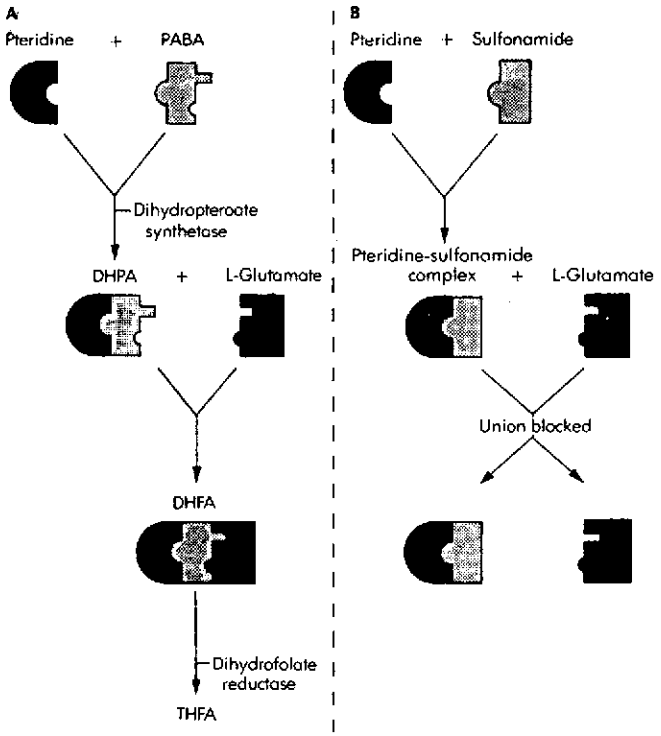


Figure 24-17. The mode of action of sulfonamides in inhibition of tetrahydrofolic acid synthesis. (*Erwin F. Lessel, illustrator.*)

1940, PABA was unknown as a bacterial metabolite, Woods predicted the mode of action described above.

ANTIFUNGAL ANTIBIOTICS

Nystatin is an antifungal agent useful in the therapy of nonsystemic fungal infections. It is produced during fermentation by a strain of *Streptomyces noursei*. This antibiotic was discovered in 1950 by Elizabeth Hazen and Rachel Brown (see Fig. 24-18).

Nystatin

The antimicrobial activity of nystatin is restricted to yeasts and other fungi, e.g., *Candida*, *Aspergillus*, *Penicillium*, and *Botrytis*; it is fungicidal in action. Chemically, nystatin is a polyene with an empirical formula of $C_{46}H_{75}NO_{18}$.

Griseofulvin

Griseofulvin is obtained from *Penicillium griseofulvin*. It is used in the treatment of many superficial fungus infections of the skin and body surfaces and is also effective in the treatment of some systemic (deep-seated) mycoses. The drug is administered orally.

ANTIVIRAL CHEMOTHERAPEUTIC AGENTS

Antibiotics such as those that we have discussed are generally not effective against viruses. You will recall that viruses are intracellular, and hence the

Figure 24-18. Elizabeth Hazen (left) and Rachel Brown examine early samples of the first antifungal antibiotic, nystatin. (*Courtesy of Research Corporation.*)

chemotherapeutic agent, in order to attack the virus, must enter the host cells. Also the agent must not be toxic to the host cell while exerting an inhibiting action on the virus. This demands a high level of selective toxicity. In cases of infection by bacteria, fungi, or protozoa the infectious agent is acted upon outside the host cells. Additionally, there are many more metabolic processes that can be interrupted with these microorganisms.

Among the more promising of the chemotherapeutic agents for treating viral diseases is interferon. Interferons are small glycoprotein substances of which two types are leukocytic interferon and fibroblast interferon. Cells exposed to interferon develop antiviral properties. The antiviral action of interferon is attributed to interference of protein synthesis.

Natural interferons are in very short supply and are expensive. Recent advances in recombinant DNA techniques (genetically engineered bacteria like *Escherichia coli* to produce interferon on a large scale commercially) have increased the availability of interferon for both chemotherapeutic and experimental use.

Acycloguanosine is a nucleoside analog which is active against the herpes virus in animals. Its mode of action appears to be that of inhibition of nucleotide utilization. A synthetic nucleotide analog, 5′-iododeoxyuridine (see Fig. 24-19),

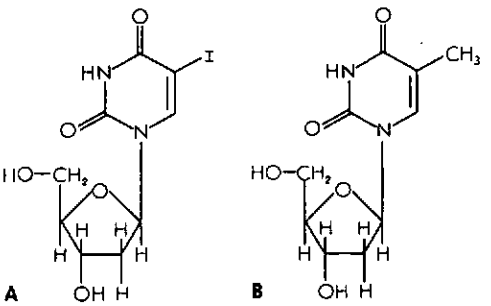


Figure 24-19. (A) The pyrimidine analogue 5′-iododeoxyuridine. (B) Thymidine, the pyrimidine which the analog resembles. This pyrimidine analog exhibits antiviral activity.

has been shown to have antiviral activity and promise as an antiviral chemotherapeutic agent. Its mode of action is most likely that of inhibition of nucleic acid synthesis—preventing the incorporation of thymidine into DNA.

Amantadine is a low-molecular-weight compound which is very effective against influenza A virus; it is not effective against influenza B. The incidence of influenza A infections is greatly reduced by use of this drug. The mode of action of amantadine is that of interfering with the uncoating of virus particles and the subsequent release of their nucleic acids.

ANTITUMOR ANTIBIOTICS

Some antibiotics have been found to possess antitumor activity. The **anthramycin** group (anthramycin, sibromycin, tomaymycin, and neothramycin) is an example of potent antitumor agents. See Fig. 24-20. One of the complicating factors associated with the potential use of these anticancer agents is that they are also cardiotoxic, a fact that illustrates the need for a high level of specificity in a chemotherapeutic agent. The antitumor action of these antibiotics is directed toward DNA structure and function. One of the problems is that of determining whether, through the manipulation of the structure of an antibiotic, e.g. anthramycin, one can cut out the cardiotoxic property without destroying the antitumor property.

SYNTHETIC CHEMOTHERAPEUTIC AGENTS

Nitrofurans

The **nitrofurans** are antimicrobial drugs which differ from the antibiotics in that they do not occur naturally. The prototype of the nitrofuran derivatives is furfural, which can be prepared from corncobs and cornstalks, oat hulls, beet pulp, peanut hulls, and other vegetable by-products. Furfural, an aldehyde derivative of furan chemically known as 2-furaldehyde, was identified in 1832 as an accidental finding during sugar-distillation studies.

However, it was not until 1944 that the American investigators Dodd and Stillman reported on the discovery of the antimicrobial properties of nitrated

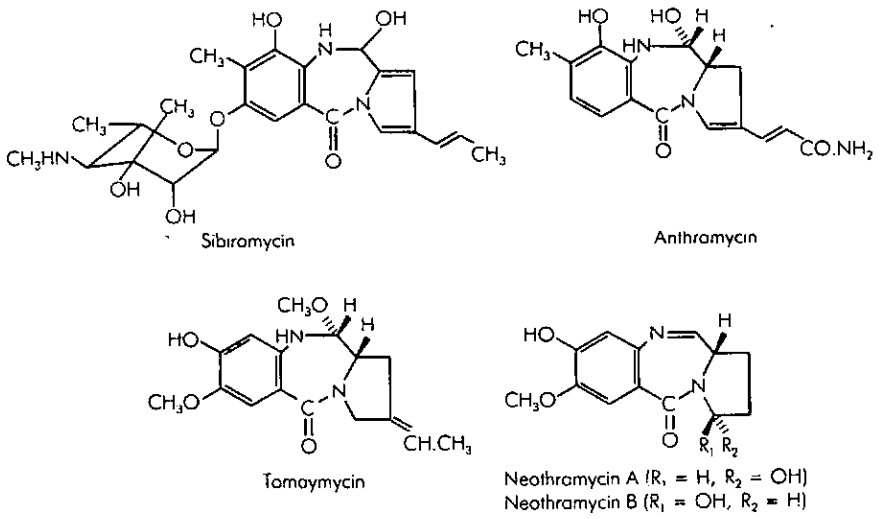


Figure 24-20. Structure of the anthramycin group of antitumor antibiotics.

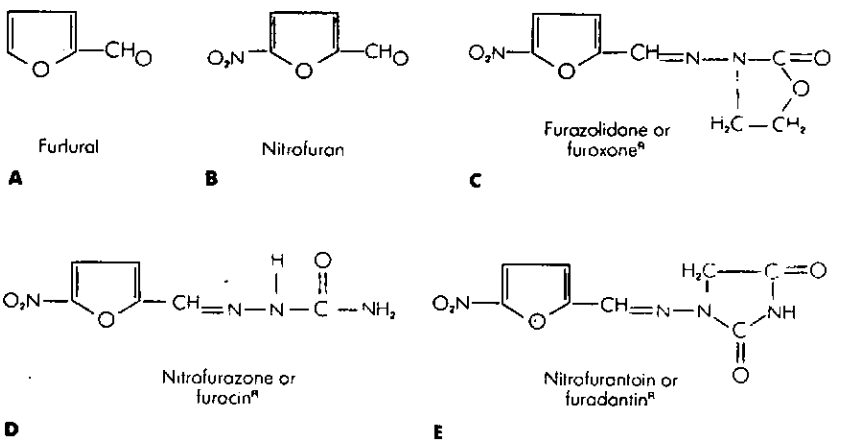


Figure 24-21. Furfural (A) is the prototype of nitrofuran compounds, and (B), (C), (D), and (E) are chemotherapeutic derivatives of furfural.

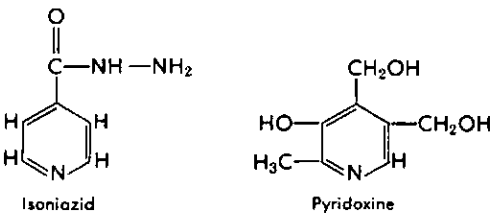


Figure 24-22. Isoniazid, a structural analog of pyridoxine (vitamin B_6), may prevent the growth of microorganisms by blocking pyridoxine-catalyzed reactions in the microbial cell.

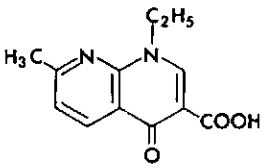


Figure 24-23. Nalidixic acid is a synthetic antibacterial drug with a selective action against bacterial DNA synthesis.

furan derivatives. They found that a surprisingly high antibacterial effect was conferred upon furans by the addition of a nitro group in the 5-position of the furan ring. Variations in the side chain in the 2-position of 5-nitrofuran provide a wide spectrum of compounds in this group. Over 1,000 compounds have been synthesized and studied.

The chemical structures of some chemotherapeutic nitrofurans are shown in Fig. 24-21. As a class, the nitrofurans generally are effective against a broad spectrum of both Gram-positive and Gram-negative bacteria, several pathogenic protozoa, and some fungi which cause superficial infections in both humans and other animals.

Isonicotinic Acid Hydrazide (Isoniazid)

Isoniazid has an important, though restricted, application in the therapy of disease. It is an example of competitive inhibition affecting a restricted group of microorganisms, the mycobacteria. It has proved to be very useful in the control of tuberculosis in humans and is more effective when given alternately with streptomycin. Because it is a structural analog of pyridoxine, or vitamin B_6 (see Fig. 24-22) and nicotinamide, isoniazid can block pyridoxine- and nicotinamide-catalyzed reactions. This may account for its antimicrobial activity.

Nalidixic Acid

Nalidixic acid is a synthetic chemical with a structural formula as shown in Fig. 24-23. It is a useful chemotherapeutic agent for urinary-tract infections caused by Gram-negative bacteria. Its antimicrobial activity is attributed, at least in part, to inhibition of DNA synthesis.

DEVELOPMENT OF RESISTANCE TO ANTIBIOTICS

Drug resistance is one of nature's never-ending processes whereby organisms develop a tolerance for new environmental conditions. Drug resistance may be due to a preexisting factor in the microorganism, or it may be due to some acquired factor(s). Penicillin resistance, for example, may result from the production of penicillinase by resistant organisms, which converts penicillin into inactive penicilloic acid. On the other hand, some normally susceptible strains of bacteria may acquire resistance to penicillin. Acquired resistance is also due to penicillinase production in genetically adapted varieties of microorganisms. In cultures of penicillin-sensitive bacteria, perhaps one organism in a hundred million may be a penicillin-resistant mutant. Normally the ratio of sensitive to resistant organisms is maintained, and no problem develops. When penicillin is present, the sensitive strains do not reproduce whereas the resistant mutants do and eventually dominate the population. This has important clinical implications and is one of the practical reasons why research effort has been made to develop synthetic penicillins which are resistant to the action of penicillinase.

Many organisms which do not produce penicillinase are also resistant to penicillin. This suggests an alternative metabolic pathway or enzyme reaction not susceptible to inhibition by penicillin.

Other mechanisms of drug or antibiotic resistance may be due to (1) competitive inhibition between an essential metabolite and a metabolic analog (drug), (2) development of an alternate metabolic pathway which bypasses some reaction that would normally be inhibited by the drug, (3) production of an enzyme altered in such a way that it functions on behalf of the cell but is not affected by the drug, (4) synthesis of excess enzyme over the amount that can be inactivated by the antibiotic or drug, (5) inability of the drug to penetrate the cell due to some alteration of the cell membrane, (6) alteration of ribosomal protein structure.

An illustration of the pattern of resistance of bacteria to antibiotics over a three-year period is shown in Table 24-2.

Table 24-2. Occurrence of Single and Multiple Resistance in S. aureus in Hospitalized Patients during the Period 1977–1980

Resistance Pattern*	Resistant, %	Resistance Pattern*	Resistant, %
XX	91	XX CT	3.6
CT	4	XX CX	4.6
TC	44	XX EM FU LM	0.012
EM	10	XX EM FU LM GM	0.00083
SU	94	XX LM	0.91
CX	5	CX LM	0.05
FU	13	CX GM	0.35
LM	1	CT EM	0.40
GM	7	TC EM	4.4
XX TC EM	4	TC EM SU	4.1
XX TC CX	1.9	TC GM	3.1
XX TC EM CX	0.20	TC FU	5.7
XX TC EM CX FU	0.026	EM CX	0.5
XX EM	9.1	EM LM	0.10
XX TC	40	FU LM	0.13

* XX = β-lactamase production; CT = cephalothin; TC = oxytetracycline; EM = erythromycin; SU = sulfadimidine; CX = isoxazolylpenicillins (and methicillin); FU = fusidic acid; LM = lincomycin; GM = gentamicin; CL = chloramphenicol.

SOURCE: T. Bergan and J. Lernestedt, "Antibiotic Resistance in Staphylococci from a Hospital Environment," *Chemotherapy*, 29:28–36, 1983.

Transmission of Drug Resistance

When chemotherapeutic agents such as the sulfonamides and antibiotics were first used, development of bacterial resistance was rather infrequent. Resistance became much more of a problem as the widespread use of antibiotics led to the elimination of sensitive organisms from the population with the accompanying increase in the numbers of resistant organisms.

The initial appearance of resistant organisms was thought to be the result of a change in a single bacterial gene that conferred resistance to the bacterium. The evidence that this takes place during sulfonamide therapy is convincing. Another, more recent, explanation for the development of resistance, at least in some Gram-negative bacteria, is that resistant organisms have an additional gene whose function is to protect the bacterium from the bactericidal effect of the drug or antibiotic. For example, such a gene is responsible for penicillinase production by penicillin-resistant staphylococci. In some instances bacteria carry the resistant gene at the time of infection, and their propagation is encouraged while sensitive strains are inhibited or killed. At other times the resistant gene is transmitted by conjugation from other bacteria during treatment.

