MICROBIAL ANTAGONISMS AND ANTIBIOTIC SUBSTANCES

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Penicillin crystals

Microbial Antagonisms

AND

Antibiotic Substances

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"*La vie empêche la vie*"—Pasteur

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This book is affectionately dedicated to

BOBILI

who has stimulated me in moments of depression, who has been at all times an inspiration in the search for the unknown, my constant associate and antagonist

PREFACE

On the basis of their relation to man, the microscopic forms of life may be classified in two major groups: pathogenic forms that attack living systems, especially those useful to man and to his domesticated plants and animals; and saprophytic forms that attack inanimate matter, including the universal scavengers and the organisms utilized in industry and in the preparation of foodstuffs. Between true parasitism—one organism living in or upon the body of another—and true saprophytism —one organism merely destroying the waste products and the dead cells of another—are groups of relationships that may be designated as antagonistic and associative. In the first of these, one organism is injured or even destroyed by the other, whereas in the second, one organism assists the other and may in turn be benefited by it.

The antagonistic interrelationships among microorganisms have attracted attention since the early days of bacteriology. Following the discovery by Pasteur that microbes are responsible for certain human, animal, and plant diseases, it was established that other organisms, later designated as antagonists, are able to combat and even destroy the disease-producing agents. At first the soil was believed to be the natural habitat of the bacteria that cause epidemics and disease as a whole, but after careful study the fact was definitely established that very few of these bacteria survive for long in the soil. On the contrary, the soil was found to be the natural medium for the development of antagonists chiefly responsible for the destruction of pathogens. The saprophytic organisms that influence in various ways the disease-producing bacteria and fungi were found to inhabit, in addition to the soil, various other natural substrates, such as manure heaps and water basins.

The activities and potentialities of these antagonistic microbes still present many problems. Little is known about the nature and mode of formation of the antibiotic substances they produce, and even less about the mode of their action. The substances vary greatly in their physical and chemical properties. Some are soluble in water, others in ether, alcohol, or other solvents. Some are thermolabile, others are thermostable. Some are sensitive to alkalies, others are not. Some are readily oxidized

and destroyed, others are not. Some are subject to destruction by specific enzymes. The substances are largely bacteriostatic in action, to a lesser extent bactericidal; some are also fungistatic and fungicidal.

Some of the substances are highly toxic to animals. Others are either nontoxic or of limited toxicity and are active *in vivo*. Some hemolyze red blood cells, others do not. Those that are hemolytic and moderately toxic may be useful for application to local infections. Those that are neither hemolytic nor toxic and are active *in vivo* may have great importance in combating certain diseases in animals and man.

Some substances are formed by only a few specific organisms, others may be formed under proper conditions of nutrition by many different organisms. Some antagonists produce only one type of antibiotic substance, others form two or even more chemically and biologically different substances.

The ability of an antagonist or its products—antibiotic substances—to destroy a parasitic microorganism *in vivo* is influenced by the nature of the host as well as by the type and degree of the infection. The manner in which antagonists destroy or modify parasites varies greatly, depending frequently upon the nature of the antibiotic substances produced.

It is thus clear that the subject is extremely complicated, involving numerous interrelationships among different biological systems of both higher and lower forms of life.

In the following pages an attempt is made to present the broad interrelationships among microorganisms living in association, either in simple mixed cultures or in complex natural populations, with special attention to the antagonistic effects. Emphasis is laid upon the significance of these associations in natural processes and upon their relation to disease production in man and in his domesticated plants and animals. The chemical nature of the active—antibiotic—substances produced by various antagonists is described and the nature of the antagonistic action as well as its utilization for practical purposes of disease control is discussed. However, because concepts of the significance of these phenomena are changing so rapidly, no pretense has been made of examining completely the practical applications of this important subject.

Due to the fact that more detailed studies have been made on the production, nature, and utilization of penicillin, more information is

presented about this than about any of the other substances. However, this should not be construed as desire on the author's part to emphasize this substance.

The subject of antagonistic effects of microorganisms has been reviewed in both general treatises (83, 229) and special papers (134, 166, 251, 256, 354, 355, 539, 540, 584, 616, 796, 799, 800, 838); special attention has been paid to the occurrence of such organisms in the soil (620, 794). Advantage was taken of these reviews in the preparation of the comprehensive bibliography presented at the end of this monograph. Attention is directed also to a recent complete review of the literature on the nature and formation of penicillin, the historical development of our knowledge of this agent, method of assaying, and clinical application (678).

The author expresses his sincere appreciation to the members of the staff of the Microbiology Department, New Jersey Agricultural Experiment Station; to members of the Department of Research and Development of Merck & Co. and of the Merck Institute for permission to use reproductions of their work; to Dr. G. A. Harrop of E. R. Squibb & Sons for supplying the photograph of the penicillin-sodium crystals used as the frontispiece to this volume; to Mrs. Herminie B. Kitchen for her careful editing of the manuscript; and to the many investigators in the field whose work has been freely cited both in the form of text or tabular matter and as illustrative material.

S. A. W.

November 15, 1944

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CHAPTER I

SOILS AND WATER BASINS AS HABITATS OF MICROORGANISMS

Although microorganisms inhabit a variety of substrates, from the dust in the atmosphere, the surface of living plants and plant residues, and numerous foodstuffs to the living systems of plants and animals, their natural habitations are soils and water basins.

The soil is by no means an inert mass of organic and inorganic debris. On the contrary, it fairly teems with life. The organisms inhabiting the soil range from those of ultramicroscopic size to those readily recognizable with the naked eye. Many thousands of species, capable of a great variety of activities, are represented in the soil. The physical nature and chemical composition of the soil, the climate, the plant vegetation, and the topography influence greatly both the composition of the microbiological population of the soil and its relative abundance. One gram of soil contains hundreds, even thousands, of millions of bacteria, fungi, actinomycetes, protozoa, and other groups of microorganisms. Under certain conditions, especially when the supply of fresh organic matter in the form of plant and animal residues is increased, the number may be much greater. This varied microbiological population renders the soil capable of bringing about a great variety of chemical and biological reactions.

Through its diverse activities, the microscopic population inhabiting soils and water basins forms one of the most important links in the chain of life on earth. However, its great influence upon numerous phases of human endeavor has been recognized only within recent years. All plants and all animals, including man himself, are dependent upon these organisms to bring about some of the processes essential to the continuation of life. The growth of annual and perennial plants, the supply of food for man and animals, and the provision of clothing and shelter depend largely upon the activities of these microorganisms, especially the transformations brought about in the state of such elements as carbon, nitrogen, sulfur, and phosphorus.

Soils and water basins may be regarded as the primary reservoirs for all living systems inhabiting this planet. Whereas the great majority of microorganisms are saprophytic in nature, living upon inorganic elements and compounds and upon the dead residues of plant and animal life, others have become adapted to a parasitic form of existence and have learned to thrive upon the living tissues of plants and animals. Many of these parasites find their way into the soil and into water basins and may be able to survive there for long periods of time or even indefinitely.

Although the following discussion is limited primarily to the microbiological population of the soil, it also applies, to a greater or lesser extent, to the microorganisms that inhabit manures made up of animal excreta, household wastes, and artificially prepared composts and to those that inhabit water basins, including rivers, lakes, and seas. There are, however, marked differences in the nature of the microbial population of waters and of soils because of the physical and chemical differences in the composition of these two substrates. Nevertheless, some of the underlying principles apply to all substrates. There are, for example, marked differences in the nature and abundance of the populations of soil and water and those of milk, sewage, and foodstuffs. Whereas microorganisms multiply in the latter substrates at a very rapid rate, those in the soil and in water basins are more nearly static, since the rate of their multiplication is much slower except under very special conditions, such as the addition of fresh, undecomposed plant and animal residues or a change in the environment or in the chemical nature of the substrate.

PHYSICAL PROPERTIES OF SOILS

The soil—the surface layer of the earth's crust—comprises three distinct phases, the gaseous, the liquid, and the solid. The last is largely inorganic in nature, with varying concentrations of organic constituents originating from plant and animal residues and found in the soil in different stages of decomposition. The organic substances together with the living and dead cells of microorganisms that inhabit the soil make

up what is known as soil organic matter or, more often, soil humus. The soil as a medium for the development of microorganisms is thus markedly different from the common artificial laboratory media, whether these be synthetic or consist of products of animal or plant life, upon which these organisms are grown.

The inorganic soil particles are surrounded by films of colloidal materials, which are both inorganic and organic in nature. As a rule, the microorganisms inhabiting the soil adhere to these films, although some move freely in the water surrounding the particles. Water and air play essential roles in the soil system and control the nature and extent of the soil population. The nature and size of the mineral and organic soil fractions, as well as the phenomena of adsorption, also influence the abundance, nature, and distribution of microorganisms in the soil. Sandy soils are better aerated than heavy clay soils; they are, therefore, more favorable for the growth of aerobic bacteria and fungi. However, since such soils lack the high water-holding capacity of the heavier soils, they are more readily subject to the process of drying out, which may result in a reduction in microbial activities.

Oxygen, another important factor in microbial development in the soil, becomes available to microorganisms by gaseous diffusion. The oxygen supply diminishes with increase in depth of the soil. When an excess of free water is present in the soil, gaseous oxygen cannot penetrate very deeply and soil organisms then become dependent upon the dissolved oxygen which diffuses into the soil solution. Since the rate of oxygen diffusion is extremely slow, waterlogged soils tend to become depleted of oxygen. Under these conditions, there is marked change in the microbiological population of the soil: the fungi and actinomycetes tend to decrease, and the bacteria, especially the anaerobic types, predominate. Peat bogs are examples of soils in a perpetual anaerobic state; the microbial population is quite distinct from that of mineral soils. Semiarid soils, with a much greater diffusion of oxygen into the deeper soil layers, possess a population which is largely aerobic; in these and other mineral soils the abundance and nature of the organic matter exert a decided influence upon the abundance and nature of the microorganisms present.

The microbiological populations of soils, composts, and water basins are also influenced markedly by seasonal and temperature changes. Certain microorganisms are capable of active life at temperatures approaching the freezing point of water; others, known as thermophilic forms, can withstand very high temperatures, some being active even at 60° to 70° C.

The reaction of the soil is also a factor influencing the nature of the population. Many microorganisms are active within a very limited range of *p*H values; others, notably many of the fungi, are adapted to much wider ranges of reaction. In acid soils, larger numbers of fungi are present, because of the fact that they tolerate more readily the more acid reactions, which limit bacterial competition. On the other hand, actinomycetes comprise a large percentage of the microbial population of dry and alkaline soils.

CHEMICAL COMPOSITION OF SOILS

The solid part of the upper or surface layer (20 to 30 cm.) of the soil commonly is made up of 1 to 10 per cent organic matter and 90 to 99 per cent inorganic or mineral matter. The concentration of organic matter may be even less than 1 per cent, as in desert and poor sandy soils, or more than 10 per cent, as in certain virgin prairie soils and, especially, peat lands which consist of 50 to 99 per cent organic matter, on a dry basis.