Gene transfer between cells has been explained in Chap. 12 as being accomplished by transformation, transduction, or conjugation. Here we are interested in the transfer of antibiotic resistance by conjugation. This phenomenon was first reported independently in 1958 by two Japanese scientists, Akiba and Ochiai. They isolated both antibiotic-sensitive and antibiotic-resistant organisms of the same serotype from patients with enteric infections being treated with sulfonamides, tetracyclines, streptomycin, or chloramphenicol. They went on to demonstrate that this was due to resistant genes in a reservoir of *Escherichia coli* in the intestinal tract being transferred to *Shigella dysenteriae* that caused the infection. Since then transfer of antibiotic resistance by bacterial conjugation has been observed in other organisms and in other parts of the world.

We now know that this resistance factor, or R factor, is present in plasmids, which are small extrachromosomal self-replicating extranuclear DNA units (Chap. 12). The transmission of resistance factors in enteric infections is especially important in places where such infections are common. Organisms that are good recipients of the R factor from an *E. coli* donor include species of *Enterobacter*, *Klebsiella*, *Salmonella*, and *Shigella*. Weak recipients are species of *Pasteurella*, *Proteus*, and *Serratia*.

Antibiotic resistance represents a serious problem for clinicians, and great effort is being made to understand the mechanisms involved and to prevent its occurrence. The development of resistance can be minimized by (1) avoiding the indiscriminate use of antibiotics where they are of no real clinical value, (2) refraining from the use of antibiotics commonly employed for generalized infections for topical applications, (3) using correct dosages of the proper antibiotic to overcome an infection quickly, (4) using combinations of antibiotics of proved effectiveness, and (5) using a different antibiotic when an organism gives evidence of becoming resistant to the one used initially.

MICROBIOLOGICAL ASSAY OF ANTIBIOTICS

The potency of antibiotic content in samples can be determined by chemical, physical, and biological means. An assay is made to determine the ability of an

antibiotic to kill or inhibit the growth of living microorganisms. Biological tests offer the most convenient means of making an assay.

Chemical Assay

Where antibiotics exist in pure chemical form, their concentration can be expressed in micrograms of the pure chemical per milligram of the specimen. To be of value, such tests must give results that correlate well with those obtained in biological assays. Chemical-assay methods are generally more accurate and require less time than biological methods, but they are less sensitive, and caution must be used lest biologically inactive degradation products give misleading results.

Biological Assay

Biological potency is expressed in terms of either micrograms or units determined by comparing the amount of killing, or bacteriostasis, of a test organism caused by the substance under test with that caused by a standard preparation under rigidly controlled conditions (see Fig. 24-24).

Although the unit of measurement for some antibiotics is arbitrary, it is established by international agreement for some antibiotics and by the FDA regulation for others. For example, the international unit of penicillin is the

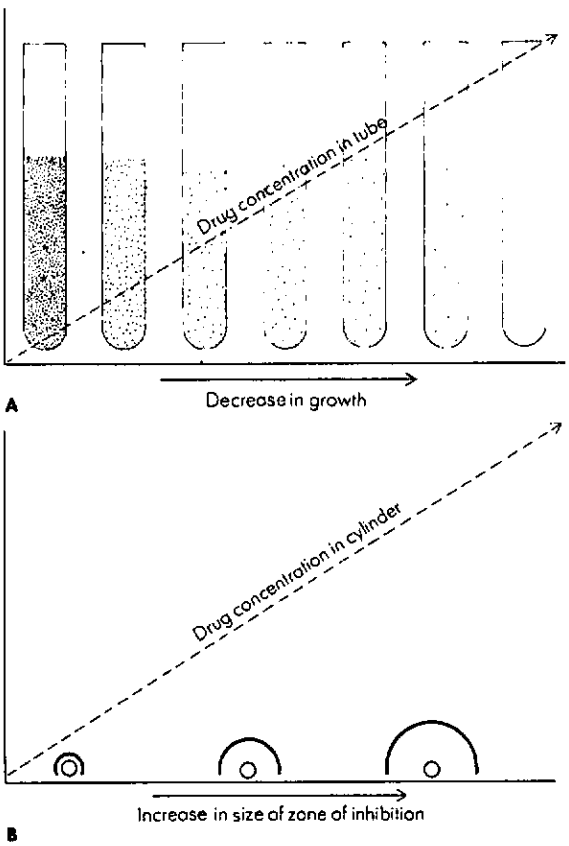


Figure 24-24. Microbiological assay of antibiotics and some other chemotherapeutic agents is accomplished by either the tube-dilution or the cylinder-plate method (a variation of the paper-disk-plate technique). (A) In the tube-dilution technique, the inhibition of growth (decrease in turbidity) produced by the unknown sample is compared with that produced by the known or standard sample. The amount of antibiotic present in the unknown sample can then be calculated. (B) The cylinder-plate technique follows much the same procedure for determining antibiotic potency, except that inhibition of growth is measured in terms of the size of the zones of inhibition.

amount of activity produced under defined conditions by 0.5988 μg of the International Standard, which is a sample of pure benzyl-penicillin (1 mg = 1,667 units). Some modifications of the method referred to above measure the interference of the antibiotic with the production by the test organism of a characteristic metabolic product such as acid, hemolysin, or the enzyme reductase.

The assay of antibiotics in blood serum, urine, tissues, and other similar substances presents some special problems because (1) the amounts present are generally very small as compared with other substances, (2) the antibiotic may be bound to proteinaceous materials in the specimen, (3) normal inhibitory substances may be present in the blood or other body fluids. Therefore, the techniques described above are modified to make them more sensitive for these specimens.

Table 24-3. Basic Sets of Antimicrobial Agents to be Tested Routinely Against Rapidly Growing Aerobic and Facultatively Anaerobic Bacteria*

Antimicrobics	Staphylococci and Streptococci	Enterococci	Enteric Gram-Negative Bacilli Other than *P. aeruginosa*		*P. aeruginosa*
			Urinary	Other	
Penicillins					
Ampicillin		1	1	1	
Carbenicillin			1	1	1
Nafcillin, oxacillin, or methicillin†	1				
Penicillin G	1	1			
Cephalosporins					
Cefamandole	2		1‡	1‡	
Cefoxitin	2		1‡	1‡	
Cephalothin	1		1	1	
Chloramphenicol	2	2		2	
Clindamycin	1				
Erythromycin	1	1			
Aminoglycosides					
Amikacin			1	1	1
Gentamicin	2		1	1	1
Kanamycin	2		1	1	
Tobramycin			1	1	1
Polymyxin B or E			2	2	1
Tetracycline	2	2	1	1	
Vancomycin	2				
Urinary tract agents					
Nalidixic acid			1		
Nitrofurantoin			1		
Sulfonamides			1		
Sulfamethoxazole/trimethoprim			1		

* 1 = primary set; 2 = secondary drugs.

† Oxacillin or nafcillin is preferable for detecting heteroresistant methicillin-resistant S. aureus.

‡ Cefamandole and cefoxitin may be reserved for testing cephalothin-resistant organisms only.

SOURCE: E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant (eds.): *Manual of Clinical Microbiology*, 3d ed., American Society for Microbiology, Washington, 1980.

MICROBIAL SUSCEPTIBILITY TO CHEMOTHERAPEUTIC AGENTS

Species and strains of species of microorganisms have varying degrees of susceptibility to different antibiotics. Furthermore, the susceptibility of an organism to a given antibiotic may change, especially during treatment. It is therefore important for the clinician to know the identity of the microbe and the specific antibiotic which may be expected to give the most satisfactory results in treatment (Table 24-3). For this information the microbiologist will be called upon to make an accurate microbiological diagnosis and to determine the susceptibility of the organism to various antibiotics. From time to time during the course of therapy the microbiologist may be required to make estimates of any change in the susceptibility of the pathogen to the drug, and possibly even to assay the antibiotic concentration in the body fluids.

Tube-Dilution Technique

The susceptibility of a microorganism to antibiotics and other chemotherapeutic agents can be determined by either the **tube-dilution** or the **paper-disk-plate** technique. By the tube-dilution technique, one can determine the smallest amount of chemotherapeutic agent required to inhibit the growth of the organism in vitro (see Fig. 24-25). This amount is referred to as the **MIC (minimal inhibitory concentration).**

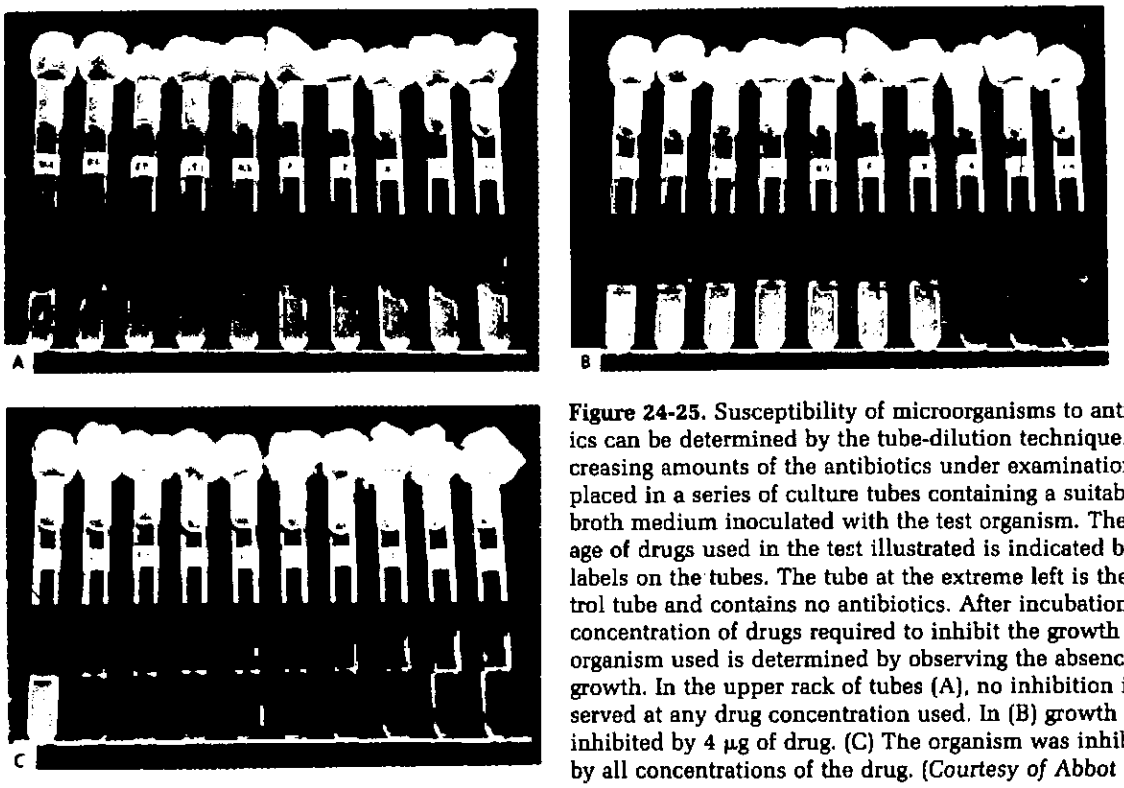


Figure 24-25. Susceptibility of microorganisms to antibiotics can be determined by the tube-dilution technique. Increasing amounts of the antibiotics under examination are placed in a series of culture tubes containing a suitable broth medium inoculated with the test organism. The dosage of drugs used in the test illustrated is indicated by the labels on the tubes. The tube at the extreme left is the control tube and contains no antibiotics. After incubation, the concentration of drugs required to inhibit the growth of the organism used is determined by observing the absence of growth. In the upper rack of tubes (A), no inhibition is observed at any drug concentration used. In (B) growth was inhibited by 4 μg of drug. (C) The organism was inhibited by all concentrations of the drug. (*Courtesy of Abbot Laboratories.*)

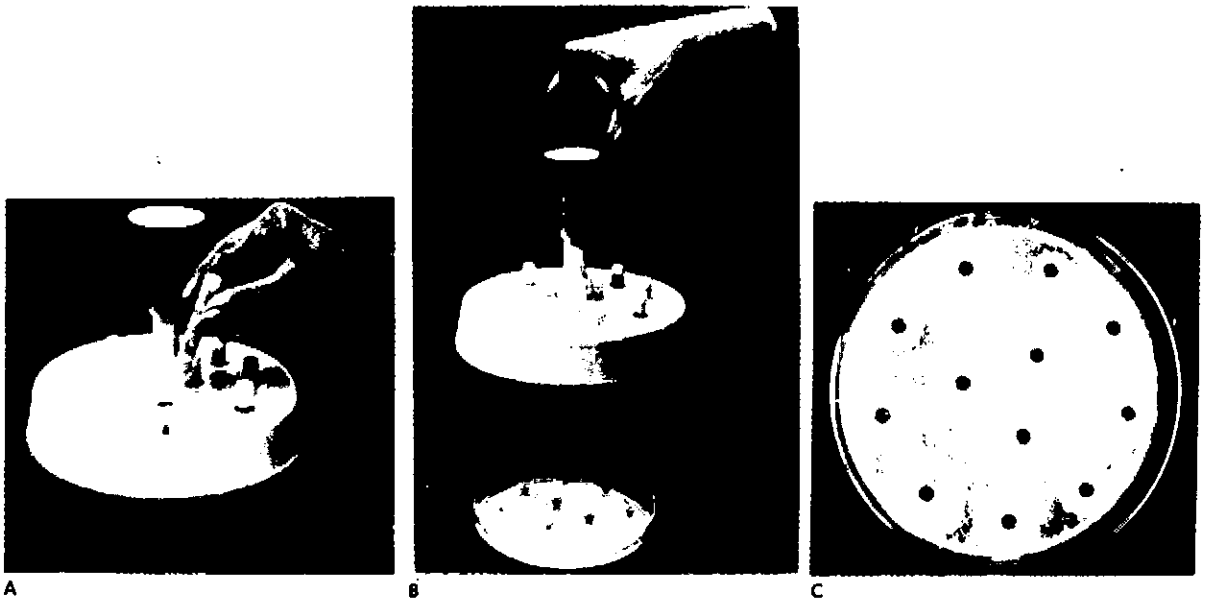


Figure 24-26. The paper-disk-plate method for determining the susceptibility of microorganisms to antibiotics. (A) Automatic dispenser of paper disks impregnated with antibiotics. (B) Disks positioned on inoculated Petri dish before incubation. (C) Zones of inhibition develop after incubation around each disk that contains an antibiotic that inhibits growth of microoganisms. (*Courtesy of Becton-Dickinson, BBL Microbiology Systems.*)

Disk-Plate Technique

The paper-disk-plate method is the most commonly used technique for determining susceptibility of microorganisms to chemotherapeutic agents. Small paper disks impregnated with known amounts of chemotherapeutic agents are placed upon the surface of an inoculated plate. After incubation, the plates are observed for any zones of inhibition surrounding the disks (see Fig. 24-26). A zone of inhibition (a clear area) around the disk indicates that the organism was inhibited by the drug, which diffused into the agar from the disk.

The single-disk method for susceptibility testing currently recommended by the FDA is a slight modification of the procedure developed by Bauer, Kirby, Sherris, and Turck in 1966. This is a highly standardized technique; the amount of antimicrobial agent contained in the disk is specified as well as the test medium, size of the inoculum, conditions of incubation, and other details. When the susceptibility test is performed in conformity with the FDA procedure, one can correlate the sizes of the zones of inhibition with the MIC of the drug for the microorganism in question; it is possible to determine whether the microorganism is resistant or susceptible to the antimicrobial agent. The relationship of MIC's to zone-of-inhibition diameters for the antibiotic cephalothin against several bacteria is shown in Fig. 24-27.

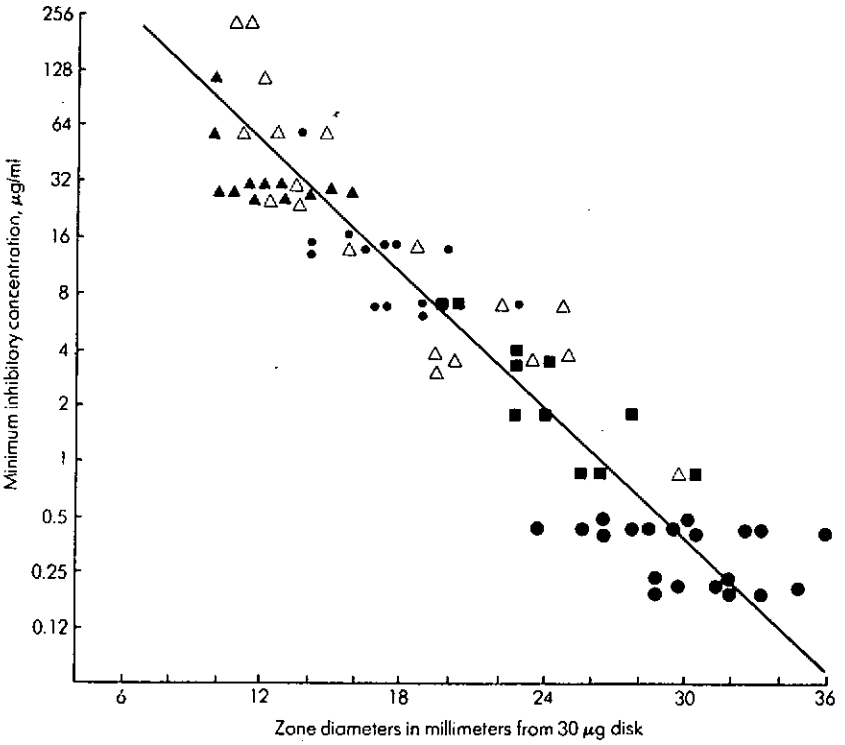


Figure 24-27. The relationship between the dilution and diffusion methods of testing the ability of an antibiotic to inhibit bacterial growth is demonstrated here for cephalothin. The size of the inhibition zone produced by an antibiotic disk goes up as the MIC goes down. All test conditions must be held constant. ▲ Enterococci, ● *Staphylococcus aureus*, • *Escherichia coli*, △ *Enterobacter-Klebsiella*, *Haemophilus*. (*Courtesy of K. J. Ryan, F. D. Schoenknecht, and W. M. M. Kirby, Hosp Pract, p. 99, 1970.*)

NONMEDICAL USES OF ANTIBIOTICS

Antibiotics are used as growth stimulants in poultry and livestock feeds. After the discovery that many domestic food-producing animals require vitamin B_{12} for optimum growth when fed a diet consisting of plant protein, it developed that by adding wastes from fermentation by-products to feeds, growth was stimulated more than could be accounted for by B_{12} alone. Even when adequate amounts of B_{12} were present in the diet, more rapid growth of young animals was noted when they were fed mash from the antibiotic fermenters. Use of pure antibiotics has given similar results. Commercially, the addition of aureomycin, terramycin, or penicillin to swine or poultry feeds at the rate of 5 to 20 g per ton of feed increases the rate of growth of young animals by at least 10 percent and sometimes by as much as 50 percent.

The stimulating effect of antibiotics on growth of domestic animals may be explained in several ways:

1 The antibiotics may destroy bacteria and other intestinal parasites that cause subclinical disease and retard growth and development. For example, it has been suggested that pigs respond dramatically to the addition of oxytetracycline to their diet because the antibiotic inhibits the growth of *Clostridium perfringens* in their intestines and prevents or reduces a chronic but subclinical toxemia.
2 Removal of the saprophytic bacteria from the intestinal tract may have a beneficial effect on the nutrition of the animals.

3 Streptomycin may have a "sparing effect" on the B_{12} in the diet, making it available in greater quantities for utilization by the animals.

The practice of supplementing animal feed with antibiotics has raised the issue of widespread development of bacterial resistance. The broad exposure of microorganisms to antibiotics provided by antibiotic supplemental feeds has led to restrictions on this practice.

Because they inhibit the growth of some bacteria and do not affect others, antibiotics have been widely used to prevent bacterial growth in tissue and culture fluids and in chick embryos used for the cultivation of viruses. Fleming's first use of penicillin was to add the crude mold-culture filtrate to media used for the isolation of *Haemophilus influenzae* from nose-and-throat washings. The antibiotic inhibited the Gram-positive cocci present but permitted *H. influenzae* to grow.

Some antibiotics are effective against plant pathogens and are attractive for treatment of plant diseases. The extent of this practice is limited mainly by economic factors, i.e., the cost of the antibiotic.

QUESTIONS

1 Define the following terms: chemotherapeutic agent, chemotherapy, antibiotics, and antibiosis.
2 Are all antibiotics useful as chemotherapeutic agents? Explain.
3 What are the characteristics of an ideal chemotherapeutic agent?
4 What contributions did each of the following persons make to the field of chemotherapy: Ehrlich, Domagk, Fleming, Waksman, Dubos, Hazen, and Brown?
5 Antibiotics are generally effective against bacterial infections and ineffective against viral infections. What are some of the reasons for this?
6 What are the major modes of antibacterial action of chemotherapeutic agents?
7 Describe the mode of antimicrobial action of the following chemotherapeutic agents: penicillins, cephalosporins, streptomycin, chlortetracycline, bacitracin, and sulfonamides.
8 How can the potency (or units) of an unknown sample of penicillin be determined?
9 Why is it important to determine the susceptibility of an infectious microorganism to chemotherapeutic agents? How can this be done?
10 Why are some antibiotics used to supplement animal foods?
11 In terms of their mode of action, explain why some chemotherapeutic agents are bacteriostatic and others bactericidal.
12 Which genera of microorganisms produce the most antibiotics?
13 What is the objection to using antibiotics as a food preservative?
14 Outline an experiment by which you could proceed to search for new antibiotics.
15 Assuming that you discovered one or more new antibiotics, what more would you need to know to ascertain whether any of them were good for chemotherapy?

REFERENCES

Abraham, E. P.: "Some Aspects of the Development of the Penicillins and Cephalosporins," in E. Don Murray and A. W. Bourquin (eds.), *Developments in Industrial Microbiology*, Society for Industrial Microbiology, Washington, D.C., 1974. *An interesting short story of the early development of antibiotics.*

Franklin, D. J., and G. A. Snow: *Biochemistry of Antimicrobial Action*, 3d ed., Chapman and Hall, Ltd., London, 1981. *A short book (215 pages) which presents in a very direct manner the characteristics of medically important antimicrobial compounds with a description of their mode of action.*

Gale, E. F., et al.: *The Molecular Basis of Antibiotic Action*, 2d ed., Wiley, New York, 1981. *Highly technical discussion of the mode of action of antibiotics in terms of modern molecular biology.*

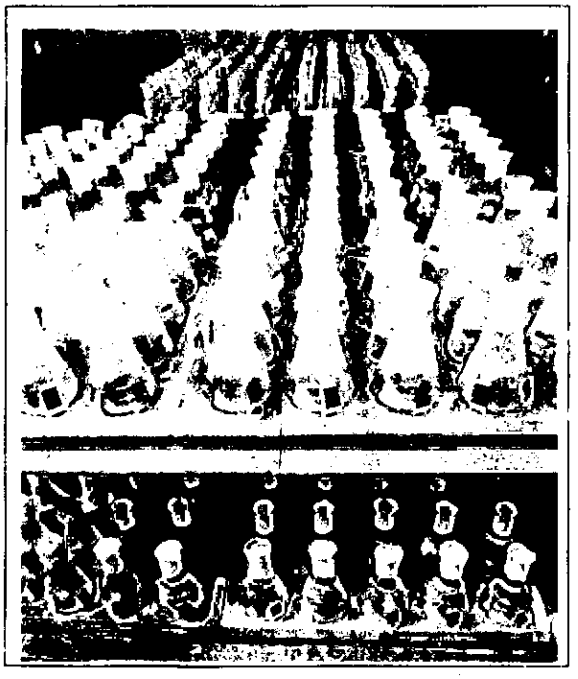
Garrod, L. P., H. P. Lambert, and F. O'Grady (eds.): *Antibiotics and Chemotherapy*, 5th ed., Churchill Livingstone, Edinburgh, 1981. *Mainly about antibiotics, but it also deals with sulfonamides and other synthetic drugs used in the treatment of infectious diseases. The discussion of modes of action of antibiotics and chemotherapeutic drugs is very instructive.*

Korzybski, Tadeusz, et al.: *Antibiotics: Origin, Nature, and Properties*, American Society for Microbiology, Washington, D.C., 1978. *A systematic presentation of a large amount of information on the sources, varieties, and properties of antibiotics in a three-volume publication.*

Lancini, G., and F. Parenti, with M. Starr (ed.): *Antibiotics: An Integrated View* (translated from the Italian by Betty Rubin), Springer-Verlag, New York, 1982. *A small, concise book (253 pages), suitable for students, in which the authors attempt to present the basic facts and concepts on all aspects of the science of antibiotics.*

Lennette, E. H., A. Balows, W., J. Hausler, Jr., J. P. Truant, and H. J. Shadomy (eds.): *Manual of Clinical Microbiology*, 4th ed., American Society for Microbiology, Washington, D.C., 1985. *Section eleven of this comprehensive manual covers in considerable detail the methodology of determining the susceptibility of microorganisms to antimicrobial agents.*

PART SEVEN ENVIRONMENTAL AND INDUSTRIAL MICROBIOLOGY



Life under extreme conditions

New discoveries are continually being made that expand our ideas about the ability of life to exist under harsh environmental conditions. For instance, although the surface waters of the ocean contain many microorganisms, it has long been thought that relatively few bacteria exist on the deep ocean floor because of the low temperature of the water, the scarcity of nutrients, and absence of light to support the growth of phototrophic organisms, and the enormous hydrostatic pressure due to the great depth of the overlying water column. This is indeed true for most regions of the ocean floor, but a startling exception was found in the late 1970s during exploration of deep-sea hydrothermal "vents" (hot submarine springs) located along submarine tectonic rifts and ridges of the ocean floor at a depth of 2,500 to 2,600 meters. In the areas surrounding these vents, living organisms were discovered in amazing and unexpected abundance, ranging from various bacteria to invertebrate animals such as giant clams, mussels, and tube worms.

The occurrence of this proliferation of organisms was partially accounted for by the increased temperature of the water in the regions surrounding the vents (10 to 20°C above the normal seawater temperature of 2.1°C), but what was the primary source of carbon and energy on which all of this life depended? The answer came when further studies indicated that the vents discharge heated water rich in geothermally produced H_2S and other reduced inorganic compounds into the surrounding waters. Moreover, geological and geochemical evidence indicated that oxygenated seawater percolates through nearby porous lava and mixes with the heated water issuing from the vents. Thus the supply of a reduced compound (H_2S) as well as the additional occurrence of oxygen might support the growth of certain chemolithotrophic bacteria that obtain energy by oxidizing H_2S and which use CO_2 as their carbon source.

Further support for this idea came when samples were obtained from the seawater and from the invertebrate animals near vent discharges by means of a submersible, deep-sea research vessel. From these samples, microbiologists from the Woods Hole Oceanographic Institution isolated cultures of chemolithotropic, sulfide-oxidizing bacteria such as *Thiomicrospira* and *Thiobacillus*-like organisms. It is likely that these and possibly other autotrophic bacteria represent the predominant or sole primary food source for the various animal populations living near the vents.

The discovery of an unexpected abundance of life occurring in these remote regions of the ocean floor has opened an entirely new and exciting area for biological research and is currently the subject of intense investigation.

Preceding page. Cultures of microorganisms being incubated on shaker platforms prior to testing for pharmaceutical products. (*Courtesy Cetus Corporation.*)

Chapter 25 Microbiology of Soil

OUTLINE

However dead the earth may look and be considered in our thoughtless moments, the experience of man far back beyond his written records has led him to associate trouble capable of multiplying itself as coming from dirt. Bacillus tetanus, amoebic dysentery, thermophilic spoilage, actinomycosis, and botulism are new terms, but the need of freedom of earth in wounds, in food, and in clothing is no recent discovery. The demonstration that soil, instead of being all dead, harbors millions of organisms releases that flight of imagination which pictures the soil as a sort of Lilliputian Zoo in which some magic hand has eliminated all barriers and set free every grade of minute but rapacious monster to go roaring after the next lesser grade as its lawful prey. Thus the soil is pictured to us in terms that lead us to ask what manner of thing it is.

This paragraph is from the introduction of an address entitled "A Microbiologist Digs in the Soil" given by the late Charles Thom, one of America's great mycologists and soil microbiologists.

Directly or indirectly the wastes of humans and other animals, their bodies,

and the tissues of plants are dumped onto or buried in the soil. Somehow they all disappear, transformed into the substances that make up the soil. It is the microbes that make these changes—the conversion of organic matter into simple inorganic substances that provide the nutrient material for the plant world. Thus microorganisms play a key role in maintaining life on earth as we know it.

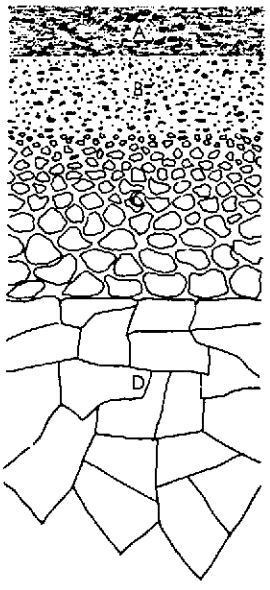
PHYSICAL CHARACTERISTICS OF SOIL

Soil has been defined as *that region on the earth's crust where geology and biology meet*. From a functional viewpoint, the soil may be considered as the land surface of the earth which provides the substratum for plant and animal life. The characteristics of the soil environment vary with locale and climate. Soils differ in depth, physical properties, chemical composition, and origin. A profile of soil is shown in Fig. 25-1.

Mineral Particles

The dominant mineral particles in most soils are compounds of silicon, aluminum, and iron, and lesser amounts of other minerals, including calcium, magnesium, potassium, titanium, manganese, sodium, nitrogen, phosphorus, and sulfur. The mineral constituents of soil range in size from small clay particles (0.002 mm or less) to large pebbles and gravel. The physical structure, aeration, water-holding capacity, and availability of nutrients are determined by the proportion of these particles, which are formed by the weathering of rock and the degradative metabolic activities of microorganisms.

Soils can be classified as **mineral soils,** which have solid matter that is largely inorganic, and **organic soils,** which have very little inorganic material. The latter are typically found in bogs and marshes. Most of the information in this chapter is concerned with mineral soils.



Horizon A: Organic debris in various stages of decomposition and minerals

Horizon B: Fine particles and minerals

Horizon C: Weathered mineral materials excluding bedrock

Horizon D: Unweathered rock, bedrock

Figure 25-1. A schematic illustration showing the profiles of soil.

Organic Residue

The plant and animal remains deposited on or in the soil contribute organic substances. Their decomposition will be described later in this chapter. In the last stages of decomposition, such material is referred to as **humus**, a dark-colored, amorphous substance composed of residual organic matter not readily decomposed by microorganisms. Indeed, the microbial population, both dead cells and living cells, is of such a large magnitude that it contributes significantly to the organic matter of soil. Certain agriculturally important properties are contributed to the soil by humus, which improves the texture and structure of the soil, contributes to its buffering capacity, and increases its water-holding capacity.