The organic matter of the soil is markedly different in chemical nature from that of plant and animal materials. It contains much less cellulose and hemicelluloses than the majority of plants and is higher in lignins and proteins. It is characterized by a narrow ratio of the two important elements carbon and nitrogen, usually about 10:1; it is much more resistant to microbial decomposition than are plant and animal residues. It is black, is soluble to a considerable extent in alkalies, and is partly reprecipitated by acids. These alkali-soluble constituents have often been designated as "humic acids" or "humic bodies," thus imparting the idea that soil organic matter is made up largely of these "acids" (922).

The inorganic constituents of the soil comprise largely sand, silt, clay, and, to a more limited extent, a number of soluble and insoluble salts, notably phosphates, sulfates, and silicates of calcium, magnesium, potassium, iron, aluminum, manganese, zinc, copper, and others. Some of the chemical elements comprise the framework of the soil and are used to only a limited extent by plant and microbial life. Others form important nutrients (for example, C, N, S, P, H, and O) or serve as catalysts for the continuation of life (Zn, Fe, Mn, Cu, Mo, B, and even K are often considered as belonging in this category). The function of most of these elements in the life of microorganisms is not fully understood. In view of the fact that some of the elements in the latter group have been found to form important constituents of certain enzyme systems, the difference between the two functions is not significant.

BIOLOGICAL STATE OF THE SOIL

The abundance of higher plant and animal life in and upon the surface of the soil influences considerably the nature and extent of the microbiological population. Certain plants harbor in their roots specific microorganisms that act as true symbionts; this is true of the root nodule bacteria of leguminous plants and the mycorrhiza-forming fungi found in orchids, evergreens, and many other plants (919). Higher plants also offer a favorable environment for the growth of certain other types of bacteria and fungi, this specific environment being designated as the rhizosphere. The bacterial population of the rhizosphere is not very different qualitatively from that found some distance away from the plants, except that certain types of bacteria are more prominently represented.

The growth of plants results in the production of waste materials and residues left in and upon the soil in the form of roots, leaves, needles, and other products, all of which offer favorable nutrients for microbial development. The root systems of plants also bring about better aeration of the soil, thus making conditions more favorable for the development of aerobic organisms. The presence of higher plants often leads to the development of certain types of bacteria, fungi, and nema-

todes that are pathogenic to the plants, such as the causative agents of root rots, damping-off diseases, root-galls, and various others. Some of the pathogens may become well established in the soil and may persist there long after the specific host plants have been removed. They may even be able to attack other hosts. Plant life thus exerts a variety of influences upon the nature and abundance of the soil-inhabiting microorganisms.

Higher animals also influence the soil microbiological population. Cattle and horses on pastures contribute, through their droppings, energy sources and various other essential nutrients for the development of microorganisms. After death, the bodies of animals, from the smallest insects to man, the lord of creation, also offer available nutrients for the growth of numerous microorganisms. Many animals living in the soil, such as insects and rodents, become carriers of certain bacteria and fungi that are destructive to their hosts; this phenomenon is often utilized for combating injurious animals. Finally, the numerous animals living on the surface of the soil leave waste products rich in bacteria, fungi, and invertebrate animals, some of which are capable of causing serious animal diseases.

NATURE AND COMPOSITION OF THE SOIL MICROBIOLOGICAL POPULATION

The microorganisms inhabiting the soil can be divided, on the basis of their systematic position in the biological kingdom, into the following eight groups: bacteria, actinomycetes, fungi, algae, protozoa, worms, insects and other near-microscopic animals, and ultramicroscopic forms. The last group comprises bodies that range from living systems to products of living organisms; they possess the property of activating similar substances and imparting to them their specific activities, as in the case of phages and viruses.

Five methods are commonly employed for determining the abundance of the various groups of microorganisms inhabiting the soil; namely, plate culture, selective culture, direct microscopic methods, contact slide, and mechanical separation. Each of these has certain ad-

vantages and certain limitations. In many cases, special methods have been devised to supplement the more common methods.

The plate method is based upon principles similar to those employed in other branches of bacteriology. Various media are used, both organic and synthetic. The soil microbiologist has attempted to produce media that either allow the development of the greatest number and the greatest variety of organisms or are particularly favorable for the growth of certain special types of organisms. None of the media so far employed allows the growth of the total soil population. The plate method is often supplemented by the selective culture method, in which a great variety of media are used in order to obtain a representative picture of the soil population. Since the number of media required to enable all soil microorganisms to develop is virtually limitless, the enrichment methods can only give a proximate idea of the nature and abundance of the microbiological population. Because of the development on the plate of certain organisms that exert a toxic effect upon others, the plate method often shows excessive variation in the numbers of bacteria and fungi (257).

The microscopic methods have been introduced to fill this gap, since by them the relative abundance of the various groups of organisms found in soils, composts, or other natural substrates can be established. Unfortunately, these methods do not allow any differentiation between living and dead cells, nor do they permit a differentiation between the various physiological types of microorganisms such as pathogens and nonpathogens. A further limitation, especially of the contact slide, is that the fast-growing forms cannot be prevented from overgrowing the slide and repressing the slow-growing types.

The mechanical separation methods are based upon the use of special sieves or water emulsions and are utilized for the study of the larger forms such as insect larvae and nematodes.

The relative abundance of the different groups of microorganisms in a given soil, as determined by any one of the foregoing methods, varies with the nature of the soil, amount of organic matter, oxygen supply, moisture content, temperature, acidity, and buffering capacity (Table 1), as well as with the nature of the higher plants growing in

the given soil (Table 2). Despite all these factors, the microbiological population of the soil throughout the world has certain definite and common characteristics and comprises certain well-defined, specific

TABLE 1. INFLUENCE OF SOIL TREATMENT ON NUMBER OF MICROORGANISMS

TREATMENT OF SOIL	REACTION OF SOIL	MICROORGANISMS FOUND*		
	*p*H	Bacteria	Actinomycetes	Fungi
Unfertilized and unlimed	4.6	3,000	1,150	60
Lime only added	6.4	5,410	2,410	23
Potassium salts and phosphates added	5.5	5,360	1,520	38
Salts and ammonium sulfate added	4.1	2,690	370	112
Salts, ammonium sulfate, and lime added	5.8	6,990	2,520	39
Salts and sodium nitrate added	5.5	7,600	2,530	47
Stable manure and salts added	5.4	8,800	2,920	73

From Waksman (925).
* In thousands per gram of soil as determined by plate method.

TABLE 2. INFLUENCE OF GROWING PLANTS ON NUMBER OF MICROORGANISMS IN THE SOIL

PLANT	SAMPLE OF SOIL TAKEN	MICROORGANISMS FOUND*		
		Bacteria	Actinomycetes	Fungi
Rye	Near roots	28,600	4,400	216
	Away from roots	13,200	3,200	162
Corn	Near roots	41,000	13,400	178
	Away from roots	24,300	8,800	134
Sugar beet	Near roots	57,800	15,000	222
	Away from roots	32,100	12,200	176
Alfalfa	Near roots	93,800	9,000	268
	Away from roots	17,800	3,300	254

From Starkey (848).
* In thousands per gram of soil.

types. The bacteria usually range in number from a few hundred thousand to several hundred million per gram of soil, though many species do not develop on the common plate. Fungi are found in the form of mycelial filaments and as spores and may therefore constitute as large a mass of living matter as do the bacteria; their actual number, as determined by the plate method, may vary from a few thousand to several hundred thousand per gram of soil. The significance of these results is not always clear, since a given colony may have originated from a hyphal filament, a mass of mycelium, or a single spore. Determination by the plate method of the number of actinomycetes is subject to the same limitations; these organisms usually constitute from 10 to 50 per cent of the colonies appearing on common bacterial agar plates.

Algae are numerous in the surface layers of soil only. Protozoa are present in the soil in an active vegetative or trophic state and in the form of cysts. The active cells appear when excessive water is present, even for a few hours; in dry soil, the cysts predominate. Flagellates are represented by the largest numbers, sometimes approaching a million individuals per gram of soil; amebae are next in abundance; ciliates are usually found to the extent of a few hundred to several thousand per gram of soil. Nematodes, rotifers, earthworms, and larvae of numerous insects are also abundant, often forming a large part of the bulk of the living mass of cell substance.

By means of the selective and enrichment culture methods, several physiological classifications of bacteria have been recognized. The following descriptive terms are commonly used to designate these groups: autotrophic vs. heterotrophic, aerobic vs. anaerobic, motile vs. nonmotile, pathogenic vs. saprophytic, psychrophilic and mesophylic vs. thermophilic, symbiotic vs. nonsymbiotic, and antagonistic vs. nonantagonistic.

The fungi may be classified into three types: saprophytic and free-living, mycorrhiza-producing, and plant pathogenic. The most common groups of soil fungi are found in the genera *Rhizopus, Mucor, Penicillium, Aspergillus, Trichoderma, Fusarium, Cladosporium,* and *Cephalosporium.* The soil often harbors an abundant population of yeasts and fleshy or mushroom fungi. The latter may produce an ex-

tensive mycelium in the soil, binding the particles together and preventing their falling apart.

Various bacteriolytic agents, including specific phages, have also been demonstrated in the soil. The phage of root-nodule bacteria is of particular interest. It is readily adsorbed by the soil, but its presence can easily be established. The repression of spore-forming bacteria and the abundance of *Pseudomonas fluorescens* (139, 140) may be due to the antagonistic action of the latter.

SOILS AND WATER BASINS AS CULTURE MEDIA

Microorganisms require for their growth and respiration certain energy sources and certain nutrients, as well as certain conditions favorable for their development. Different organisms show considerable variation in this respect. The mineral elements required for growth and multiplication are almost invariably present in the soil and to a large extent also in many water basins. The available energy supply may be limited, however, and thus usually becomes the most important factor regulating the abundance and activities of microorganisms in natural substrates. The autotrophic bacteria depend on the supply of oxidizable minerals such as ammonium salts, nitrite, sulfur, iron, and manganese, the oxidation of which makes energy available for their growth. The heterotrophic organisms are dependent on the carbon compounds brought into the soil in the form of plant and animal residues as well as the bodies of many insects, earthworms, and other small animals. The roots of plants also supply an abundance of easily available substances for microbial nutrition.

Every organic compound produced in nature finds its way, sooner or later, into the soil or into lakes and rivers, where it serves as a source of energy for microorganisms. This energy becomes available to some of the organisms through anaerobic or fermentative transformation and to others through aerobic or oxidative processes. The net change in the energy produced by any one organism or group of organisms is accompanied by a loss of free energy by the system to which the culture is

confined. The synthesis of new cell material by microorganisms is accompanied by a gain of free energy, which must be supplied by other chemical transformations. Ordinary soils, however, contain microbial nutrients in concentrations sufficient to support a large number of living cells. This can be illustrated by the fact that when a soil is sterilized and then inoculated with a pure culture of bacterium rapid multiplication takes place (Table 3). When fresh water taken from a lake or the sea is kept in the laboratory for one or two days, a great increase in its bacterial population occurs.