Water

The amount of water in soil depends on the amount of precipitation and other climatic conditions, drainage, soil composition, and the living population of the soil. Water is retained as free H_2O in the spaces between soil particles and adsorbed to the surfaces of particles. Various organic and inorganic components of soil are dissolved in soil water and thus are made available as nutrients for soil inhabitants.

Gases

The soil atmosphere is derived from air but differs in composition from it because of the biological processes occurring in soil. The gaseous phase of soil consists mainly of carbon dioxide, oxygen, and nitrogen. These gases exist primarily in the spaces between soil particles which are not filled with water, although a small amount of gas, especially carbon dioxide, is dissolved in water. Obviously, then, the amount of gases in the soil is related to the amount of moisture.

MICROBIAL FLORA OF SOIL

Fertile soil is inhabited by the root systems of higher plants, by many animal forms (e.g., rodents, insects and worms), and by tremendous numbers of microorganisms.

The vast differences in the composition of soils, together with differences in their physical characteristics and the agricultural practices by which they are cultivated, result in corresponding large differences in the microbial population both in total numbers and in kinds.

The conditions described earlier as influencing the growth of organisms under laboratory cultivation are equally applicable to the soil. With specific reference to soil, these conditions can be summarized as follows: (1) amount and type of nutrients, (2) available moisture, (3) degree of aeration, (4) temperature, (5) pH, (6) practices and occurrences which contribute large numbers of organisms to the soil, e.g., floods or addition of manure. The existence of roots and the extensiveness of the root system in soil also influence the numbers and kinds of microorganisms present.

Variations of climatic conditions may selectively favor certain physiological types. Interactions between and among microbial species no doubt has an important effect on the members of the population. This is an extremely complex situation. Predatory protozoa and antibiotic-producing actinomycetes may eliminate certain groups of microorganisms. Cellulolytic and proteolytic organisms, on the other hand, may provide nutrients for less versatile biochemical species.

Few environments on earth have as great a variety of microorganisms as fertile soil. Bacteria, fungi, algae, protozoa, and viruses make up this microscopic menagerie, which may reach a total of billions of organisms per gram (Table 25-1). The great diversity of the microbial flora makes it extremely difficult to determine accurately the total number of microorganisms present. Cultural methods will reveal only those physiological and nutritional types compatible with the cultural environment. Direct microscopic counts theoretically should permit enumeration of all except the viruses, but this technique also has its limitations, especially in distinguishing living from dead microorganisms. Very often the microbiological analysis of soil is concerned with the isolation and identification of specific physiological types of microorganisms. For this purpose enrichment-culture techniques are appropriate.

Bacteria

The bacterial population of the soil exceeds the population of all other groups of microorganisms in both number and variety (see Table 25-2). Direct microscopic counts as high as several billions per gram have been reported; plate counts from the same samples yield only a fraction of this number (millions). The reason for this discrepancy is that there is such a great variety of nutritional and physiological types of bacteria in soil that no single laboratory environment (i.e., composition of medium and conditions of incubation) supports the growth of every viable cell in the inoculum. The following are all likely to be found in soil: autotrophs and heterotrophs; mesophiles, thermophiles, and psychrophiles; aerobes and anaerobes; cellulose digesters and sulfur oxidizers; nitrogen fixers and protein digesters; and other kinds of bacteria. It is generally agreed that there are many species of bacteria in soil yet to be discovered.

Table 25-1. Soil Population in a Fertile Agricultural Soil

Type	Number per Gram
Bacteria:	
Direct count	2,500,000,000
Dilution plate	15,000,000
Actinomycetes	700,000
Fungi	400,000
Algae	50,000
Protozoa	30,000

SOURCE: A. Burges, *Microorganisms in the Soil*, Hutchinson, London, 1958.

Large numbers of actinomycetes, as many as millions per gram, are present in dry warm soils. The most predominant genera of this group are *Nocardia*, *Streptomyces*, and *Micromonospora*. These organisms are responsible for the characteristic musty or earthy odor of a freshly plowed field. They are capable of degrading many complex organic substances and consequently play an important role in building soil fertility. The actinomycetes are also noted for their ability to synthesize and excrete antibiotics. The presence of antibiotic substances in soil can rarely be detected, but this does not exclude the possibility that they may be present and active in the microenvironment.

Fungi

Hundreds of different species of fungi inhabit the soil. They are most abundant near the surface, where an aerobic condition is likely to prevail. They exist in both the mycelial and spore stage. Since growth can take place from either a spore or a fragment of a mycelium, it is difficult to estimate their numbers; however, counts ranging from thousands to hundreds of thousands per gram of soil have been reported. Fungi are active in decomposing the major constituents of plant tissues, namely, cellulose, lignin, and pectin. The physical structure of soil is improved by the accumulation of mold mycelium within it. One of the characteristics of soil of considerable agricultural importance is its the binding together of fine soil particles to form water-stable aggregates. This is accomplished by the penetration of mycelium through the soil, forming a network which entangles the small particles.

Table 25-2. Physiological Groups of Bacteria in Various Types of Soil (Numbers of Bacteria per Gram of Soil)

Soil Type	Garden	Field	Meadow	Coniferous Forest	Marshland
Moisture content in percent of moist soil	17.9	18.1	17.0	21.2	37.2
Percent calcium carbonate	4.7	5.0	11.4	0	7.6
Bacteria developing on nutrient-gelatin plates	8,400,000	8,100,000	8,100,000	1,500,000	1,500,000
Bacteria developing on nutrient-agar plates	2,800,000	3,500,000	3,000,000	900,000	1,700,000
Bacteria growing in deep cultures of glucose agar (anaerobes)	280,000	137,000	620,000	345,000	2,180,000
Urea-decomposing bacteria	37,000	8,500	5,200	8,800	2,500
Denitrifying bacteria	830	400	850	380	370
Pectin-decomposing bacteria	535,000	70,000	235,000	810,000	3,700
Anaerobic butyric acid bacteria	368,000	50,300	83,500	203,000	235,000
Anaerobic protein-decomposing bacteria	35,000	22,000	36,800	17,000	2,000
Anaerobic cellulose-decomposing bacteria	367	350	367	17.7	1
Aerobic nitrogen-fixing bacteria	2,350	1,885	18	0	17
Anaerobic nitrogen-fixing bacteria	5,500	700	370,000	2,020	67
Nitrifying bacteria	880	1,701	37	0	34

SOURCE: M. Düggeli in S. A. Waksman, *Principles of Soil Microbiology*, Williams & Wilkins, Baltimore, 1932.

Yeasts are likely to be more prevalent in soils of vineyards, orchards, and apiaries, where special conditions, particularly the presence of sugars, favor their growth.

Algae

The population of algae in soil is generally smaller than that of either bacteria or fungi. The major types present are the green algae and diatoms. Their photosynthetic nature accounts for their predominance on the surface or just below the surface layer of soil. In a rich fertile soil, the biochemical activities of algae are dwarfed by those of bacteria and fungi. In some situations, however, algae perform prominent and beneficial changes. For example, on barren and eroded lands they may initiate the accumulation of organic matter because of their ability to carry out photosynthesis and other metabolic activities. This has been observed in some desert soils.

Cyanobacteria, the oxygenic photosynthetic bacteria, are known to grow on the surfaces of freshly exposed rocks where the accumulation of their cells results in simultaneous deposition of organic matter. This establishes a nutrient base that will support growth of other bacterial species. The growth and activities of the initial algae and bacteria pave the way for the growth of other bacteria and fungi. The mineral nutrients of the rock are slowly dissolved by acids resulting from microbial metabolism. This process continues with a gradual accumulation of organic matter and dissolved minerals until a condition results that supports growth of lichens, then mosses, then higher plants. The cyanobacteria play a key role in the transformation of rock to soil, a first step in rock-plant succession.

Protozoa

Most soil protozoa are flagellates or amoebas; the number per gram of soil ranges from a few hundred to several hundred thousand in moist soils rich in organic matter. From a microbiological standpoint they are of significance since their dominant mode of nutrition involves ingestion of bacteria. Of academic interest

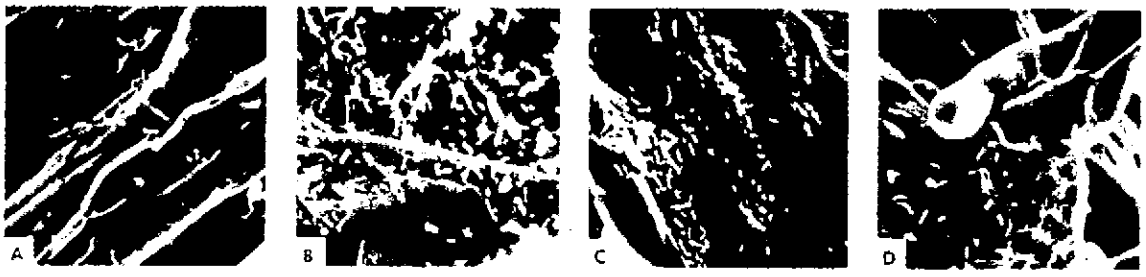


Figure 25-2. The microbial flora in the rhizosphere as seen in scanning electron micrographs of epidermal root cells. In this study plants were removed from a forest preserve, and special precautions were observed to recover root systems with as little mechanical damage as possible to the tissues. The root systems were dissected from the plants and representative specimens selected for electron microscopy. (A and B) Fungal hyphae and bacteria on the epidermal cells of *Ammophilia arenaria*. [(A) × 900; (B) × 800.] (C) Bacteria on the epidermis of a barley root, × 1,000. (D) Root hair, epidermal cell, and bacteria, × 850. (*Courtesy of K. M. Old and New Phytol*, **74**:51, *1975*.)

is the fact that they demonstrate a preference for certain microbial species. Since not all bacteria are suitable as food for protozoa, the protozoa may be a factor in maintaining some equilibrium of microorganisms in soil.

Viruses

Bacterial viruses (bacteriophages), as well as plant and animal viruses, periodically find their way into soils through additions of plant and animal wastes. Also, soil microorganisms themselves may harbor viruses.

The Rhizosphere

The region where the soil and roots make contact is designated the **rhizosphere**. The microbial population on and around roots is considerably higher than that of root-free soil; the differences are both quantitative and qualitative. Bacteria predominate, and their growth is enhanced by nutritional substances released from the plant tissue, e.g., amino acids, vitamins, and other nutrients; the growth of the plant is influenced by the products of microbial metabolism that are released into the soil. It has been reported that amino acid–requiring bacteria exist in the rhizosphere in larger numbers than in the root-free soil. It has been demonstrated that the microbiota of the rhizosphere is more active physiologically than that of nonrhizosphere soil. The rhizosphere represents a tremendously complex biological system, and there is a great deal yet to be learned about the interactions which occur between the plant and the microorganisms intimately associated with its root system.

Electron-microscope techniques have been developed to observe microorganisms directly on the root surfaces (see Fig. 25-2).

INTERACTIONS AMONG SOIL MICROORGANISMS

The microbial **ecosystem** of soil includes the total microbial flora together with the physical composition and physical characteristics of the soil. It is the sum of the **biotic** and the **abiotic** components of soil.

The microorganisms that inhabit the soil exhibit many different types of associations or interactions. Some of the associations are indifferent or neutral; some are beneficial or positive; others are detrimental or negative. As each different type of association or interaction is discovered, it has been given a specific descriptive label. As you might presume, many of these associations do not fall neatly into discrete categories. Furthermore, and likewise not unexpected, there is the existence of some confusion and contradiction in the use of terms. The term **symbiosis**, for example, as first proposed, referred to the "living together of dissimilarly named organisms"; it was used as a general term. Later it took on a more specific meaning, namely, an association between bacteria and plants referred to as symbiotic nitrogen fixation which is described later in this chapter. Currently the trend is to use the term *symbiosis* as originally intended, that is, merely as a condition in which the individuals of a species live in close association with individuals of another species. We shall describe the following types of microbial associations:

Neutral: neutralism
Positive or beneficial: mutualism, commensalism
Negative or detrimental: antagonism, competition, parasitism, predation

Neutral Associations

Neutralism

It is conceivable that two different species of microorganisms occupy the same environment without affecting each other. For example, each could utilize different nutrients without producing metabolic end products that are inhibitory. Such a condition might be transitory; as conditions change in the environment, particularly availability of nutrients, the relationship might change.

Positive Associations

Mutualism

Mutualism is an example of a symbiotic relationship in which each organism benefits from the association. The manner in which benefit is derived varies. One type of mutualistic association is that involving the exchange of nutrients between two species, a phenomenon called **syntrophism**. Many microorganisms synthesize vitamins and amino acids in excess of their nutritional requirements. Others have a requirement for one or more of these nutrients. Still others synthesize a particular essential nutrient in suboptimal amounts. Hence, certain combinations of species will grow together but not apart when nutrient levels are very low.

Another mutalistic association is characterized by different metabolic products from the association as compared with the sum of the products of the separate species. Figure 25-3A illustrates a mutualism between *Thiobacillus ferrooxidans* and *Beijerinckia lacticogenes* in a medium which lacks carbon and nitrogen sources. The growth of the two species in association and the resulting effect on the rate and extent of leaching copper from an ore is shown. Leaching is one of the processes for recovering metals from ore; microorganisms play the important role of oxidizing insoluble metal sulfides to soluble sulfates. The interactions that occur in this mutualistic association are shown in Fig. 25-3B.

Commensalism

The phenomenon of **commensalism** refers to a relationship between organisms in which one species of a pair benefits; the other is not affected. This occurs commonly in soil with respect to degradation of complex molecules like cellulose and lignin. For example, many fungi are able to dissimilate cellulose to

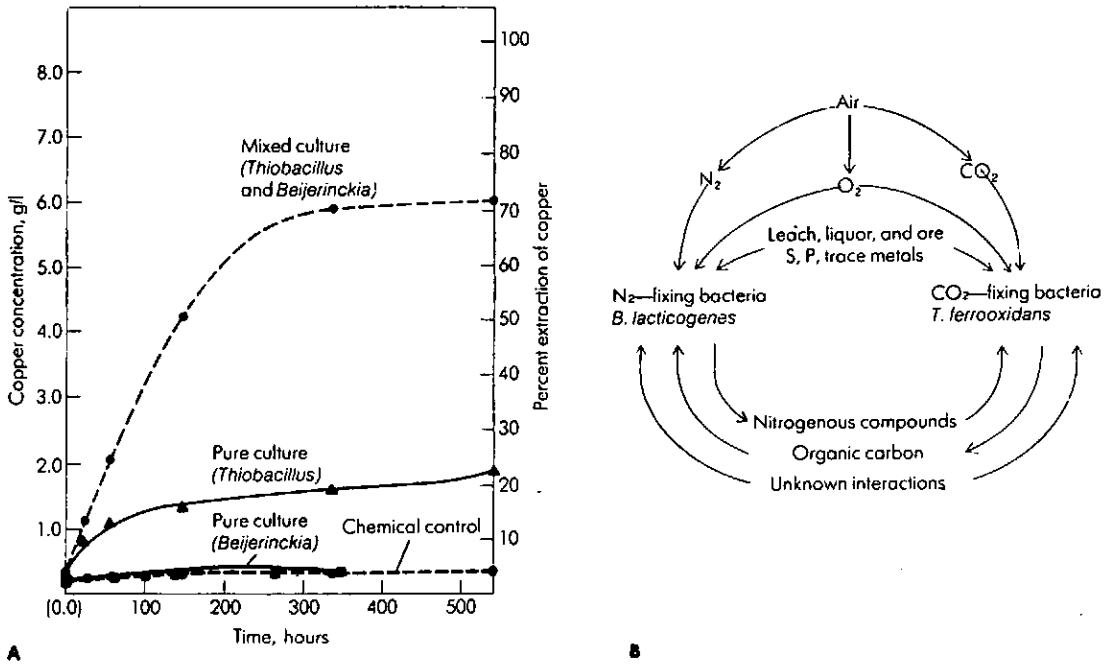


Figure 25-3. An example of microbial mutualism in ore leaching. *Thiobacillus ferrooxidans* and *Beijerinckia lacticogenes* were inoculated into a medium free of added carbon and nitrogen sources; sterile ore concentrate was added to the medium. (A) Results of leaching of copper with pure and mixed culture. (B) Proposed mutualistic interactions between the two species in a leaching environment devoid of fixed carbon or fixed nitrogen. (*Courtesy of N. C. Trivedi and H. M. Tsuchiya, Int J Miner Process, 2:1, 1975.*)

glucose and beyond (they are cellulolytic). Many bacteria are unable to utilize cellulose, but they can and do utilize the fungal breakdown products of cellulose, e.g. glucose and organic acids. The existence of many nutritionally fastidious bacteria in soil suggests that their growth and survival is dependent upon the synthesis and excretion of vitamins and amino acids by less fastidious species.