There is considerable variation in the ease with which a specific or-

TABLE 3. MULTIPLICATION OF COLIFORM BACTERIA IN STERILE SOIL

ORGANISM	BACTERIA INOCULATED*	BACTERIA RECOVERED*	
		After 10 days	After 26 days
Escherichia coli			
in soil alone	2,600	149,000,000	138,000,000
Aerobacter aerogenes			
in soil alone	109,000	48,000,000	42,600,000
in soil and glucose	109,000	1,660,000	240,000,000

From Waksman and Woodruff (949).
* Per gram of soil.

ganism can be isolated from a natural substrate and consequently in the techniques employed. Some microorganisms may be present in abundance and can be readily isolated. Others are found only in limited numbers and can be obtained only with considerable difficulty and by the use of special procedures. Still others can be isolated only after the natural substrate is treated in such a manner as to favor the multiplication of the specific organism; this can be done by enriching the soil with a nutrient or substance which the particular organism is able to utilize, or by changing conditions of reaction, by aeration, or by other treatment that would favor the rapid development of the organism in question. Special strains or races of microorganisms may often be developed as a result of such treatment, which tends to favor the adaptation of the organisms present in the soil to a particular process.

NUTRITION OF MICROORGANISMS IN NATURAL SUBSTRATES

It was at first assumed that bacteria and other microorganisms possess a simpler type of metabolism than do higher plants and animals; although some can obtain all the nutrients required for cell synthesis and energy from simple elements and compounds, others need for their nutrition certain highly complicated organic substances. Recently it has been recognized that various "growth-promoting" substances or vitamins play an important role in the nutrition of many microorganisms. It has also been established that highly complicated enzyme systems are produced by these lower forms of life, and that many interrelationships exist among their metabolic processes, the composition of the medium, and the environmental conditions. One thus begins to realize that the metabolism of these microbes is also highly complicated. Most of the information on their nutrition is based upon their growth on artificial culture media. In nature, however, these organisms live in associations and vary considerably in the degree of their interdependence. As yet no laboratory method has been developed that duplicates these conditions.

Microorganisms vary considerably in their nutrition and energy utilization, as well as in the breakdown and transformation of the available nutrients. Certain elements or compounds are required for cell synthesis. In some cases, certain trace elements as well as varying concentrations of growth-promoting substances are also essential. Among the nutrient elements, nitrogen occupies a prominent place. Considerable variation exists in the ability of microorganisms to utilize different types of nitrogen compounds: some can obtain their nitrogen from a wide variety of substances; others are restricted to the use of a single group of compounds such as proteins, amino acids, urea, ammonia, or nitrate; a few are able to use atmospheric nitrogen. The variety of organic nitrogenous bodies supplied to microorganisms in soils and in water basins is limited only by the number of such compounds synthesized by plants and animals. The complex forms of nitrogen are broken down to simpler compounds; these may be assimilated by organisms and again built up into complex forms, or they may be utilized only by other organisms. Microbial activity thus regulates the state of the nitro-

gen in natural substrates and is responsible for the continuous stream of ammonia and nitrate forming the available sources of nitrogen that makes possible the growth of higher plants.

THE GROWTH OF THE MICROBIAL CELL IN PURE CULTURE AND IN MIXED POPULATIONS

When nutrients are available in sufficient concentration and when the environmental conditions are favorable for the development of the microbial cell in pure culture, growth follows a definite sigmoid-shaped curve. Slow multiplication is followed by rapid development, until a certain maximum number of cells within a given volume of medium is reached; the rate of growth then diminishes. The maximum population of *Aerobacter aerogenes* grown in a medium containing lactose and ammonium tartrate increases at first in proportion to the concentrations of these nutrients but later becomes independent of them. The onset of the stationary phase may be due to several factors: exhaustion of substances necessary for growth, change in the reaction of the medium to one unfavorable for further development, accumulation of toxic products. When the nutrients in the medium are exhausted, addition will restore growth. When an unfavorable change in reaction has taken place, the addition of acid or alkali will render the medium again favorable. The production of toxic substances in the medium can be counteracted usually by the use of heat or by treatment with charcoal, though some of the injurious bodies may be heat-resistant.

In the presence of other microorganisms, a certain organism may show reactions markedly different from those obtained in pure culture: it may produce substances that are either favorable or injurious to the other cells, it may compete with the other organisms for the available nutrients or it may render the medium more favorable for their development. It has been shown (936), for example, that certain bacteria like *Bacillus cereus* can attack native proteins but not amino acids, whereas others like *Pseudomonas fluorescens* can attack amino acids but not proteins; when these two organisms were placed together in the same medium, their activities supplemented one another. Numerous

other instances are found in soil and water of an organism preparing the substrate for another, ranging from distinct symbioticism, where one organism depends absolutely for its living processes upon the activities of another (symbiosis), to association, where one organism merely is favored by the growth of another (metabiosis), to the injury of one organism by another (antagonism), and finally, to the actual destruction of one by another (parasitism).

INTRODUCTION OF DISEASE-PRODUCING MICROORGANISMS INTO THE SOIL

Ever since higher forms of life first made their appearance on this planet they have been subject to attack by microbes. These microscopic organisms must have gained, at an early stage in the development of the higher forms, the capacity of attacking them in one manner or another. There is no plant or animal now living that is not subject to infection by different bacteria, fungi, and protozoa. The more advanced the animal body is in the stage of evolution, the more numerous are its ills, most of which are caused directly or indirectly by microorganisms.

The microbial agents causing thousands of diseases of plant and animal life have now been recognized and even isolated and described. In many cases these disease-producing agents are closely related morphologically to those which lead a harmless existence in soils or water basins; many of the saprophytes, for instance, are found to be of great benefit to man and to his domesticated plants and animals. This suggests the probability that pathogenic microorganisms represent certain strains of soil and water-inhabiting types that have become adjusted to a parasitic existence. During their life in the host, they multiply at a rapid rate and produce substances toxic to the body of the host. The result is that the host is incapacitated for a certain period of time, until it succeeds in building up resistance against the invading organisms. It may thus overcome the injurious effect of the pathogen or it may be killed if such resistance cannot be effected. In the first instance, a temporary or permanent immunity against the specific disease-producing microbe or its close relatives may result. The host is often able to survive the attack without being able to destroy the invading microbes; if

it again attains a normal form of life, it is designated as a carrier of the disease-producing agent.

Pathogenic organisms pass their existence in the living body of the plant or animal. They spread from one host to another by contact or through a neutral medium, such as water, milk, or dust where they may remain alive and active for varying lengths of time, or they reach the soil or water basins in the excreta of the host. If the host is killed by the infecting microbes, they may survive for some time upon the remnants of what was once a living animal or plant and thus find their way into the soil and water basins.

Considering the millions of years that animals and plants have existed on this planet, one can only surmise the great numbers of microbes causing the numerous diseases of all forms of life that must have found their way into the soil or into streams and rivers. What has become of all these pathogenic bacteria? This question was first raised by medical bacteriologists in the eighties of the last century. The soil was searched for bacterial agents of infectious diseases. It was soon found that, with very few exceptions, organisms pathogenic to man and animals do not survive very long. This was at first believed to be due to the filtration effect of the soil upon the bacteria (32). It came to be recognized, however, that certain biological agents are responsible for the destruction of the pathogenic organisms. These investigations led to the conclusion that the soil can hardly be considered as a carrier of most of the infectious diseases of man and animals. The fact that many pathogens can grow readily in sterilized soil but do not survive long in normal fresh soil tends to add weight to the theory of the destructive effect upon pathogens of the microbiological population in normal soil.

INTRODUCTION OF SAPROPHYTIC ORGANISMS INTO THE SOIL

It often becomes necessary to inoculate the soil with organisms not usually found there. The common practice of inoculating soil with bacteria capable of forming root nodules on leguminous plants is a case in point. It is essential, therefore, to know how long these organisms will survive. The survival period is influenced greatly by the presence of a

host plant that protects the specific bacteria from attack by antagonistic organisms. In the absence of the host plant, the bacteria seem to disappear gradually, and reinoculation becomes advisable when the host is again planted in the given soil. It has been observed also that specific strains of bacteria tend to deteriorate in the soil, and that it is necessary to reinoculate the soil with more vigorous strains of the organisms in question.

The ability of other bacteria, notably members of the *Azotobacter* group, to fix nitrogen independently of host plants and the fact that these organisms are absent in many soils led to the suggestion that such soils might benefit from inoculation. It has been found, however, that when soils and peats are inoculated with *Azotobacter chroococcum* large-scale destruction of the latter often occurs (779). This is believed to be due to the presence in the soil of antagonistic organisms as well as toxic substances (477, 648, 951).

Certain fungi are unable to grow in fresh nonsterilized soil but are capable of growing in heated soil. This was found to be due to the fact that normal soils contain certain substances that render the growth of the fungus impossible; these substances are destroyed by heating. An extract of fresh soil was found to act injuriously upon the growth of the fungus *Pyronema;* the injurious effect was partly removed on boiling (500).

The survival of microorganisms added to soil or water is thus influenced by the nature of the native soil or water population, the organisms added, the composition of the substrate, and various environmental conditions.

SAPROPHYTIC AND PATHOGENIC NATURE OF CERTAIN SOIL MICROORGANISMS

Various fungi and actinomycetes causing animal diseases, notably skin infections, appear to resemble very closely the corresponding soil saprophytes. It was therefore suggested that many of the dermatophytic fungi normally lead a saprophytic existence in the soil but are also capable of developing on epidermal tissue, and bringing about infection of the tissues. This was found to be true especially of species of

Sporotrichum, various actinomycetes such as those causing lumpy jaw of cattle, and certain other organisms. Henrici (396) divided fungus infections of animals into two groups: first, superficial mycoses, comprising moniliases and dermatomycoses, that are caused by a variety of fungi widely distributed in nature; and, second, deep-seated infections, namely, aspergillosis, sporotrichosis, and blastomycosis, with a marked tendency to restricted distribution. The latter were said to be caused primarily by saprophytic forms, including varieties capable of chance survival and of multiplication when accidentally introduced into animal tissues.

Walker (952) suggested that the partly acid-fast coccoid, diphtheroid, and actinomycoid organisms that have been cultivated repeatedly from leprosy are merely different stages in the life cycle of the same form. The causative agent of leprosy, like certain pathogenic actinomycetes, is believed to be a facultatively parasitic soil organism, probably of wide but irregular distribution. Leprosy was thus looked upon primarily as a soil infection, brought about presumably through wounds; a secondary means of infection by contagion was not excluded. A comparison of cultures obtained from rat leprosy, human leprosy, and bacteria of soil origin led to the conclusion that the strains from all three sources were identical; human and rat leprosy were said to have the same etiology and endemiology, finding a normal habitat in the soil.

An interesting relationship has been shown to exist between Texas fever and the capacity of cattle tick (*Boophilus bovis*), the parasite carrier, to persist in the soil (836). The causative agent is an organism with protozoan characteristics. It persists in southern pastures where the carriers survive from one season to the next and keep the cattle continuously infected. The disease is of little importance in northern regions, the ticks being destroyed during the winter. When northern cattle are moved to southern pastures, they become subject to the disease.

Pathogenic microorganisms capable of surviving in the soil have presented important economic problems to farmers raising hogs, cattle, poultry, and other domestic animals, but disease incidence through this source has been greatly diminished by the proper practice of sanitation. The rotation of crops has been utilized for the purpose of overcoming these conditions, several years usually being required to render infected

pastures safe for use. The fact that most pathogenic organisms rapidly disappear when added to the soil makes this problem rather simple; the prevention of infectious diseases would have presented far more difficult problems were the infecting agents to remain indefinitely virulent in the soil. The few disease-producing agents that are capable of persisting, such as anthrax, blackleg, and coccidiosis, have been the cause, however, of considerable damage to animals.