Another example of commensalism is that of a change in the substrate produced by a combination of species and not by individual species. For example, lignin, a major constituent of wood, is generally resistant to degradation by pure cultures of microorganisms under laboratory conditions. However, the lignin in forest soil is degraded by the soil microbial flora, particularly fungi.

Negative Associations

Antagonism

When one species adversely affects the environment for another species, it is said to be antagonistic. Such organisms may be of great practical importance, since they often produce antibiotics or other inhibitory substances which affect the normal growth processes or survival of other organisms. Antagonistic rela-

tionships are quite common in nature. For instance, both *Staphylococcus aureus* and *Pseudomonas aeruginosa* are antagonistic toward *Aspergillus terreus*. Certain *Pseudomonas* pigments inhibit germination of *Aspergillus* spores. *S. aureus* produces a diffusable antifungal material that causes distortions and hyphal swellings in *A. terreous* (Fig. 25-4). Although microorganisms from a variety of natural habitats produce antibiotics, soil microorganisms are the most common producers. It is not unusual for one organism to produce five or six different antimicrobial agents. There is some question about the role of antibiotics in nature; production in the laboratory is under conditions quite different from those in nature. Production of antibiotics in soil may enable the antibiotic-producing organism to thrive successfully in a competitive environment. For example, large populations of actinomycetes have been found in the chitinaceous shells of dead crustaceans in the sea. Their existence, in the environment free of other microorganisms, is likely due to the antibiotics they produce.

Organisms that elaborate antibiotics represent the classic example of this phenomenon: however, antibiosis may result from a variety of other conditions

Figure 25-4. *Aspergillus-Staphylococcus* interaction. (A) *Aspergillus terreus* 20-h culture in nutrient broth with *Staphyloccus aureus* added after 8 h (X500). (B) *A. terreus* and *S. aureus* as in (A) in glucose-peptone broth (X600). (C) *A. fumigatus* and *S. aureus* in glucose-peptone broth (X600). (D) *A. terreus* 20-h culture in nutrient broth (X800). (*Courtesy of A. Mangan, J Gen Microbiol,* **58**:261, 1969.)

operative in mixed populations. Cyanide is produced by certain fungi in concentrations toxic to other microorganisms, and the algae elaborate fatty acids which exhibit a marked antibacterial activity. Other metabolic products that may result from microbial activity in the soil which are likely to be inhibitory to other species are methane, sulfides, and other volatile sulfur compounds.

Many soil microorganisms—important examples are the myxobacteria (slime bacteria) and streptomycetes—are antagonistic because they secrete potent lytic enzymes which destroy other cells by digesting their cell wall or other protective surface layers, as shown in Fig. 25-5. Presumably the degraded cellular material, as well as the released protoplasmic material, serves as nutrients. Although it might be assumed that organisms producing lytic substances have a selective advantage over sensitive microbes, microbial interactions of this type are difficult to interpret. It appears that in the natural environment producers of lytic substances are often found in close proximity with sensitive organisms and do not predominate over them.

Competition

A negative association may result from competition among species for essential nutrients. In such situations the best adapted microbial species will predominate or, in fact, eliminate other species which are dependent upon the same limited nutrient substance.

Parasitism

Parasitism is defined as a relationship between organisms in which one organism lives in or on another organism. The parasite feeds on the cells, tissues, or fluids of another organism, the **host**, which is commonly harmed in the process. The parasite is dependent upon the host and lives in intimate physical and metabolic contact with the host. All major groups of plants, animals, and microorganisms are susceptible to attack by microbial parasites.

An interesting example of a parasitic relationship between microbes is the bacterial parasite of Gram-negative bacteria named *Bdellovibrio bacteriovorus*, which is widespread in soil and sewage. This unusual motile bacterium attaches

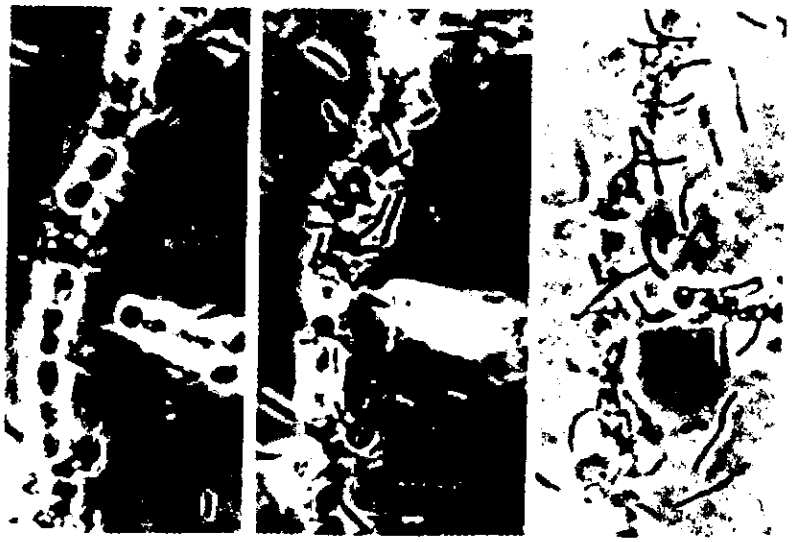


Figure 25-5. Lysis of cyanobacteria by a myxobacter. Shown in this series is a sequence of lysis of *Nostoc* filament by the myxobacter. The myxobacter culture used in this experiment was isolated from fish ponds and is capable of lysing many species of bacteria. (*Courtesy of Mirian Shilo, J Bacteriol*, **104**:453, 1970.)

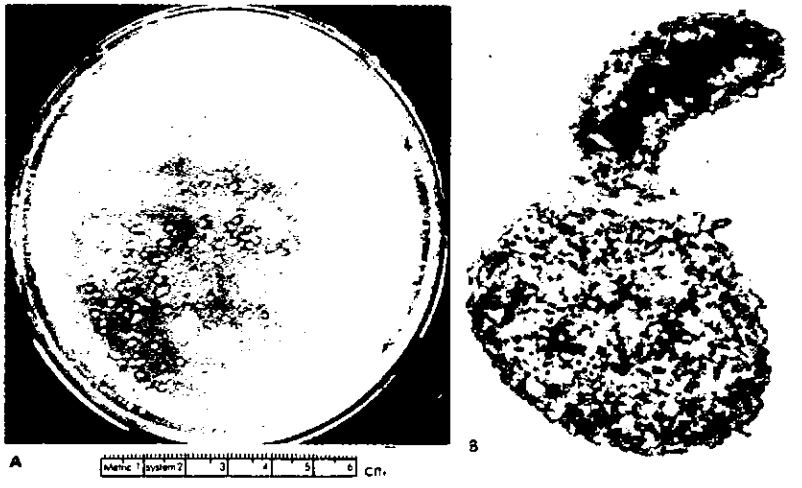


Figure 25-6. Bacteriolysis produced by *Bdellovibrio bacteriovorus*. (A) Plate culture of *B. bacteriovorus* on a lawn of *E. coli* showing whitish-gray colony surrounded by circular plaque-like clearing zone. Central colony consists of bdellovibrios and the clear zone contains a few intact *E. coli* cells and spheroplasts of the host cells (*E. coli*). (B) Electron micrograph thin section showing *B. bacteriovorus* penetration into *E. coli* cell (X48,000). (*Courtesy of J. C. Burnham, T. Hashimoto, and S. F. Conti, J Bacteriol*, 96:1366, 1968.)

to a host cell at a special region and eventually causes the lysis of that cell (see Chap. 13). As a consequence, plaquelike areas of lysis (Fig. 25-6) appear when these parasites are plated along with their host bacteria. There are also many strains of fungi which are parasitic on algae and other fungi by penetration into the host.

Viruses which attack bacteria, fungi, and algae are strict intracellular parasites since they cannot be cultivated as free-living forms. The phenomenon of lysogeny is quite important because of the possibility for genetic recombination in natural populations and the subsequent expression of new characteristics.

BIOGEOCHEMICAL ROLE OF SOIL MICROORGANISMS

Soil microorganisms serve as biogeochemical agents for the conversion of complex organic compounds into simple inorganic compounds or into their constituent elements. The overall process is called **mineralization**. This conversion of complex organic compounds into inorganic compounds or elements provides for the continuity of elements (or their compounds) as nutrients for plants and animals including people.

It is possible to construct a sequence of reactions to illustrate that microorganisms perform an essential role in maintaining a cyclic process for the reutilization of elements under natural conditions. In this respect we can view the planet earth as a closed system dependent upon the process of recycling for maintenance of life as we know it.

In the following paragraphs we shall discuss the role of soil microorganisms with respect to the transformations they bring about on nitrogen, carbon, sulfur, phosphorus, and their compounds.

BIOCHEMICAL TRANSFORMATIONS OF NITROGEN AND NITROGEN COMPOUNDS: THE NITROGEN CYCLE

Because of the importance of nitrogen for plant nutrition, the biochemical events that make up the nitrogen cycle have been studied in considerable detail.

The sequence of changes from free atmospheric nitrogen to fixed inorganic nitrogen, to simple organic compounds, to complex organic compounds in the tissues of plants, animals, and microorganisms, and the eventual release of this

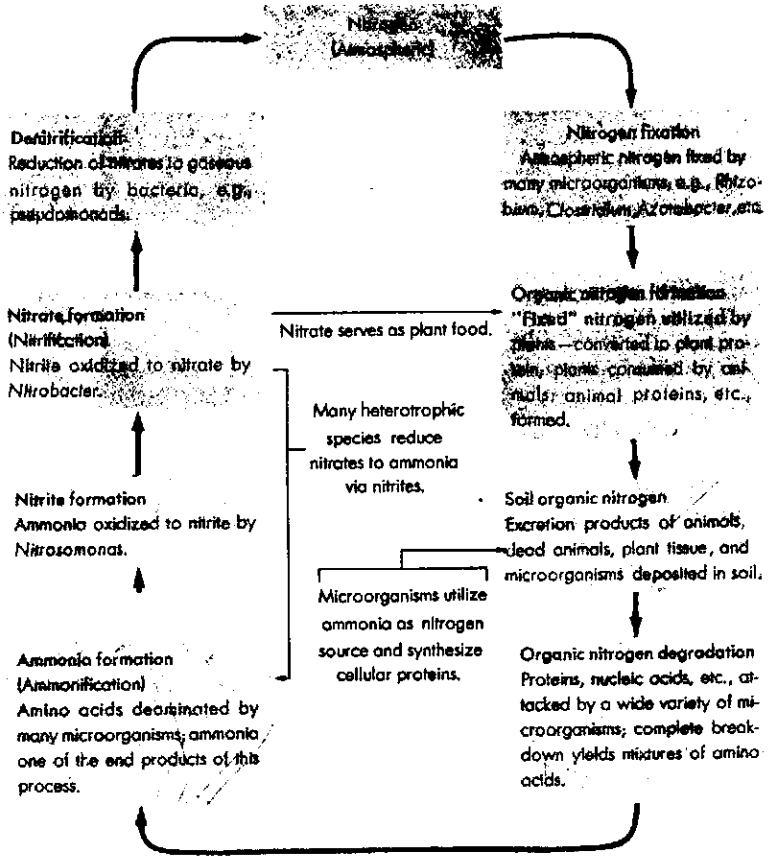


Figure 25-7. Nitrogen cycle in nature (schematic), showing the role of microorganisms.

nitrogen back to atmospheric nitrogen is summarized in Fig. 25-7, the nitrogen cycle.

Proteins, nucleic acids, purine and pyrimidine bases, and amino sugars (glucosamine and galactosamine) represent the complex organic nitrogenous substances which are deposited in soil in the form of animal and plant wastes or their tissues. Synthetic processes of microorganisms also contribute some amount of complex organic nitrogen compounds.

The simplest form of nitrogen involved in biological transformations is gaseous elementary nitrogen. The overall transformations in which microorganisms are involved range from nitrogen gas to protein. A great many intermediate products and a corresponding large number of intricate enzymatic reactions are involved in bringing about these changes.

Some of the biochemical events in the nitrogen cycle are summarized below.

Proteolysis

The nitrogen in proteins (as well as in nucleic acids) may be regarded as the end of the line as far as synthesis of nitrogenous compounds is concerned. The nitrogen in proteins is "locked" and is not available as a nutrient to plants. In

order to set this organically bound nitrogen free for reuse, the first process that must take place is the enzymatic hydrolysis of proteins (proteolysis). This is accomplished by microorganisms capable of elaborating extracellular proteinases that convert the protein to smaller units (peptides). The peptides are then attacked by peptidases, resulting ultimately in the release of individual amino acids. The overall reactions may be summarized:

$$\text{Proteins} \xrightarrow{\text{proteinases}} \text{peptides} \xrightarrow{\text{peptidases}} \text{amino acids}$$

Some bacterial species elaborate large amounts of proteolytic enzymes. Among the most active in this respect are some of the clostridia, e.g., *Clostridium histolyticum* and *C. sporogenes*; a lesser degree of activity is found in species of the genera *Proteus*, *Pseudomonas*, and *Bacillus*. Many fungi and soil actinomycetes are extremely proteolytic. Peptidases, however, occur widely in microorganisms as demonstrated by the fact that peptones (partially hydrolyzed proteins) are a common constituent of bacteriological media and provide a readily available source of nitrogen.

Amino Acid Degradation: Ammonification

The end products of proteolysis are amino acids. Their fate in the soil may be utilization as nutrients by microorganisms or degradation by microbial attack. Amino acids are subject to a variety of pathways for microbial decomposition. We are concerned here with the liberation of nitrogen from these compounds, which is accomplished by deamination, i.e., removal of the amino group. Although several variations of deamination reactions are exhibited by microorganisms, one of the end products is always ammonia, NH_3. An example of a specific deamination reaction is

$$\underset{\text{Alanine}}{CH_3CHNH_2COOH} + \tfrac{1}{2}O_2 \xrightarrow[\text{deaminase}]{\text{alanine}} \underset{\text{Pyruvic acid}}{CH_3COCOOH} + \underset{\text{Ammonia}}{NH_3}$$

This reaction is classified as an oxidative deamination. Many microorganisms can deaminate amino acids. The production of ammonia is referred to as ammonification. The fate of the ammonia thus produced varies, depending upon conditions in the soil. Ammonia is volatile and, as such, leaves the soil; however, if solubilized, NH_4^+ is formed. Some of the subsequent possibilities include accumulation and utilization by plants and microorganisms and, under favorable conditions, oxidation to nitrates.

Nitrification

Microorganisms convert ammonia to nitrate, and the process is called nitrification. The process occurs in two steps, each step performed by a different group of bacteria.

1 Oxidation of ammonia to nitrite by ammonia-oxidizing bacteria

$$2NH_3 + 3O_2 \rightarrow 2HNO_2 + 2H_2O$$

2 Oxidation of nitrite to nitrate by nitrite-oxidizing bacteria

$$HNO_2 + \tfrac{1}{2}O_2 \rightarrow HNO_3$$

Bacteria of both physiological groups, ammonia oxidizers and nitrite oxidiz-

Table 25-3. Composition of Medium for Isolation of Nitrifying Bacteria Using Enrichment Culture Technique

Ingredients	g/L
$(NH_4)_2SO_4$	2.0
K_2HPO_4	1.0
$MgSO_4$	0.5
$FeSO_4$	0.4
NaCl	0.4
$CaCO_3$	1.0
$MgCO_3$	1.0

ers, are Gram-negative chemolithotrophs. Their main source of carbon is obtained through carbon dioxide fixation; energy is derived by the oxidation of NH_3 or NO_2^- depending upon the group. Nitrifying bacteria occur widely in nature in a variety of habitats, including soil, sewage, and aquatic environments.

Nitrifying bacteria cannot be isolated directly by the usual techniques employed to isolate heterotrophic bacteria. Some of the reasons for this are: they are slow-growing compared with heterotrophs; and they may be present in very small numbers compared with other physiological types. Accordingly, enrichment cultures are used for their isolation. An example of a medium for this purpose is shown in Table 25-3. A relatively large inoculum is used, and incubation is in the dark at 25 to 30°C for a period of 1 to 4 months.

Species of ammonia-oxidizing bacteria vary in morphology (rod, spherical, spiral, or lobular) and usually have an extensive membrane system within their cytoplasm. They frequently form cysts and zooglea. See Fig. 25-8. The following species have been recognized as ammonia oxidizers:

Nitrosomonas europaea
Nitrosovibrio tenuis
Nitrosococcus nitrosus
Nitrosococcus oceanus

Species of nitrite-oxidizing bacteria exhibit some of the same morphological characteristics as the ammonia oxidizers. Only a few species have been isolated and described. These include *Nitrobacter winogradskyi* and *Nitrospina gracilis*.

An interesting historical event involving nitrification and production of gunpowder may be cited. During the Napoleonic wars, France was unable to import nitrate, which was needed for the manufacture of gunpowder. To solve this

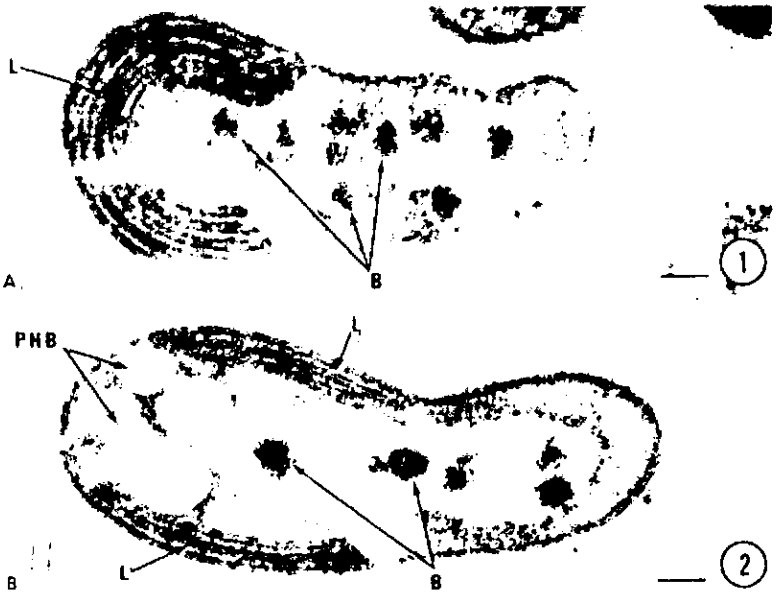


Figure 25-8. Ultrastructure of *Nitrobacter winogradskyi*. (A) Thin section from cell grown chemoautotrophically and harvested during exponential phase of growth, showing lamellar membrane system (L) at the swollen end of the cell and electron-dense polyhedral bodies (B). (B) Thin section from cell grown on nitrite mineral-salts medium supplemented with 5 mmol sodium acetate and harvested during exponential phase of growth. Section shows lamellas (L), polyhedral bodies (B), and electron-transparent bodies believed to be poly-β-hydroxybutyrate (PHB) reserve material. (*Courtesy of L. M. Pope, D. S. Hoare, and A. J. Smith, J Bacteriol, 97:936, 1969.*)

dilemma, artificial niter beds were made, consisting of soil mixed with animal waste and vegetable materials, ashes, etc. Aeration was performed by turning the heap over from time to time. After a long period of incubation, crude saltpeter (mineral nitrates) was extracted with hot water. This occurred, of course, long before the specific activities of microorganisms were known. Nitrification was discovered to be a biological process by Schloesing and Muntz in 1877; Winogradsky isolated the bacteria responsible for the process in 1890.

Reduction of Nitrate to Ammonia

Several heterotrophic bacteria are capable of converting nitrates into nitrites or ammonia. This normally occurs under anaerobic conditions, e.g., in waterlogged soil. The oxygen of the nitrate serves as an acceptor for electrons and hydrogen. The process involves several reactions, and the overall result is

$$HNO_3 + 4H_2 \rightarrow NH_3 + 3H_2O$$

This reaction is not of major significance in well-cultivated agricultural soil.

Denitrification

The transformation of nitrates to gaseous nitrogen is accomplished by microorganisms in a series of biochemical reactions. The process is known as **denitrification.** From the standpoint of agriculture, this is an undesirable process in that it results in loss of nitrogen from the soil and hence a decline in nutrients for plant growth.

Species of several genera of bacteria are capable of transforming NO_3^- to N_2, e.g., *Achromobacter, Agrobacterium, Alcaligenes, Bacillus, Chromobacterium, Flavobacterium, Hyphomicrobium, Pseudomonas, Thiobacillus,* and *Vibrio.*

The overall biochemical reaction which expresses the process of denitrification is

$$\underset{\text{Nitrate}}{2NO_3^-} \rightarrow \underset{\text{Nitrite}}{2NO_2^-} \rightarrow \underset{\text{Nitric oxide}}{2NO} \rightarrow \underset{\text{Nitrous oxide}}{N_2O} \rightarrow \underset{\text{Nitrogen}}{N_2}$$

Experimental results which illustrate the order in which products rise and fall during denitrification are shown in Fig. 25-9.

Environmental conditions in a soil have a significant effect on the level of denitrification. For example, the process is enhanced in soils (1) by an abundance of organic matter, (2) by elevated temperatures (25 to 60°C), and (3) by neutral or alkaline pH. Availability of oxygen has a dual effect. Denitrification proceeds only when the oxygen supply is limited. However, oxygen is necessary for nitrite and nitrate formation.

Nitrogen Fixation

A number of microorganisms are able to use molecular nitrogen in the atmosphere as their source of nitrogen. The conversion of molecular nitrogen into ammonia is known as **nitrogen fixation.** Two groups of microorganisms are involved in this process: (1) **nonsymbiotic** microorganisms, those living freely and independently in the soil; and (2) **symbiotic** microorganisms, those living in roots of plants. Several types of experiments are used to detect nitrogen fixation by microorganisms. One approach is to demonstrate growth in a nitrogen-free medium. More specific evidence of fixation can be obtained by cultivating the microorganism in the presence of nitrogen labeled with isotopic nitrogen. $^{15}N_2$ can be measured by using a mass spectrometer. In essence, after

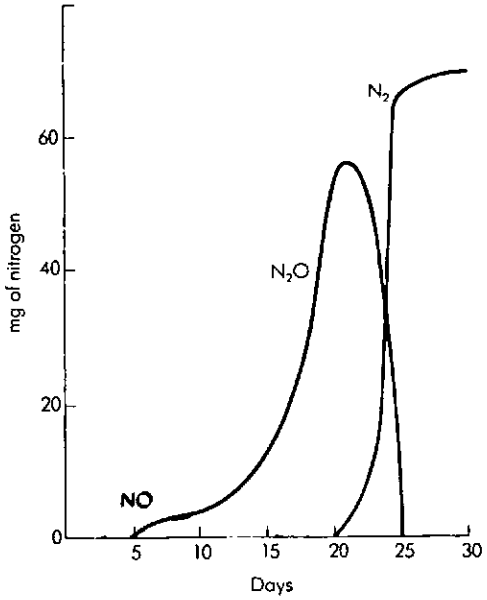


Figure 25-9. Sequence of products during denitrification in Norfolk sandy loam. (*Courtesy of F. B. Cady and W. V. Bartholomew, Soil Sci Soc Am Proc,* **24**:*477, 1960.*)

Table 25-4. Some Examples of Nitrogen-Fixing Bacteria

Cyanobacteria
- *Anabaena* spp.
- *Nostoc* spp.
- *Gloeotrichia* spp.
- *Synechococcus* spp.
- *Plectonema* spp.
- *Oscillatoria* spp

Phototrophic bacteria
- *Rhodospirillum rubrum*
- *Rhodopseudomonas palustris*
- *Rhodomicrobium vannielii*
- *Chromatium vinosum*
- *Chlorobium thiosulfatophilum*

Chemotrophic bacteria
- *Azospirillum lipoferum*
- *Azotobacter chroococcum*
- *Beijerinckia indica*
- *Rhizobium leguminosarum*
- *Methylomonas methanitrificans*
- *Escherichia coli*
- *Enterobacter aerogenes*
- *Bacillus macerans*
- *Clostridium butyricum*
- *Xanthobacter autotrophicus*

the organism is grown in the mixture of atmospheric nitrogen and $^{15}N_2$, the culture is examined for evidence of $^{15}N_2$ incorporated in any compounds. Its presence is positive proof that nitrogen has been fixed. Under suitable conditions an increase of as little as 0.001 μg of nitrogen can be detected by this technique.

The capability of the nitrogen-fixing enzyme to act upon acetylene, discovered in the mid-1960s, has led to the development of a simple, rapid, relatively inexpensive technique now widely used to measure nitrogen fixation. The test is based on the observation that the nitrogen-fixing enzyme (*nitrogenase*) interacts with triple-bonded compounds, e.g., acetylene, to form ethylene as follows:

$$\underset{\text{Acetylene}}{HC{\equiv}CH} \xrightarrow[\text{enzyme nitrogenase}]{2H} \underset{\text{Ethylene}}{H_2C{=}CH_2}$$

The comparable reaction with nitrogen is

$$\underset{\text{Nitrogen}}{N{\equiv}N} \xrightarrow[\text{enzyme nitrogenase}]{6H} \underset{\text{Ammonia}}{2NH_3}$$

The technique involves exposing the specimen being assayed for nitrogenase activity to acetylene in a suitable vessel and, after a period of incubation, analyzing the gas phase for ethylene by gas-liquid chromatography. The amount of ethylene produced is a measure of nitrogenase activity.

The essential reactants in the bacterial nitrogen fixation process are:

1 **The nitrogenase enzyme complex.** This has been characterized as two components, and neither is active without the other. Component I is nitrogenase and

component II is nitrogenase reductase. Component I is known as the MoFe protein (Mo for molybdenum, Fe for iron). Component II, which is a smaller molecule is designated the Fe protein. Both molecules contain sulfur.

2 A strong reducing agent such as ferredoxin or flavodoxin
3 ATP
4 A regulating system for NH_3 production and utilization
5 A system that protects the nitrogen-fixing system from inhibition by molecular oxygen

The overall biochemical reaction for nitrogen fixation can be expressed as:

$$N_2 + 6e^- + 12ATP + 12H_2O \xrightarrow[\text{complex}]{\text{nitrogenase}} 2NH_4^+ + 12ADP + 12P_i + 4H^+$$

Nonsymbiotic Nitrogen Fixation

Nonsymbiotic nitrogen fixation has been studied extensively with *Clostridium pasteurianum* and species of *Azotobacter*. For many years, these bacteria were the only ones known to be capable of this activity. The former is an anaerobic bacillus, and the latter are aerobic oval to spherical cells; both are widely distributed in soils. The nitrogen-fixing capacity of the *Azotobacter* species is greater than that of *Cl. pasteurianum*. In recent years many other microorganisms have been found to fix nitrogen (see Table 25-4).

It has been estimated that the amount of nitrogen fixed by the nonsymbiotic process ranges between 20 and 50 lb/acre annually. This estimate is no doubt subject to much variation depending upon the conditions peculiar to a particular soil.

Symbiotic Nitrogen Fixation

Symbiotic nitrogen fixation is accomplished by bacteria of the genus *Rhizobium* in association with legumes (plants that bear seeds in pods, e.g., soybeans, clover, and peas). Before these bacteria can fix nitrogen, they must establish themselves in the cells of root tissue of the host plant. Infection of the root system by the rhizobia bacteria is closely associated with the formation of an "infection thread" that develops in certain root hairs (see Fig. 25-10). The nitrogen-fixing bacteria invade the host plant cells via this infection thread. Some of the cells of the plant are thus infected, causing cell enlargement and an increased rate of cell division, leading to the formation of abnormal growths (nodules) on the root system. Several types of nodulation are illustrated in Fig. 25-11.

The legume, the bacteria, and the nodule constitute the system for this type of nitrogen fixation. It is a process where both the bacteria and the plant benefit by the association. The bacteria convert atmospheric nitrogen to fixed nitrogen which is available to the plant, and in turn, the bacteria derive nutrients from the tissues of the plant.

Not all species of *Rhizobium* produce nodulation and nitrogen fixation with any legume. There is a degree of specificity between the bacteria and legumes. For purposes of inoculation with commercial preparations of these bacteria, legumes are divided into seven major categories as follows: alfalfa, clover, peas and vetch, cowpeas, beans, lupines, and soybeans. *Rhizobium* species or strains effective for one group are less effective or ineffective for other groups. Even within a species, certain strains are more effective than others with a given host plant. Evidence of this specificity is demonstrated in Fig. 25-12.