Of greater economic importance than the survival in the soil of human and animal pathogenic agents is the fact that the soil harbors a number of plant pathogens, including not only fungi, bacteria, and actinomycetes, but also nematodes and insects. Fortunately, the continued development of these organisms in the soil also leads to the accumulation of saprophytic organisms destructive to them.

The extent to which virus diseases persist in the soil is still a matter for speculation. It has been demonstrated that the phage of legume bacteria may persist and become responsible for a condition designated as "alfalfa-sick soils" and "clover-sick soils" (169, 474). In order to overcome this condition, the breeding of resistant varieties of plants has been recommended.

CHAPTER 2

HUMAN AND ANIMAL WASTES

And a place shalt thou have without the camp, whither thou shalt go forth abroad: and a spade shalt thou have with thy weapons; and it shall be, when thou sittest abroad, thou shalt dig therewith, and shalt afterward cover that which cometh from thee.—Deuteronomy 23:13 and 14.

HUMAN and animal excreta and other waste products, which are or frequently become both offensive and dangerous to public health, sooner or later find their way into the soil and water basins. The soil also receives the many residues of growing crops that are annually left on the land, together with the waste materials of the farm and the home (439, 922). These wastes contain substances partly digested by man and animals, and their metabolic waste products, as well as freshly synthesized material in the form of microbial cells. The microbial population of such waste materials comprises agents of digestion, some microbes that are present accidentally, and some that possess the capacity of causing human, animal, and plant diseases.

These waste materials do not remain long in an unaltered form and do not accumulate in or on the surface of the soil or in water basins; otherwise both soil and water long ago would have been rendered unsightly, disagreeable bodies, which man would not dare to tread upon or enter. On the contrary, the soil and the water are capable of digesting all these cast-off materials and of completely destroying their undesirable characteristics. Through all past ages, the waste products of plant and animal life have disappeared, whereas the soil and the water in the rivers, lakes, and seas have remained essentially the same, except under very special conditions such as those that brought about the production of peat in water-saturated basins and, in past geological ages, the formation of coal. The capacity of soil and water to destroy these offensive wastes is due entirely to the microorganisms that inhabit the substrates. The important ultimate products of destruction are ammonia, carbon dioxide, and water; often hydrogen and methane are

produced; various mineral compounds, such as phosphates, sulfates, and potassium salts are also liberated. These mineralized substances are essential for the continuation of plant and animal life on this earth.

Largely because of the activities of the microorganisms inhabiting soils and water systems, man does not need to worry about the disposal of plant and animal wastes. These activities need only be regulated, in order to accomplish the breakdown of complex substances with the greatest efficiency and the least loss of valuable nutrient elements. The following principal objectives are usually to be attained: first, the destruction of plant and animal pathogens, including pathogenic bacteria and fungi and disease-producing protozoa, worms, and insects; second, the liberation of the essential elements required for plant nutrition in available forms, especially carbon, nitrogen, and phosphorus; and, third, the formation of certain resistant organic substances, known collectively as humus, which are essential for the improvement of the physical, chemical, and biological condition of the soil.

STABLE MANURES AND FECAL RESIDUES

Microbial Population

Fresh excreta of animals and man are rich in fecal bacteria, consisting, on the average, of 5 to 20 per cent bacterial cells (802). Lissauer (533) calculated that the bacterial substance of feces ranges from 2.5 to 15.7 per cent of the dry weight, with an average of 9 per cent. Bacteria were reported to make up as much as 9 to 42 per cent of the bulk of animal stools, the number depending on the composition of the foodstuffs, the nature of the animal and its condition of health, and other factors (364). Since 1 mg. of dry bacterial substance contains about 4 billion bacterial cells, the number of these organisms in fecal excreta can be seen to be very large, although many, if not most, of the cells are no longer in a living state. Osborne and Mendel (659) removed from the feces of white rats the residual food material and found that the bacterial cells made up 23 to 41 per cent of the total material; the nitrogen content of these cells varied from 10.7 to 12.2 per cent. Since the removal of the residual foodstuffs consisted in treatment with ether, alcohol, and acid, some of the bacterial cell constituents were also removed; the actual

concentration of bacterial substance may, therefore, have been even greater.

It has been reported (441) that 1 ml. of the intestinal contents of cattle contains 10 billion cells capable of development. By suitable methods of cultivation, human feces were found (588) to contain 18 billion bacteria per gram. Determination (542) of the number of bacteria in stable manure gave 11.6 billion cells per gram of material by the plate method, and, by the dilution method, 5 billion peptone-decomposers, 100 million urea-decomposers, and 2.5 million cellulose-decomposers. About 100 billion bacteria may be produced daily in the human intestine. Human feces are made up, on an average, of 32.4 per cent bacterial cells amounting to 2,410 millions of bacteria per milligram of moist material. Feces of healthy persons were shown (300) to contain 8.2 to 24.2 per cent bacterial cells; in those of persons suffering from intestinal disturbances the percentages were 20.1 to 40.2. With the development of the microscopic technique for counting bacteria, much larger numbers of cells were shown to be present than could be determined by the plate method.

The urine of healthy persons is sterile or very low in bacteria. Because of the ability of many bacteria to utilize the chemical constituents of urine, rapid bacterial multiplication takes place in fresh urine, especially when mixed with animal feces and bedding (775).

The microbiological population of animal excreta is characteristic. In addition to the common fecal bacteria, it contains fungi, thermophilic bacteria, and, in herbivorous animals, anaerobic cellulose-decomposing bacteria (543).

Various methods have been developed for permitting the preferential development of certain types of bacteria. Gram-negative bacteria in the feces can be repressed by certain reagents (839); gram-positive bacteria can be repressed by the addition of antibiotic substances such as actinomycin (Table 4).

The bacterial population of fresh cow manure was found (796) to consist of 47.5 per cent streptococci (*Streptococcus pyogenes, Sarcina* sp., and *Micrococcus candicans*), 21.2 per cent coli-like colonies (*Escherichia coli, A. aerogenes,* and *S. septicemiae*), and many dark colony-forming types. Other groups represented were *Bacteroides, Flavobacterium,*

TABLE 4. EFFECT OF ACTINOMYCIN ON THE MICROBIOLOGICAL POPULATION OF CERTAIN NATURAL SUBSTRATES

SUBSTRATE	DILUTION FOR PLATING	MILLIGRAMS OF ACTINOMYCIN PER 10 MILLILITERS OF AGAR	COLONIES ON PLATE	TYPES OF BACTERIA ON PLATE
Air-dry soil	1,000	0	Numerous	Largely gram-positive, many spore formers
	1,000	0.01	Fewer	Gram-negative
	1,000	0.10	96	Gram-negative
	1,000	1.00	0	None
Fresh soil	1,000	0	Numerous	Largely gram-positive
	1,000	0.01	Fewer	Gram-negative
	1,000	0.10	Few	Gram-negative
	1,000	1.00	0	None
Fresh milk	100	0	790	Gram-positive and gram-negative
	100	0.01	346	Gram-negative
	100	0.10	251	Gram-negative
	100	1.00	1	Gram-negative
Fresh sewage	1,000	0	1,248	Mostly gram-negative
	1,000	0.01	1,172	Gram-negative
	1,000	0.10	1,131	Gram-negative
	1,000	1.00	121	Gram-negative

From Waksman and Woodruff (945).

Pseudomonas, *Bacillus*, various anaerobic bacteria, *Oidium*, and many others. When the manure was allowed to decompose, yellow rods, fluorescent bacteria, and mesentericus types took the place of the streptococci.

The following heterotrophic bacteria have been demonstrated (775) in manure: *Bacillus subtilis*, *Bacillus mesentericus*, *Bacillus cereus*, *Bacillus tumescens*, *Bacillus petasites*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Salmonella enteritidis*, *Escherichia coli*, *Proteus vulgaris*, *Micrococcus luteus*, *Micrococcus candicans*, *Staphylococcus albus*, *Sarcina flava*, *Streptococcus pyogenes*, and others. Anaerobic bacteria are also abundant (329).

Pathogenic bacteria may also occur frequently in human feces and in stable manure; *Mycobacterium tuberculosis* and various hemolytic streptococci (830), as well as pathogenic anaerobes including *Clostridium welchii, Clostridium septicum, Clostridium oedematis,* and *Clostridium fallax* have been found (468).

The protozoa capable of developing in manure and in urine include not only saprophytic forms but also certain parasites, such as *Trichomastric* and *Trichomonas,* capable of living and even of multiplying in excreta. The coprophilic protozoa comprise various flagellates, certain amebae, and ciliates. The liquid part of the manure is considerably richer than the solid in total number of protozoa as well as in species, including *Polytoma uvella, Cryptochilum nigricans,* and *Tetramitus rostratus.* These protozoa nearly all feed upon bacteria. The infusoria may feed upon smaller protozoa, so that forms like *Colpidium* may not destroy bacteria at all.

Human and animal excreta also contain a large population of fungi, chiefly in a spore state. Schmidt (801) divided the manure-inhabiting fungi into three groups:

Those found only in manure; their spores are swallowed with the feed, and they pass unchanged through the digestive tract, though they are favorably influenced toward germination by the body heat and digestive fluids of the animal. Their natural multiplication by spores is impossible without the physiological action of the digestive processes.

Those that do not have to pass through the digestive tract of an animal in order to germinate and develop. The representatives of this group occur in nature only in manure, although some are able to grow also on other substrates. They can be cultivated both on manure and on other media, mostly at ordinary temperatures.

Organisms found both in manure and on other substrates. They grow readily at room temperature on a number of media.

Composition and Decomposition

The chemical composition of human and animal excreta, and of stable manures in general, varies considerably, depending on the nature of the animal, its age, mode of nutrition, and composition of food-

stuffs (454). As soon as voided, manure begins to undergo rapid decomposition. This results in the formation of ammonia (140) and various other nitrogenous degradation products (290). These give rise to offensive smells, which are controlled by the conditions of decomposition. From a sanitary point of view, it is essential that decomposition should be accompanied by the destruction of the injurious organisms present in the manure. The fecal organisms gradually disappear and their place is taken by a population concerned in the decomposition of cellulose, hemicelluloses, and proteins (922).

The decomposition of complex plant and animal residues leads to a rapid reduction in carbohydrates and is accompanied by the evolution of considerable heat, the temperature of the compost reaching as high as 80° C., as shown in Figure 1.

In order to hasten the decomposition of manure, conditions must be favorable to the activities of microorganisms. It must be properly

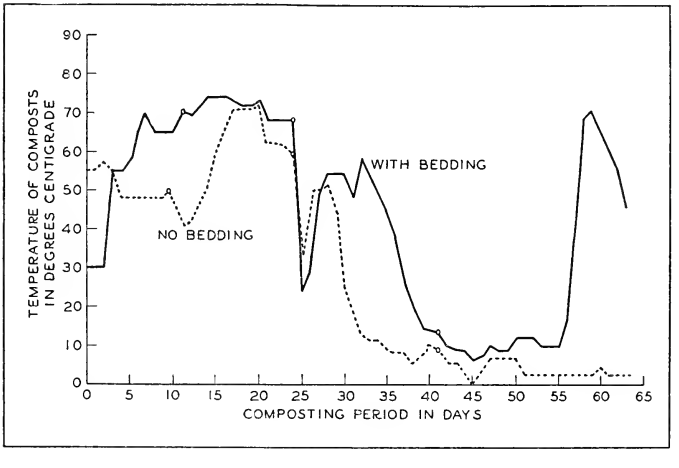


FIGURE 1. Influence of straw bedding upon temperature changes in the composting of manure. Circles indicate times of turning composts. From Waksman and Nissen (940)

aerated and well moistened but not saturated with water. By placing the manure, together with the waste materials of the farm and the home, in heaps, designated as composts, the decomposition processes can be controlled so as to lead to heat liberation; this results in the destruction of the injurious organisms and the conservation of the plant nutrient elements. When not properly regulated, the decomposition processes may be wasteful, unsanitary, and unsightly, and may even become a source of infection to man and his domesticated animals.

SEWAGE

Disposal of sewage and other home wastes is one of the important sanitary problems of men living in industrial and residential centers. Haphazard methods of disposing of sewage not only lead to conditions most unpleasant to human habitation but they are dangerous from the standpoint of infectious diseases.

Sewage abounds in microorganisms that originate not only from human excreta but also from other household and industrial wastes. The various saprophytic bacteria present in sewage rapidly attack the organic constituents and bring about their gradual mineralization. The destructive action of saprophytic organisms greatly reduces the number of pathogens (334). Activated sludge, for example, has been shown (853) to possess a definite and consistent bactericidal action against the colon bacteria. In addition to antagonistic organisms, active bacteriophages against nearly all types of intestinal bacteria are present in sewage. The destruction of pathogens by bacteriolysis thus readily finds a place in the activated-sludge method of sewage purification.

Dissolved oxygen is generally present when sewage is diluted with water. As the destruction of the organic matter proceeds rapidly, the oxygen becomes depleted, so that none is left after a few hours. The predominant bacterial flora of the water may then become anaerobic, with the result that the chemical processes of decomposition are completely changed; hydrogen sulfide, mercaptans, and other foul-smelling substances are then formed. This is accompanied by a typical anaerobic breakdown of carbohydrates, leading to the formation of vari-

ous organic acids, carbon dioxide, hydrogen, and methane. The nitrogen in the protein and urea is transformed to ammonia and various amines. When sewage is aerated, the anaerobic processes gradually give way to aerobic processes, as the oxygen diffuses into the liquids or as the sewage is diluted with water containing dissolved oxygen.

When sewage is freed from solids by sedimentation before discharge, or when it is aerated sufficiently to maintain the concentration of dissolved oxygen, decomposition proceeds rapidly without the production of the bad odors usually associated with the anaerobic breakdown. The destruction of the pathogenic bacteria results largely through the activities of the saprophytes (772, 980). For the purpose of promoting the development of aerobic bacteria, processes employing the use of intermittent sand filters, broad irrigation, contact beds, trickling filters, and activated sludge are applied.

The modern methods of sewage purification are based on the long-known fact that the soil is a destroyer of offensive wastes. In early days, in fact, the soil handled all sewage problems. Sewage disposal plants in modern cities are so operated that microorganisms found to be so efficient in the soil are able to act under optimum conditions, resulting in rapid purification. Sewage freed from most of its organic constituents can be discharged into a stream and will not deplete the water of its dissolved oxygen. Chlorine is frequently employed in the final treatment to assure the complete destruction of the pathogens.

GARBAGE

The processes involved in the disposal of garbage from the home are similar to those utilized in the disposal of stable manure rather than of sewage. At present, garbage usually is destroyed by burning, which results in great economic waste, or is dumped outside cities, thus creating centers of infection and unpleasant appearance. More logical and less wasteful processes are based upon the principle of composting. Several of these processes are now utilized in India and China, where economic pressure is greatest. By proper handling, a product is formed that is free from injurious insects, parasitic worms, and bacteria, and that has conserved all the valuable elements essential for plant growth.

DESTRUCTION OF INJURIOUS MICRO-ORGANISMS

Improper methods of disposal of human and animal wastes were responsible, in the early history of mankind, for many epidemics of cholera, typhoid, plague, and other diseases. Only in recent years, after man learned the nature of the spread of these diseases, were proper methods developed for disposing of human wastes. According to Winfield (990), fecal-borne diseases rank with venereal disease and tuberculosis as the most important infectious diseases of China, because the people do not maintain proper sanitation and because human excreta are used as fertilizers. Any successful system for the control of these diseases must be sanitary and at the same time profitable. The composting method can meet these requirements.

To illustrate this point, it is sufficient to consider an analysis (990) of the occurrence of *Ascaris lumbricoides*, its transmission, and its relation to *Entamoeba histolytica*. Of 1,190 persons examined, 81 per cent were positive for ascaris, with an average egg count of 14,000 per cubic centimeter. Children had a higher count than adults, and females a higher count than males. The life habits of the Chinese people are highly favorable for the spread of ascaris. By a special process of composting of feces, sufficient heat was produced to destroy disease-producing organisms and their reproductive bodies. The compost thus produced is highly effective as a fertilizer.

Many other natural substrates, like saliva (704), possess antibacterial properties due to the antagonistic action of their own bacterial populations. Though antagonistic microorganisms may persist in soil or in other natural substrates, substances toxic to bacteria soon tend to be destroyed (444).

SURVIVAL OF HUMAN AND ANIMAL PATHOGENS IN SOIL AND WATER

During the period 1878 to 1890 following the brilliant work of Pasteur, when bacteriology was still in its infancy, medical bacteriologists took much interest in soil microbes. This was due largely to the belief that causative agents of disease that find their way into the soil

may survive there and thus become a constant and important source of infection. The introduction by Koch, in 1881, of the gelatin plate method placed in the hands of the investigator a convenient procedure for measuring the abundance of the soil population and determining the survival in the soil of agents causing serious human diseases. In spite of the fact that this method revealed only a very small part of the soil population, it enabled the medical bacteriologist to establish beyond doubt that such organisms tend to disappear in the soil. This resulted in definite conviction on the part of the public health and medical world that the soil is seldom a source of infection. It was soon demonstrated that disease-producing agents die out in the soil at a rather rapid rate, depending on the nature of the organisms, the soil, climate, and other conditions.

Organisms that Survive for Long Periods

Only a few disease-producing microorganisms are able to survive in the soil for any considerable periods of time. These few include the organisms causing tetanus, gas gangrene, anthrax, certain skin infections, actinomycosis in cattle, coccidiosis in poultry, hookworm infections, trichinosis, enteric disorders in man, blackleg in cattle, and Texas fever. To these may be added the botulinus organism and others producing toxic substances, as well as bacteria, actinomycetes, and fungi that cause plant diseases such as potato scab, root rots, take-all disease of cereals, and damping-off diseases.

Anthrax, a scourge of cattle and sheep, is a persistent survivor in soil; spores of this organism are known to retain their vitality and virulence for fifteen years. Anthrax survives particularly well in damp regions, especially in soils rich in decomposing organic matter; the hay and feed from these lands may transmit the disease to animals. The fact that certain fields carry anthrax infection was recognized in Europe long before the nature of the disease was known. Human infection results from contact with diseased animals or animal products.

The anaerobic, spore-forming bacteria that cause gas gangrene are widely distributed in nature. They are found extensively in soils and in decomposing plant and animal residues. The causation of disease by

these organisms received particular attention during the first world war, which was fought chiefly in trenches (957).

Another important pathogenic anaerobe able to survive in soil for long periods of time is *Clostridium chauvoei*, the causative agent of blackleg in cattle; southern pastures are said to be better carriers of blackleg than northern pastures.

Clostridium tetani is also widely distributed in the soil and appears to be associated with the use of stable manures. Nicolaier (638, 639) found, in 1884, that tetanus could be produced in experimental animals by the injection of soil samples, 69 positive results being obtained from 140 inoculations. This organism is believed to occur in the soil in the form of spores; its mode of survival, however, is not sufficiently known because of a lack of careful study.

The botulinus organism not only may remain alive in the soil for a long time (602), but it may also produce there a potent toxin that causes much loss of water fowl and other wild life. Aeration of the soil results in the destruction of this toxin by aerobic bacteria (710).

Thus we see that pathogenic spore-forming bacteria are always found in the soil. Other pathogens are able to survive in the soil only for limited periods of time. They are eliminated sooner or later from the soil, either because of their inability to compete with the soil population or because of their actual destruction by the latter. Although the pathogens seem to possess considerable resistance toward unfavorable soil conditions, they are unable to multiply at rates permitting their indefinite survival in the soil. The anthrax bacillus and certain other parasites infesting domesticated and wild animals belong to this group. Certain insect and animal carriers make possible the survival and spread of many pathogens in the soil.

Organisms that Survive for Brief Periods

The great majority of disease-producing bacteria, however, are able to survive only for very brief periods outside their respective hosts, especially in soil and water. It is sufficient to cite the fact that typhoid and dysentery bacteria, which are known to contaminate watersheds and water supplies, disappear sooner or later. It has been estimated, for

example (997), that in a sewage sludge free to undergo normal digestion, typhoid bacteria probably survive for less than 7 days. It was suggested, therefore, that sludge held in a digestion tank for about 10 days might be applied to the soil for fertilizer purposes without detriment to public health.

The gram-negative bacteria of the typhoid-dysentery group die out rapidly in septic material; the typhoid bacteria survive for about 5 days, the Flexner type of dysentery for about 3 days, and the Shiga bacillus dies out even in a shorter period. If decomposition in the tank has not advanced far enough, as shown by low alkalinity, the organisms may survive for a much longer period. The efficiency of ripe tank effluent to destroy bacteria is believed to be due to both the alkaline reaction and the presence of antagonistic metabolic products. The destruction of typhoid and dysentery bacteria in the soil depends on a number of factors, chief among which are the moisture content and reaction, and the nature and abundance of the microbiological population. In moist or dry soils, most of the pathogenic bacteria were found to die within 10 days (488).

Numerous other pathogenic agents, including those causing some of the most deadly human and animal scourges—tuberculosis, leprosy, diphtheria, pneumonia, bubonic plague, cholera, influenza, mastitis and abortion in cattle, the many poxes—constantly find their way into the soil in large numbers. They disappear sooner or later, and no one now ever raises the question concerning the role of the soil as the carrier of these disease-producing agents or as the cause of severe or even minor epidemics.

This rapid disappearance of disease-producing bacteria in the soil may be due to a number of factors: (a) unfavorable environment; (b) lack of sufficient or proper food supply; (c) destruction by predacious agents such as protozoa and other animals; (d) destruction by various saprophytic bacteria and fungi considered as antagonists; (e) formation by these antagonists of specific toxic or antibiotic substances destructive to the pathogens; (f) in the case of some organisms at least, increase of the bacteriophage content of the soil resulting in the lysis of some bacteria, especially certain spore-formers (49).

The course of survival of only a few disease-producing organisms

outside the host has been studied in detail. Sufficient information has been accumulated, however, to justify certain general conclusions. When *E. coli* is added to sterile soil, it multiplies at a rapid rate, but when added to fresh, nonsterile soil it tends to die out quickly (Table 5). The rate of its disappearance is independent of the reaction of the soil and of the temperature of incubation, but a marked increase in the number of soil organisms antagonistic to *E. coli* accompanies the disappearance (Table 6).