Figure 25-10. Nodule formation by *Rhizobium* on legume plants. The first stage in the establishment of the *Rhizobium*-legume N_2-fixing symbiosis is the infection of the host legume by the appropriate *Rhizobium* species. Root hairs are the site of infection. The first microscopically visible indication of the bacteria-plant interaction is deformation and curling of the normally straight root hairs. Aseptically cultured clover seedling with undeformed root hairs is shown (A), × 40. (B) A clover seedling (× 40) inoculated with *R. trifolii*. The bacteria are in clumps (flocs) in the rhizosphere. Note the change in appearance of the root hairs. A characteristic deformation is curling at the root hair tip to produce a "shepherd's crook" (C). The bacteria enter the root hair and are enclosed in a tubular structure, the infection thread (C and D), which is the first microscopically visible sign of a successful infection. The bacteria appear to enter the root hair by a process of invagination. Root hair cell-wall growth is redirected at a localized point resulting in the wall growing back into the root hair to form the tubular infection thread. There is no direct penetration through the root hair cell wall, and the bacteria remain extracellular within the infection thread. (E, F, G, H) A serial section sequence through a root hair which had a shepherd's crook at the origin of the infection thread. (E) A diagrammatic illustration of a serial sectioned root hair showing the infection thread (IT), nucleus (N), and the initiation of sectioning (top arrow). (F) A section before the invagination showing the infection thread (IT) which contained bacteria (B). The arrows indicate the region of the root hair cell wall where the invagination process has begun (× 4,500). (G) A section through the middle of the invagination showing the infection thread wall (arrows) of the pore, bacteria (B) within the infection thread, and the root hair nucleus (× 4,500). (H) A section past the pore; the arrows point out where the wall of the pore is grazed by the knife (× 4,500). Bacteroids within a nodule (I) are surrounded by membrane which is believed to be derived from the plant. The bacteroids contain electron-dense, unidentified inclusions. (*Courtesy of C. A. Napoli and H.Hubbell, Appl Microbiol,* **30**:*1003, 1975.*)

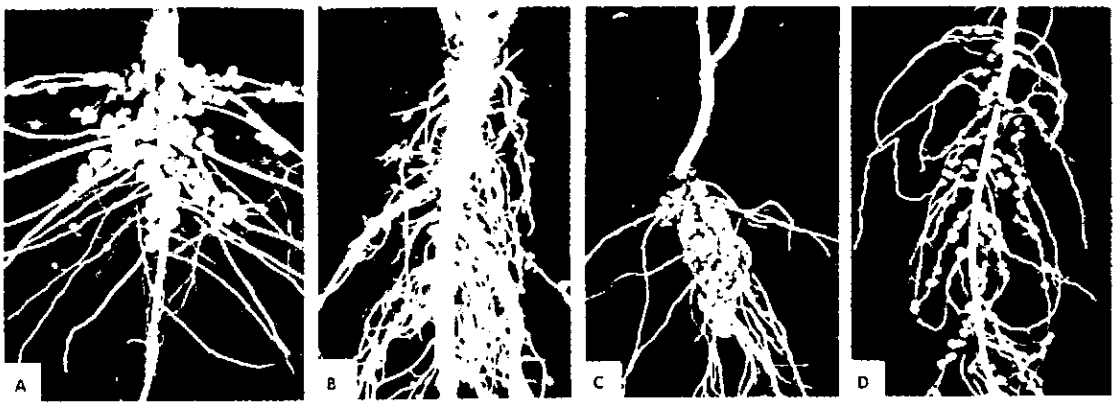


Figure 25-11. Root nodules are produced by effective strains of bacteria on several legumes: (A) soybean, (B) sweet clover, (C) pea, (D) Birdsfoot trefoil. (*Courtesy of the Nitragin Co., Inc.*)

Figure 25-12. Different strains of rhizobia have different effects on the growth of clover. Tests are carried out on Crimson clover in the following manner: Seeds are planted in sterile sand contained in a jar. The sand is then inoculated with the bacteria. Each jar contains a solution of nutrients—except nitrogen—which diffuse through the sand. Thus the extent of growth is indicative of the amount of nitrogen being supplied by the bacteria. (A) was not inoculated; (B), (C), and (D) were inoculated with different strains of rhizobia. Note the difference in growth response. (*Courtesy of L. W. Erdman, USDA.*)

Inoculation of seeds before planting is a desirable practice, since not all agricultural soils contain the right kinds of bacteria for optimum symbiotic nitrogen fixation with legume crops. Most of the commercial preparations consist of selected strains of bacteria dispensed in moist humus. This material is mixed with water and sprinkled over the seeds prior to planting.

Recombinant DNA and Nitrogen Fixation (Genetic Engineering)

The vast amount of knowledge that has accumulated in the last decade about microbial genetics, including the development of highly sophisticated techniques for gene splicing and cloning, has led to some dramatic developments in the field commonly referred to as genetic engineering. Applied aspects of this development are discussed later in Chap. 29, Industrial Microbiology. Nevertheless, we wish to mention here that many laboratories and research scientists

are concentrating their efforts on the possibility of developing new systems for nitrogen fixation using recombinant DNA technology.

One area of research is directed toward introducing the "package" of nitrogen-fixing genes from bacteria into plant cells. If this were achieved, plants might be capable of directly fixing nitrogen from the atmosphere. This would be a tremendous advance not only for agriculture but for the world at large in terms of producing food more economically and abundantly. Obviously, considerably more research is necessary before this kind of genetic engineering can be attempted at a practical level. For instance, nitrogenase is easily destroyed by oxygen, and some means of protection of this enzyme complex from oxygen would have to be provided in order for a plant cell to be able to fix nitrogen.

Alternatively, it may be possible to modify certain bacteria in a manner so that they would develop a relationship with the root system of other plants, as the *Rhizobium* species grow with legumes. For example, a symbiotic bacterial nitrogen-fixing system with cereal grains would have a tremendous effect on grain production both in yield and cost.

BIOCHEMICAL TRANSFORMATIONS OF CARBON AND CARBON COMPOUNDS: THE CARBON CYCLE

The ultimate source of organic carbon compounds in nature is the carbon dioxide present in the atmosphere (or dissolved in water). The process, carbon dioxide fixation, was discussed in Chap. 11. Although green plants and algae are the most important agents of carbon dioxide fixation, bacteria are also capable of synthesizing organic matter from inorganic carbon. The occurrence of photosynthesis among microorganisms has already been described. Other examples of carbon dioxide transformation or incorporation into organic compounds by bacteria are:

Carbon Dioxide Fixation

1 Utilization of carbon dioxide by autotrophic bacteria; the carbon dioxide represents the sole source of carbon for these organisms and is transformed by a reduction reaction to carbohydrates. The general reaction is

$$CO_2 + 4H \rightarrow (CH_2O)_x + H_2O$$

2 Carbon dioxide fixation by heterotrophic microorganisms is common among bacteria. A specific example of this type of reaction is

$$\underset{\text{Pyruvic acid}}{CH_3COCOOH} + CO_2 \rightarrow \underset{\text{Oxalacetic acid}}{HOOCCH_2COCOOH}$$

Organic Carbon Compound Degradation

The organic carbon compounds that eventually are deposited in the soil are degraded by microbial activity. The end product, carbon dioxide, is released into the air and soil. Fresh air contains approximately 0.03 percent carbon dioxide by volume. Bacteria and fungi are the principal microorganisms that degrade organic carbon compounds.

Under most natural systems of vegetation, e.g., forests, the amount of organic material in the soil remains approximately the same from year to year. This results from a balance established between the annual litter fall and death of the plants and the capacity of microorganisms to degrade these tissues.

The most abundant organic material in plants is cellulose. It is readily attacked by many species of bacteria and fungi. The initial enzymatic attack is by cel-

lulase which splits this long-chain polymer of glucose to cellobiose, which contains two glucose units. In turn, the cellobiose is split to glucose by the enzyme β-glucosidase; glucose is metabolized readily by many microorganisms. Complete oxidation yields CO_2 and H_2O. The process can be summarized as follows:

1 Cellulose $\xrightarrow[\text{cellulase}]{\text{enzyme}}$ cellobiose

2 Cellobiose $\xrightarrow[\beta\text{-glucosidase}]{\text{enzyme}}$ glucose

3 Glucose $\xrightarrow[\text{of many micro-organisms}]{\text{enzyme systems}}$ carbon dioxide, water, and/or other end products

An example of the breakdown rate of glucose (and microbial growth) by soil microorganisms is shown in Fig. 25-13. Similar degradation pathways occur for the other major plant tissue substances such as hemicellulose, lignin, and pectin. Carbon dioxide may also originate from the decarboxylation of amino acids, as well as from the dissimilation of fatty acids. All of these transformations may occur in the soil.

A general summary of the carbon cycle is shown in Fig. 25-14.

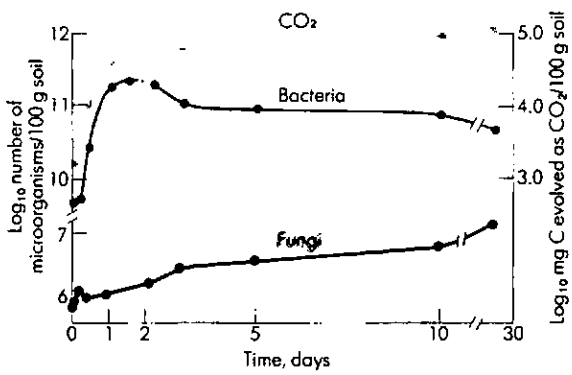


Figure 25-13. Plate counts of bacteria and fungi and cumulative CO_2 evolution during the incubation of soil treated with glucose. (*Courtesy of B. Behera and G. H. Wagner, Soil Sci Soc Am Proc*, **38**:591, 1974.)

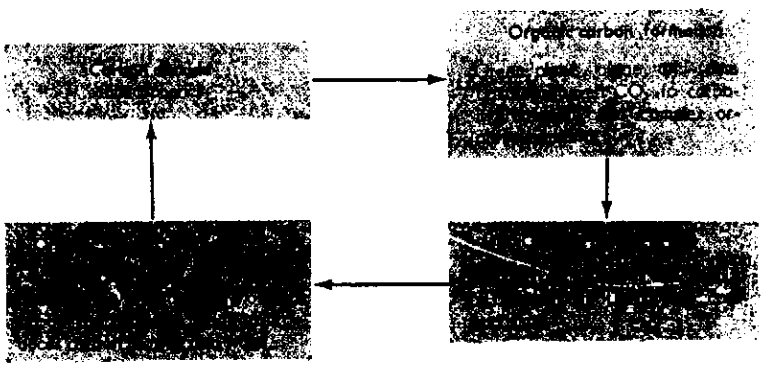


Figure 25-14. Carbon cycle (schematic), showing the role of microorganisms.

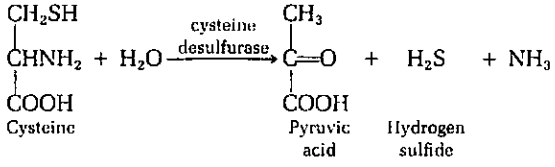
BIOCHEMICAL TRANSFORMATIONS OF SULFUR AND SULFUR COMPOUNDS: THE SULFUR CYCLE

Sulfur, like nitrogen and carbon, passes through a cycle of transformations mediated by microorganisms (see Fig. 25-15). Some species oxidize and others reduce various sulfur compounds. The microbial transformations of sulfur have counterparts in the microbial transformation of nitrogen. For example, sulfide and ammonia are reduction products of the dissimilation of some organic compounds; both may be oxidized by various bacterial species. Some of the biochemical changes by microorganisms involved in this cycle may be summarized as follows:

1 Sulfur in its elemental form cannot be utilized by plants or animals. Certain bacteria, however, are capable of oxidizing sulfur to sulfates. The classical example is *Thiobacillus thiooxidans*, an autotroph; the reaction involved is

$$2S + 2H_2O + 3O_2 \rightarrow 2H_2SO_4$$

2 Sulfate is assimilated by plants and is incorporated into sulfur-containing amino acids and then into proteins. Degradation of proteins (proteolysis) liberates amino acids, some of which contain sulfur. This sulfur is released from the amino acids by enzymatic activity of many heterotrophic bacteria. The following reaction is an example:



3 Sulfates may also be reduced to hydrogen sulfide by soil microorganisms. An example of bacteria involved in this process is the genus *Desulfotomaculum*, and the reaction suggested is

$$4H_2 + CaSO_4 \rightarrow H_2S + Ca(OH)_2 + 2H_2O$$

4 Hydrogen sulfide resulting from sulfate reduction and amino acid decomposition is oxidized to elemental sulfur. This reaction is characteristic of certain pigmented (photosynthetic) sulfur bacteria and is expressed as

$$CO_2 + 2H_2S \xrightarrow{\text{light}} \underset{\text{Carbohydrate}}{(CH_2O)_x} + H_2O + 2S$$

A laboratory technique which facilitates isolation of various sulfur-metabolizing bacteria is the Winogradsky column shown in Fig. 25-16. The column contains mud, $CaSO_4$, plant tissue (a source of carbohydrate-cellulose), and water. It is exposed to daylight and incubated at room temperature. The microbiological events can be summarized as follows:

1 A variety of heterotrophic microorganisms oxidizes various substrates, depleting the oxygen supply and creating anaerobic conditions:

$$\text{Organic matter} + O_2 \rightarrow \text{organic acids} + CO_2$$

2 Organic acids serve as the electron donors for the reduction of sulfates and sulfites to hydrogen sulfide by anaerobic sulfate-reducing bacteria, e.g., *Desulfotomaculum*:

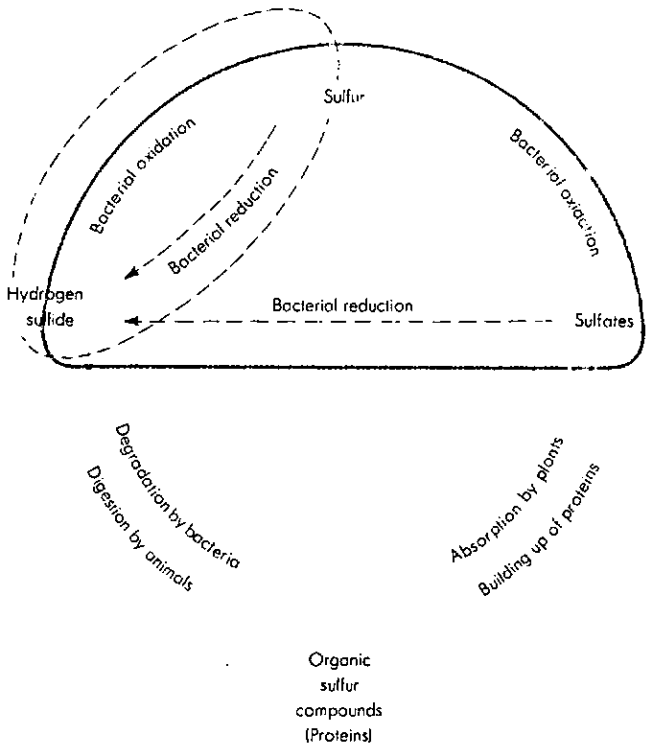


Figure 25-15. Sulfur cycle (schematic), showing the role of microorganisms. (*After Bunker.*)

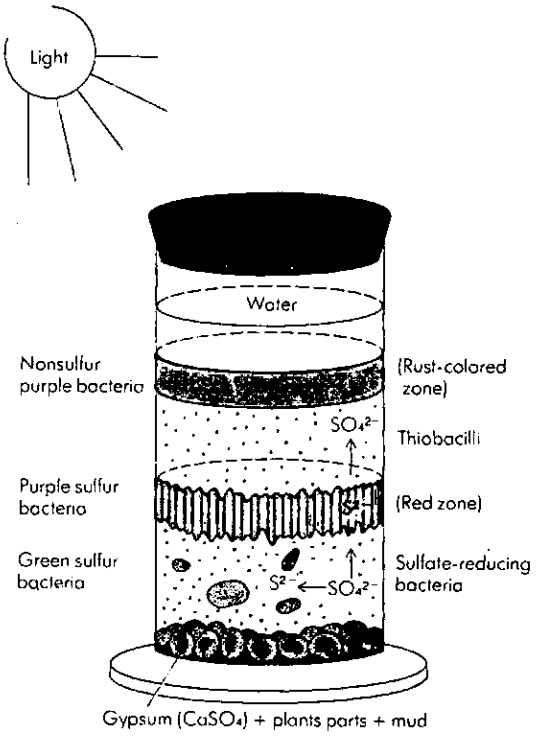


Figure 25-16. A Winogradsky column showing areas of localization of sulfur-metabolizing bacteria. See text for explanation of sequential developments. (*Courtesy of T. Hattori, Microbial Life in the Soil, Marcel Dekker, Inc., New York, 1973.*)

$$\text{Organic acids} + SO_4^{2-} \rightarrow H_2S + CO_2$$

3 Photosynthetic microorganisms such as the purple and green sulfur bacteria (*Chromatium* and *Chlorobium*) use hydrogen sulfide as the electron donor to reduce CO_2:

$$CO_2 + H_2S \xrightarrow{\text{sunlight}} (CH_2O)_x + S$$

4 The aerobic sulfur-metabolizing bacteria, *Thiobacillus* spp., develop in the upper portion of the column and oxidize reduced sulfur compounds (sulfides, elemental sulfur, sulfite). Final oxidation product is sulfate; sulfur accumulates:

$$\text{Reduced sulfur compounds} \rightarrow SO_4^{2-} + \text{accumulation of S}$$

5 The nonsulfur purple bacteria (*Rhodospirillum*, *Rhodopseudomonas*, and *Rhodomicrobium*) are facultative phototrophs; they grow aerobically in the dark and anaerobically in the light and can utilize sulfide at low levels. They are capable of utilizing hydrogen gas as an electron donor in photosynthesis:

$$CO_2 + H_2S \xrightarrow{\text{light}} (CH_2O)_x + S$$

$$CO_2 + 2H_2 \xrightarrow{\text{light}} (CH_2O)_x + H_2O$$

BIOCHEMICAL TRANSFORMATIONS OF OTHER ELEMENTS AND THEIR COMPOUNDS

The preceding discussion was concerned with transformations of nitrogen, carbon, and sulfur and their compounds. But this represents only a part of the elements and their compounds that are subject to assimilation and dissimilation by microorganisms. The metabolic activity of microorganisms (production of acids) solubilizes phosphate from insoluble calcium, iron, and aluminum phosphates. Phosphates are released from organic compounds such as nucleic acids by microbial degradation. Bacteria change insoluble oxides of iron and manganese to soluble manganous and ferrous salts. The reverse is also possible.