TABLE 5. SURVIVAL OF BACTERIA ADDED TO SOIL AND THEIR EFFECT UPON THE SOIL MICROBIOLOGICAL POPULATION

INOCULUM	INCUBATION		ORGANISMS RECOVERED*	
	Number of days	Temperature	Total	Coliform bacteria
Control soil	5	28° C.	21,400	<200
E. coli added†	5	28° C.	25,600	6,800
E. coli added‡	5	28° C.	39,700	3,500
E. coli added	5	37° C.	22,800	4,700
Control soil	33	28° C.	5,900	<10
E. coli added	33	28° C.	22,100	130
E. coli added‡	33	28° C.	17,600	140
E. coli added	33	37° C.	23,000	<10

From Waksman and Woodruff (951).
* In thousands per gram of soil.
† Washed suspension of *E. coli* cells added at start and after 5 days.
‡ $CaCO_3$ added to soil.

TABLE 6. INFLUENCE OF ENRICHMENT OF SOIL WITH ESCHERICHIA COLI ON NUMBER OF ANTAGONISTIC MICROORGANISMS

	ANTAGONISTS* PER GRAM OF SOIL		TOTAL MICROORGANISMS PER GRAM OF SOIL
	After 65 days	After 117 days	After 117 days
Control soil	500,000	1,150,000	9,100,000
Enriched soil	4,000,000	5,700,000	40,000,000
Enriched soil + $CaCO_3$†	6,000,000	4,700,000	36,300,000

From Waksman and Woodruff (949).
* An antagonistic colony is one surrounded by a halo on the *E. coli* plate.
† This container received fewer enrichments with *E. coli* than the one without $CaCO_3$.

In order to illustrate the fate of certain important disease-producing bacteria which find their way into the soil or into natural water basins, it is sufficient to draw attention to reports of experiments made on a few typical pathogens.

The Colon-Typhoid Group of Bacteria

Frankland (295, 296) was the first to establish that *Eberthella typhosa* may survive in sterilized polluted water or in pure deep-well water for 20 to 51 days although it died out in 9 to 13 days in unsterile surface water. In other studies (464) it was found that the typhoid organism is able to survive in sterilized tap water for 15 to 25 days, as against 4 to 7 days in fresh water; the bacteria died off even more rapidly in raw river or canal water, the survival time being reduced to 1 to 4 days. The degree of survival of the typhoid organism in water was found to be in inverse ratio to the degree of contamination of the water, the saprophytic bacteria in the water being apparently responsible for the destruction of the pathogen. These conclusions were later confirmed (777). Freshly isolated cultures of *E. typhosa* survived a shorter time than laboratory cultures, higher temperatures (37° C.) being more destructive than lower ones (438). Although some investigators (1007) claimed that *E. coli* may survive in soil for 4 years, others (789) found that it disappeared rapidly. Viable typhoid bacteria were recovered (774) from polluted soil after 100 days in unsterilized soil, and after 16 months in sterilized soil. Sedgwick and Winslow (811) reported that cells of *E. coli* rapidly die out in the soil, 99 per cent destruction occurring in dry soil in 2 weeks, with a longer survival in moist soil.

In general *E. typhosa* is able to survive only a short time in unsterilized soil, but much longer in sterile soil (831). S. Martin (586), for example, observed that typhoid bacteria survived and grew readily in sterile soil; however, when added to a well-moistened and cultivated soil they were rapidly destroyed. The same phenomenon occurred when the pathogens were added to a culture of a soil organism in a nutrient medium. Only in certain soils were conditions favorable for the prolonged survival of the pathogen. The conclusion was reached that the typhoid organism is destroyed by the products of decomposition taking place in the soil. It was further concluded that an antagonis-

tic relation appeared to exist in some soils but not in others and that this was due to the action of specific antagonistic bacteria present in the particular soils.

Frost (302) also reported that typhoid bacteria are rapidly destroyed when added to the soil. In 6 days, 98 per cent of the cells were killed, and in the course of a few more days all the cells tended to disappear entirely from the soil. Under conditions less favorable to the growth of antagonists, the typhoid organism survived not only for many days, but even for months. The conclusion was reached that when soil bacteria are given a chance to develop by-products, there results a marked destruction of typhoid organisms brought into contact with them.

The survival of typhoid and dysentery bacteria in soil has been the subject of many other investigations (256, 577, 685).

Among the factors responsible for the disappearance of *E. typhosa* in water, the presence of certain water bacteria was found to be of special importance (899). Rochaix and Vieux (760) demonstrated that when an achromogenic strain of *Pseudomonas aeruginosa* was present in drinking water, it was not accompanied by any other bacteria. Media inoculated with this organism and *E. coli* gave, after 13 days' incubation, only cultures of the former. That the two organisms could coexist, however, was shown by inoculation into sterilized water. Only the actual development of the antagonist led to the repression of the fecal organism. The oxygen supply of the water is important (975). *E. typhosa* added to activated sludge increased within the first 4 to 6 hours; this was followed by a reduction in 24 hours, and a 99 per cent destruction in several days (411). The survival period was shorter in sewage-polluted than in unpolluted waters, especially when the sewage was aerated. About 80 per cent reduction of typhoid bacteria was obtained in the Netherlands East Indies by the passage of sewage through Imhoff tanks. Digestion of sludge reduced the number further but did not eliminate the bacteria completely; after the sludge was dried no typhoid bacteria could be found (613).

A study of microorganisms antagonistic to *E. coli* resulted in the isolation of organisms from 5 of 44 samples of well water, 1 of 12 samples of spring water, and 6 of 16 samples of surface water. The antagonists included 3 strains of *Pseudomonas,* 1 each of *Sarcina, Micro-*

coccus, *Flavobacterium*, and yeast, 2 actinomycetes, and 3 unidentified nonspore-forming, gram-negative rods (445).

The survival of *E. typhosa* in manure and in soil is known to be repressed decidedly by various saprophytic bacteria. When a carrier was induced to urinate on a soil, *E. typhosa* could be recovered within 6 hours from the washings of the soil; however, after 30 hours the organism could no longer be demonstrated, although the soil was still moist with the urine (616). In the absence of sunlight, the organism was recovered after 24 hours but not later. When the urine was allowed to dry on towels, the bacterial cells survived for 10 days because saprophytic microorganisms failed to develop on the dry towels. Other evidence was submitted that *E. typhosa* is destroyed by bacteria grown in association with it (382). Moisture was found (785) to be the most important factor influencing the longevity of typhoid bacteria in the soil; 50 per cent of the bacteria died during the first 48 hours, the survival of the remainder extending over a period of months.

E. coli was rapidly crowded out by other organisms in manure piles (623). The addition of 9 million cells of *E. coli* and 13 million cells of *A. aerogenes* to a soil resulted, in 106 days, in reductions to 6,000 and 25,000 respectively; in 248 days, both organisms had completely disappeared (828). The occurrence of coliform bacteria in soil depends entirely on the degree of pollution; soil relatively free from pollution contains no coliform bacteria or only a small number. No evidence of multiplication of these bacteria in the soil could be detected (873).

Sea water, as well, appears to have a bactericidal effect upon organisms added to it (937, 1014). This is believed to be due to the presence of some substance other than salt. Dysentery and typhoid organisms were found to disappear from sea water in 12 and 16 hours, whereas paratyphoid organisms survived for 21 and 23 days (887). Protozoa were found to be at least partly responsible for the destruction of the typhoid organism added to water systems (250, 440, 709, 815).

Mycobacterium tuberculosis

The fate outside the hosts of the bacteria causing tuberculosis in man and in animals has also been studied extensively. Considerable diffi-

culty has often been encountered, however, in demonstrating the presence of this pathogen, which must be detected usually by guinea pig inoculation methods (894). The organism was found to be alive in a dark room after 157 to 170 days, but not after 172 to 188 days; in diffused light, the longevity was only 124 days; in the incubator, the organism retained its virulence for 33 days, but not for 100 days; on ice, virulence was still evident after 102 days but not after 153 days (618).

Pure cultures of the bovine organism mixed with cow manure and exposed in a 2-inch layer in a pasture remained virulent for 2 months in sunlight and longer in the shade. Tubercle bacteria were still alive in a garden soil on the 213th day and dead on the 230th day. They were alive in buried tuberculous guinea pigs on the 71st day, and dead on the 99th day. In running water, they survived for more than a year (85). *Mycobacterium tuberculosis* survived for 309 days in sputum kept in darkness, even when completely desiccated; in decomposing sputum, living organisms could be isolated after 20 but not after 25 days (792, 842). Under conditions prevailing in southern England, it was found (987) that the tubercle organism may remain alive and virulent in cow's feces exposed on pasture land for at least 5 months during winter, 2 months during spring, and 4 months during autumn; in summer, no living organisms were demonstrated even after 2 months. Under protection from direct sunlight, the survival period was longer. Feces protected from earthworms yielded viable cells even after 5 months. Virulent bacteria were still present in stored liquid manure at least 4 months after infection, though during this time a gradual reduction in virulence of the organism was observed.

The addition of manure to soil was found to favor the survival of the tubercle bacteria, as indicated by a higher proportion of test animals becoming tuberculous when the amount of manure added to the soil was increased (574). Positive tests were obtained for soil and manure after 178 days, but not later. The organism survived on grass for at least 49 days. Samples of milk of tuberculous cows, kept frozen and examined periodically, gave positive tests even after 2 years and 8 months (551). Rhines (746) found that *M. tuberculosis* multiplied in sterile soil as well as in the presence of certain pure cultures of bacteria; however, a fungus was found to check the development of the pathogen, especially

in manured soil. In nonsterile soil, the pathogen was slowly destroyed, the plate count being reduced to about one sixth of the original in 1 month. In a study of the survival of avian tubercle bacteria in sewage and in stream water, there was a reduction, in 73 days, from 48,000 to 1,400 per milliliter in sewage and to 4,200 in water (745).

Other Disease-producing Microorganisms

A study of the viability of *Brucella melitensis* in soil and in water in Malta brought out the fact that the organism survived in sterile tap water 42 days and in unsterile tap water only 7 days. It survived 25 days in soil and 69 days in dry sterile soil, but only 20 days in unsterile manured soil, 28 days in dry natural road dust, 20 days in dry sterile sand, and 80 days on dry cloth (326, 430, 431, 432).

The rapid destruction of cholera bacteria added to soil was first pointed out by Houston (437). Similar rapid destruction of the diphtheria organism was also noted. *Serratia,* however, retained its vitality for 158 days. *Vibrio comma* also survived for a short time only in feces (358), different strains showing considerable variability; temperature was an important factor. During the hot season in Calcutta, the viable period was somewhat longer than a day, as compared to 7 or 8 days during the cold season; the critical cholera months were found to follow directly the cool months. The organism did not survive very long in fresh water, although the time appeared to be long enough to cause occasional serious epidemics. It remained alive for 47 days in sea water (450). The conclusion was reached that although the organism is ordinarily destroyed rapidly in water as a result of competition with other microbes, it may survive in certain instances for some time.

THE SOIL POPULATION

The nature of the soil population can thus be considered as more or less dynamic, its modification being controlled by the addition of organic matter and by soil treatments. The introduction of foreign organisms tends not to change the nature of the population, but merely to stimulate the development of such members as are capable of destroying the foreign organisms. The production of antibiotic substances by mem-

bers of the soil population may also be directly responsible for the rapid destruction of the added organisms. It has been shown (635), for example, that subsurface soils contain microbiological populations that are smaller, less versatile, and less adaptable than surface soil populations. Some factor in the subsurface soils was believed to cause the prevention of the rapid development of the introduced organisms. Antibiotic or inhibitory substances were said to be responsible for this effect, these substances being of microbial origin. Aqueous extracts of soil did not adversely affect the growth of soil bacteria *in vitro*, but alcohol extracts reduced their activity in the soil and in artificial culture media.

CHAPTER 3

INTERRELATIONSHIPS AMONG MICROORGANISMS IN MIXED POPULATIONS

It must not be forgotten that there are extremes in another direction, where one of the two associated organisms is injuring the other, as exemplified by many parasites, but these cases I leave out of account here. This state of affairs has been termed antibiosis.—*H. M. Ward.*

SYMBIOSIS AND ANTIBIOSIS

Microbes grow and bring about many metabolic reactions in natural substrates, such as soils and water basins, in a manner quite different from those in pure cultures where they are not influenced by the growth of other organisms. In artificial and natural media, whether these be synthetic materials, complex organic mashes and infusions used for the preparation of industrially essential products, or the bodies of plants and animals, pure cultures of microbes are free from the associative and competitive effects of other microbes found in natural substrates. In mixed populations, a number of reactions that do not commonly take place in pure cultures are involved. Even in the case of mixed infections, a pathogen may be preceded or followed by one or more saprophytes, whereby the processes of destruction brought about in the living animal or plant body are alleviated or hastened. In the mixed populations found in natural substrates, the ecological relationships are largely responsible for many of the essential differences in the behavior and metabolism of the microbes, as compared with the same organisms growing in pure culture.

Almost all microorganisms inhabiting a natural milieu, such as soil or water, are subject to numerous antagonistic as well as associative, or even symbiotic, interrelations. Every organism is influenced, directly or indirectly, by one or more of the other constituent members of the complex population. These influences were at first visualized as due primarily to competition for nutrients (526). This was well expressed

by Pfeffer (684), who said that "the entire world and all the friendly and antagonistic relationships of different organisms are primarily regulated by the necessity of obtaining food." It was soon recognized, however, that this explanation does not account fully for all the complex interrelations among microorganisms in nature.

De Bary (165) was the first to emphasize, in 1879, the significance of the antagonistic interrelations among microorganisms; when two organisms are grown on the same substrate, sooner or later one overcomes the other and even kills it. This phenomenon was designated "antibiosis" (42, 953). Symbiotic, or mutualistic, and antagonistic relationships among microorganisms indicate whether advantages or disadvantages will result to the organisms from the particular association; the first are beneficial and the second are injurious and may even be parasitic. Kruse (508) asserted that, when two organisms are capable of utilizing the same nutrients but are differently affected by environmental conditions such as reaction, air supply, and temperature, the one that finds conditions more suitable for its development will grow more rapidly and in time be able to suppress the other. According to Porter (695), the effects produced by fungi in mixed culture are due either to exhaustion of nutrients or to the formation of detrimental or beneficial products. E. F. Smith (829) emphasized that when two or more organisms live in close proximity they may exert antagonistic, indifferent, or favorable effects upon one another. These potentialities were later enlarged (1011) to include stimulating, inhibiting, overgrowing, and noninfluencing effects. After considerable experimentation and speculation, Lasseur (513, 514) came to the conclusion that antagonism is a very complex phenomenon and is a result of numerous and often little-known activities. Antagonism influences the morphology of the organisms, their capacity of pigment production, and other physiological processes.

No sharp lines of demarcation can be drawn between associative and antagonistic effects. Well-defined effects of two symbionts may change during the various stages of their life cycles or as a result of changes in the environment. It is often difficult to separate strictly symbiotic phenomena from associations of less intimate nature, frequently desig-

nated as commensalisms. The various stages of transition from obligate parasitism to true saprophytism can be represented as follows:

Obligate parasitism (certain bacteria, smut fungi)	→	Facultative parasitism (species of *Fusarium, Rhizoctonia*, and *Actinomyces*)	→	Modified parasitism; hosts may derive some benefit (certain mycorrhiza)
Balanced parasitism (various mycorrhiza)	→	True symbiosis (root-nodule bacteria, lichen formations)	→	True saprophytism (autotrophic and heterotrophic bacteria and fungi).

The phenomena of antagonism do not fit exactly into the above scheme but are parallel with it: the injurious effects of one organism upon another range from antagonism of varying degrees of intensity to the actual living or preying of one organism upon another. The latter may be classified with the phenomena of parasitism and disease production.

Microorganisms inhabiting the soil live in a state of equilibrium (943). Any disturbance of this equilibrium results in a number of changes in the microbial population, both qualitative and quantitative. The ecological nature of this population found under certain specific conditions, as well as the resulting activities, can be understood only when the particular interrelationships among the microorganisms are recognized. Because of its complexity, the soil population cannot be treated as a whole, but some of the processes as well as some of the interrelations of specific groups of organisms can be examined as separate entities. Some have received particular attention, as the relations between the nonspore-forming bacteria and the spore-formers, the actinomycetes and the bacteria, the bacteria and the fungi, the protozoa and the bacteria, and the relations of the bacteria and the fungi to the insects.

The term "synergism" has been used to designate the living together of two organisms, resulting in a change that could not be brought about by either organism alone (425). Microbes living in association frequently develop characteristics which they do not possess when living in pure culture. Schiller (797, 798), for example, found that when beer yeasts are placed together with tubercle bacteria in a sugar-containing but nitrogen-free medium, the yeasts develop antagonistic properties

toward the bacteria and use the latter as a source of nitrogen; the yeasts secrete a bacteriolytic substance that is also active outside their cells. Various bacteria are able to kill yeasts when they are inoculated into suspensions of the latter in distilled water. The destruction of the fungus *Ophiobolus*, the causative agent of the take-all disease of cereals, by soil organisms was believed (312) to be a result of the need of a source of nitrogen by the latter.

The term "autoantibiosis" has been used (670) to designate the phenomenon of self-inhibition or "staling" of medium as a result of the previous growth of the organism in this medium.

THE NATURE OF A MIXED MICROBIAL POPULATION

A mixed microbial population is made up of a great variety of bacteria, and often also of fungi, actinomycetes, and protozoa; to these are added, under certain conditions, various algae, diatoms, nematodes and other worms, and insects. The specific nature and relative abundance of the various microorganisms making up a complex population in either a natural or an artificial environment depend upon a number of factors, which can be briefly summarized as follows:

The physical nature of the medium in which the population lives: soil, compost, or manure pile; river, lake, or ocean; sewage system; or peat bog.

The nature, concentration, and availability of the chemical constituents of the medium used by the microbes as nutrients, especially the materials used as sources of energy and for the building of cell substance. Various organic and inorganic substances, whether complex or simple in chemical composition, favor the development of specific groups of microorganisms capable of utilizing them. For example, sulfur favors the development of specific sulfur bacteria, and cellulose favors such organisms as are capable of attacking this complex carbohydrate as a source of energy. In many instances there is considerable competition for the available food material. Organisms that possess a greater capacity of attacking the particular compound, or are capable of preventing the development of other organisms by the formation of substances injurious to the latter, usually become pre-

dominant. Proteins, starches, and sugars can be acted upon by a great variety of microorganisms. The predominance of one group may depend not only upon the chance presence of the particular organism or its capacity for more rapid growth, but also upon its ability to form alcohols, acids, and other products that influence the growth of other organisms.

Environmental conditions favorable or unfavorable to the development of specific organisms. Of particular importance in this connection are temperature (thermophilic vs. mesophilic organisms), oxygen supply (aerobic vs. anaerobic organisms), moisture content (bacteria and fungi vs. actinomycetes), reaction (acid-sensitive vs. acid-tolerant forms), as well as the physical conditions of the substrate as a whole.

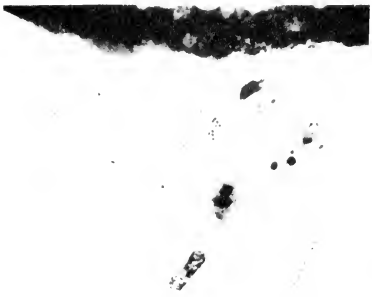
The presence and abundance of organisms that produce substances having a favorable and stimulating or an injurious and toxic effect upon other organisms, or that may compete for the available nutrients. The equilibrium in the microbiological population in a natural medium such as soil or water may be upset by the introduction of specific nutrients, as well as by treatment with chemical and physical agents whereby certain organisms are destroyed and others stimulated.

The presence of specific microorganisms in a natural medium may be considerably influenced by the presence of certain parasitic or phagocytic agents. The role of protozoa in controlling bacterial activities by consuming the cells of the bacteria has been a subject of much speculation. The presence of bacteria, fungi, and nematodes capable of destroying insects is of great importance in human economy. Many other relationships, such as the presence of phages against specific organisms, are often found greatly to influence the nature and composition of a specific population.

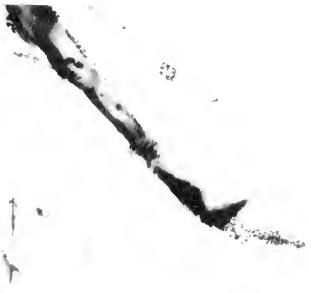
ASSOCIATIVE INTERRELATIONSHIPS

Numerous instances of associative interrelationships among microorganisms are found in nature. These may be grouped as follows:

Preparation or modification of the substrate by one organism whereby it is rendered more favorable or more readily available for the growth of another organism. As an illustration one may cite the breakdown



Large root with bacterial cells and filaments of actinomycetes

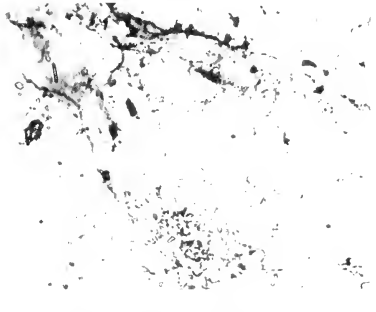


Root hair with rod-shaped bacteria in colonies and short chains

Terminal portion of root hair showing bacteria in form of mantle

Root hair undergoing attack by bacteria and actinomycetes

Masses of coccoid bacteria growing about a fungus filament



Dense colonies of bacteria surrounding root hairs

FIGURE 2. Relationships between microorganisms and root systems of higher plants. From Starkey (848).

of cellulose by specific bacteria, thereby making the particular energy source available to noncellulose-decomposing organisms, including not only certain bacteria and fungi but also higher forms of life such as ruminant animals (herbivores) and insects (termites, cockroaches), which carry an extensive cellulose-decomposing microbiological population in their digestive systems. Another illustration is the breakdown of complex proteins by proteolytic bacteria, resulting in the formation of amino acids and polypeptides, which form favorable substrates for peptolytic bacteria. The ammonia liberated from proteins and amino acids supplies a source of energy for nitrifying bacteria and a source of nitrogen for many fungi. The ability of bacteria to concentrate in solution those nutrients that are present only in mere traces enables animal forms (protozoa) to exist at the expense of the bacteria (102).