From these examples of biogeochemical changes that take place in the soil, it should be apparent that microorganisms do, indeed, perform numerous and essential functions that contribute to the productivity of soil.

BIODEGRADATION OF HERBICIDES AND PESTICIDES

Herbicides are chemical substances that kill plants, especially weeds; pesticides, as the term denotes, are chemical substances that destroy pests. In the context of soil, we think of those pests which adversely affect economic crops—weeds, insects, and pathogenic microorganisms. Thus a more specific nomenclature for substances classified as pesticides would be herbicides, insecticides, fungicides, and nematocides.

The wide-scale application of herbicides and pesticides, while improving the crop yield, raises questions as to the short- and long-range effects as they are deposited in the soil. Are they degraded by soil microorganisms, and if so how rapidly? Do they have a temporary (or permanent) effect upon the soil microbiota? Do they constitute a form of runoff pollution to streams and rivers and as such affect aquatic plant life? These are some of the questions that concern the soil microbiologist as well as other soil scientists, biologists, and environmentalists. Naturally, a major research effort is directed toward answering the questions asked above, as well as others. An ideal pesticide compound would be one that destroys the pest quickly, and, in turn, the pesticide compound would be degraded to more elementary nontoxic substances. The soil is the

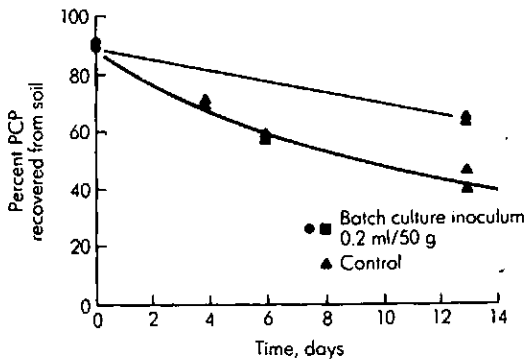
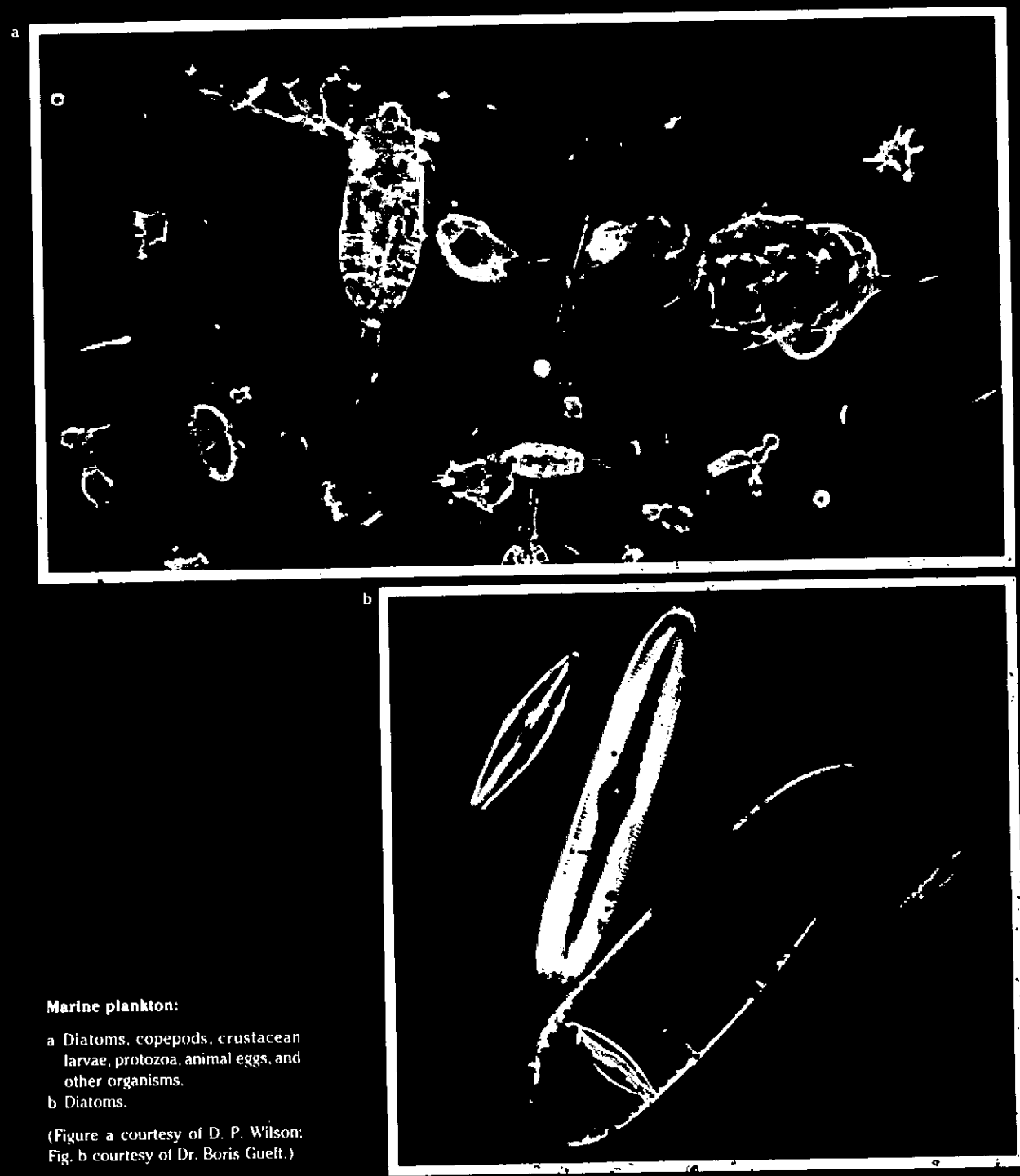


Figure 25-17. Degradation of PCP (pentachlorophenol) in soil by indigenous and inoculated bacteria under laboratory conditions at 30°C. (*Courtesy of R. U. Edgehill and R. K. Finn, Appl Environ Microbiol*, **45**:1122, 1983.)



Marine plankton:

a Diatoms, copepods, crustacean larvae, protozoa, animal eggs, and other organisms.
b Diatoms.

(Figure a courtesy of D. P. Wilson; Fig. b courtesy of Dr. Boris Gueft.)

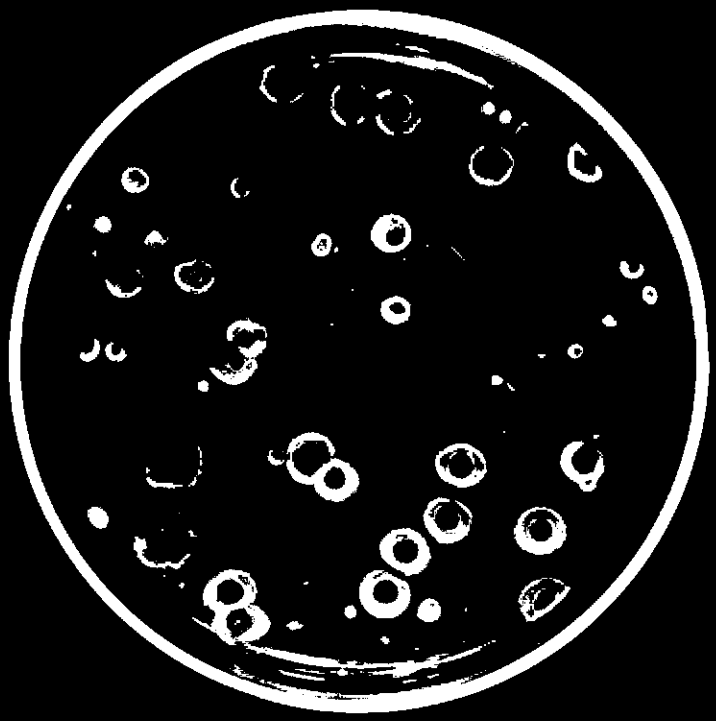
Room air samples:

a Exposure of agar medium in petri dish to room air

b Collection of microorganisms by special air sampling device.

(Figures a and b courtesy of Environmental Services Branch, National Institutes of Health, Public Health Service.)

a



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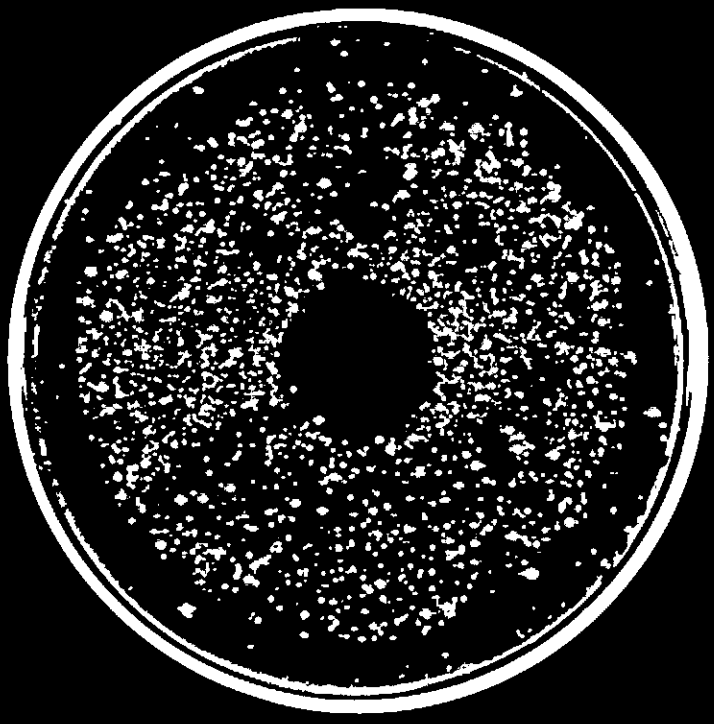

"sink" which receives the pesticide, and it is the soil microbiota that we depend upon to degrade the compound. As an example to illustrate the results of research on this topic, Fig. 25-17 shows the rate of disappearance (degradation) of a pesticide deposited in the soil. This aspect of soil microbiology, namely, the impact of, and the fate of, pesticides deposited in the soil, is a subject of growing concern.

QUESTIONS

1 Describe how the physical composition of soil influences the magnitude and diversity of the microbial flora.
2 Describe one contribution made by Winogradsky and one by Beijerinck to our knowledge of soil microbiology.
3 Assume that you made a microscopic count on a soil sample and a standard nutrient agar plate count from the same sample. What generalizations are likely with respect to the comparability of the counts?
4 How could one proceed to enumerate, by cultural techniques, the various physiological groups of microorganisms present in soil?
5 Compare the microbial flora of soil in the region of the rhizosphere to that in an area at a distance from the rhizosphere.
6 What is meant by the term *mineralization*? Give an example.
7 Assume that some protein material is buried in the soil. Trace the changes it may undergo as a result of microbial attack. Identify bacteria capable of bringing about each of the changes.
8 Distinguish between symbiotic and nonsymbiotic nitrogen fixation. Name several genera of bacteria that are nonsymbiotic nitrogen fixers.
9 What are the components of the bacterial nitrogen-fixing system?
10 How may the process of nitrogen fixation be determined experimentally?
11 Describe the process by which *Rhizobium* spp. invade the root system of a leguminous plant.
12 How is recombinant DNA technology being explored to develop new means of nitrogen fixation?
13 Tremendous amounts of plant material, largely cellulose, are deposited annually on the earth's surface. Insofar as microbiological events are concerned, what happens to this cellulose?
14 Illustrate, with reactions, the manner in which organically bound sulfur is released by microbial dissimilation.
15 Describe the mineralization process as related to the dissimilation of organic phosphorous compounds.

REFERENCES

Alexander, M.: *Introduction to Soil Microbiology*, Wiley, New York, 1977. *A general book useful for more detailed information on the topics presented in this chapter as well as other subjects related to soil microbiology.*

Gray, T. R. G., and D. Parkinson (eds.): *The Ecology of Soil Bacteria: An International Symposium*, University of Toronto Press, Toronto, 1968. *The papers include identification of soil bacteria, factors influencing their development, effects on soil and metabolic interrelations between bacteria and between bacteria and other microorganisms, as well as the microbial flora in the plant-root environment.*

Griffen, D. M.: *Ecology of Soil Fungi*, Syracuse University Press, Syracuse, New York, 1972. A view of general problems of soil fungal ecology is presented in the first section. The second part provides a critical review on the physical ecology of soil fungi.

Hattori, Tsutomu: *Microbial Life in the Soil, an Introduction*, Marcel Dekker, New York, 1973. The subject matter is presented by interweaving relevant material from other disciplines. The coverage of microbial distribution, physiology, and interactions is good, and interrelationships between colloidal systems of the soil and microbes are examined in depth.

Payne, W. J.: *Denitrification*, Wiley, New York, 1981. A small book (214 pages) which provides an in-depth discussion of microbiological denitrification.

Richards, B. N.: *Introduction to the Soil Ecosystem*, Longman, Inc., 1974. A good overview of the characteristics and activities of organisms found in soil is given. The ecosystem concept and soil as an ecosystem are presented in detail.

Starr, M. P., et al. (eds.): *The Prokaryotes: A Handbook on Habitats, Isolation and Identification of Bacteria*, vols. 1 and 2, Springer-Verlag, New York, 1981. These two volumes provide extremely comprehensive coverage of all groups of procaryotes. One can obtain good coverage of groups of soil bacteria in these volumes.

Stewart, W. D. P. (ed.): *Nitrogen Fixation of Free-Living Microorganisms*, Cambridge, New York, 1976. A series of papers discussing the distribution of the nitrogenase enzyme in certain bacteria and blue-green algae and their role in the soil and aquatic ecosystems and methods of measuring nitrogen fixation.