Influence upon the oxygen concentration available for respiration. This involves the phenomenon first observed by Pasteur (673) of consumption of oxygen by aerobic bacteria, thus making conditions favorable for the development of anaerobes (650–652, 843).

Symbiotic interrelationships, where both organisms benefit from the association. The three most important examples found in nature are: (a) the phenomenon of symbiosis between root-nodule bacteria and leguminous plants; (b) mycorrhiza formations between certain fungi and higher plants; (c) lichen formation between algae and fungi. Certain other interrelations are not strictly symbiotic, but are found to fall between groups a and c; here belong nitrate reduction accompanied by cellulose decomposition and nitrogen-fixation with cellulose decomposition, carried out in each case by two specific groups of organisms.

Production by one organism of growth-promoting substances that favor the development of other organisms. The formation of riboflavin by anaerobic bacteria in the digestive tract of herbivorous animals is an interesting and highly important phenomenon in the nutrition of such animals. The production of bacterial growth stimulants by yeasts and the beneficial action of mixed populations upon nitrogen-fixation by *Azotobacter* are other illustrations of this general phenomenon. The presence of specific bacteria has been found necessary for the sporulation of certain yeasts and for the formation of perithecia by various Aspergilli (612, 787). Various other processes of association have also been recognized (920).

Destruction by one microorganism of toxic substances produced by another, thereby enabling the continued development of various members of the microbiological population.

Modification of the physiology of one organism by another. In the presence of certain bacteria, *Clostridium granulobacter-pectinovorum* forms lactic acid instead of butyl alcohol (845). The presence of *Clostridium acetobutylicum* in cultures of bacteria producing dextro-lactic acid and laevo-lactic acid causes such bacteria to form the inactive lactic acid (870); intimate contact of the bacteria is essential, the use of membranes preventing this effect. The presence of *A. aerogenes* modifies the optimum temperature for nitrogen-fixation by *Azotobacter* (749). Pigment formation by *Ps. aeruginosa* may be weakened when the latter is grown together with other organisms. *E. coli* may lose the property of fermenting sugars when grown in the presence of paratyphoid organisms (453).

Some associations of microorganisms are not so simple. The complex system of animal infection by more than one organism, with the resulting complex reactions in the animal body, is a case in point.

The effect of one organism upon the activities of another can be illustrated by the results of the decomposition of complex plant material by pure and mixed cultures of microbes (Table 7). *Trichoderma*, a fungus

TABLE 7. DECOMPOSITION OF ALFALFA BY PURE AND MIXED CULTURES OF MICROORGANISMS

ORGANISM	TOTAL ALFALFA DECOMPOSED	HEMICELLULOSES DECOMPOSED	CELLULOSE DECOMPOSED	NH_3–N PRODUCED
	Per cent	Per cent	Per cent	mgm.
Trichoderma	9.3	4.7	0	61
Rhizopus	6.6	12.8	2.9	53
Trichoderma + *Rhizopus*	13.7	22.6	10.6	63
Trichoderma + *Cunninghamella*	15.0	15.4	5.7	47
Trichoderma + *Ps. fluorescens*	10.5	14.5	6.4	32
Streptomyces 3065	16.6	43.0	23.2	52
Trichoderma + *Streptomyces* 3065	12.5	14.6	4.8	56
Soil infusion	28.4	40.9	50.8	21

From Waksman and Hutchings (938).

known to be an active cellulose-decomposing organism, did not attack at all the cellulose of alfalfa and decomposed the hemicelluloses only to a limited extent; however, the organism utilized the proteins rapidly, as illustrated by the amount of ammonia liberated. *Rhizopus*, a noncellulose-decomposing fungus, attacked largely the hemicelluloses in the alfalfa and some of the protein; a small reduction in cellulose was recorded, probably because of an analytical error. When *Trichoderma* was combined with *Rhizopus*, the former attacked readily both the cellulose and the hemicelluloses. The same effect upon the activity of *Trichoderma* was exerted by other noncellulose-decomposing organisms, such as the fungus *Cunninghamella* and the bacterium *Ps. fluorescens*. On the other hand, when *Trichoderma* was combined with a cellulose-decomposing *Streptomyces*, there was considerable reduction in the decomposition of the total plant material as well as of the cellulose and hemicelluloses. These results further emphasize the fact that two organisms may either supplement and stimulate each other or exert antagonistic effects. The total soil population is far more active than any of the simple combinations of microorganisms.

COMPETITIVE INTERRELATIONSHIPS

The following competitive relations among the microscopic forms of life inhabiting the sea have been recognized (18):

Competition among chlorophyol-bearing diatoms for the available nutrient elements in the water
Competition among the copepods for the available particulate food materials, notably the diatoms
Competition between individuals belonging to one species and individuals belonging to another
Competition between young, growing, and reproducing cells and older, respiring cells
Food competition and space competition
Competition between transitory and permanent populations
Competition between sedentary or sessile organisms and free-moving forms

This list has been enlarged (924) to include other factors that are particularly prominent in nonaquatic environments:

Degree of tolerance of the immune or resistant varieties and of the less resistant or more sensitive forms to attack by disease-producing organisms

Fitness for survival of microbes that are able to adapt to a symbiotic form of life, such as leguminous plants or mycorrhiza-producing plants, and those that are not so adapted

Survival of parasitic forms that require living hosts for their development, as contrasted with saprophytes that obtain their nutrients from mineral elements or from dead plant, animal, and microbial residues

Various special types of competition, for example, competition between strains of root-nodule bacteria (*Rhizobium*), whereby one strain checks completely the multiplication of other strains, even outside the plant, the dominant strain then becoming responsible for all the nodules produced, as shown by Nicol and Thornton (637).

These phenomena of competition are found not only in natural substrates, such as soil and water, but also in artificial media. When several microbes are growing in the same culture medium, some will be repressed in course of time whereas others will survive and take their place. This is due to the fact that these microbes compete for the use of the same nutrients or that conditions, such as reaction, oxygen supply, and temperature, are more favorable to some organisms than to others. Another phenomenon may also be involved, that some organisms may produce toxic substances that repress the growth of others. In artificial media, slowly growing tubercle bacteria, diphtheria organisms, and others will be repressed by the rapidly growing saprophytes. Under aerobic conditions, aerobic bacteria and fungi will repress yeasts and anaerobic bacteria, whereas under anaerobic conditions the reverse will take place. An alkaline reaction will favor the development of bacteria, an acid reaction will favor the growth of fungi.

ANTAGONISTIC INTERRELATIONSHIPS

When two or more organisms live together, one may become antagonistic to the others. The composition of the medium and the conditions of growth influence the nature and the action of the antagonist; its metabolism and cell structure may become modified or the cell itself may be destroyed (174). In urine, for example, staphylococci may be-

come antagonistic to *E. coli* or vice versa, depending on the initial numbers of the two groups, on the formation of metabolic products, or on the exhaustion of nutrients (247). The toxic substances produced by the antagonists comprise a variety of compounds, ranging from simple organic acids and alcohols to highly complex bodies of protein or polypeptide nature.

Various types of antagonism are recognized. Nakhimovskaia (627) concluded that all phenomena of antagonism among microorganisms can be conveniently classified into four groups of reactions:

1. Antagonism *in vivo* vs. antagonism *in vitro*. According to some investigators (513, 514), only the inhibitive forms of antagonism (*in vitro*) may be designated as true antagonisms; the *in vivo* forms were designated as phenomena of antibiosis. Usually, however, this differentiation is not recognized.
2. Repressive, bactericidal, and lytic forms of antagonism. One may further distinguish between bacteriostatic and bactericidal, fungistatic and fungicidal forms of antagonism, as well as between antagonism of function and antagonism of growth.
3. Direct, indirect, and true antagonism.
4. One-sided and two-sided antagonism; antagonism between strains of the same species and antagonism among strains of different species (228).

Duclaux (208) was the first to demonstrate that the growth of a fungus upon a certain medium renders the medium unfavorable for the further growth of the same organism. Küster (509) has shown that culture solutions in which fungi have grown are not suitable for the germination of freshly inoculated spores but are improved by boiling. This effect was observed as a result of the growth not only of the same organism but also of other species. Similar observations were made for bacteria: Marmorek (583) reported, in 1902, that the growth of hemolytic streptococci in broth rendered the medium unsuitable for subsequent growth of the same organism. The production of spores by bacteria was believed to be caused by the formation of toxic, thermolabile organic substances; upon the destruction of these by boiling, the medium was made again favorable for the growth of bacteria and bacterial spores were able to germinate again. Some of the toxic substances

appeared to be thermostable; Nadson and Adamovic (625) showed that certain metabolic products of microorganisms, even when heated to 120° C., may have a strong influence upon the subsequent growth of the organisms.

Fungi are capable of producing not only growth-inhibiting but also growth-promoting substances (509, 547). By means of certain procedures, it was found possible to separate the two (654). The tendency of fungus hyphae to turn away from the region in which other hyphae of the same fungus were growing was explained as a negative reaction to chemical substances produced by the growing fungus (304). This negative chemotropism was shown to be due to thermolabile staling substances (352). The phenomenon of staling was often spoken of as vaccination of medium (45), and was ascribed to the action of protein degradation products.

These and other experiments led to the conclusion that many microorganisms are capable of producing substances that are injurious to their own development (iso-antagonistic) or, and sometimes much more so, to other organisms growing close to them (hetero-antagonistic). The growth of certain fungi and bacteria in practically pure culture, even in a nonsterile environment, was believed to be due to this phenomenon. It is sufficient to mention the lactic and butyric acid bacteria, the citric acid-producing species of *Aspergillus,* the lactic and fumaric acid-producing species of *Rhizopus,* and the alcohol-producing yeasts. The chemical substances produced by these organisms in natural substrates may be looked upon as protective metabolic products of microorganisms in their struggle for existence. Such products play a highly significant part in the metabolism of various organisms, especially those that grow parasitically upon living plant and animal bodies.

Among the various types of antagonism, the one resulting in the production of active substances that can be isolated and purified has received the greatest consideration recently. These substances have been designated as toxins, poisons, antagonistic agents, bacteriostatics, and antibiotics. The chemical nature of some has been elucidated, but that of many others is still unknown. Some of these substances are destroyed by boiling, by exposure to light, or by filtration, whereas others are resistant to heat and to ultraviolet rays; some are readily adsorbed by

certain filters, from which they can be removed by the use of special solvents such as ether, alcohol, chloroform, and acetone. The concentration of the antagonistic substance produced by many fungi and bacteria was found (240, 641) to be greatly influenced by the energy and nitrogen sources in the medium and by environmental conditions, such as temperature and aeration.

The three important types of antagonism are (a) the repressive, inhibitive, or bacteriostatic, (b) the bactericidal, and (c) the bacteriolytic. When one bacterium is inoculated into the filtrate of another, the growth of the first is slower than that of the control (299). Certain types of antagonism express themselves in the destruction by the antagonist of the other organisms present in the mixed culture, with or without producing a lytic effect. *B. mesentericus*, for example, is capable not only of depressing but also of killing the cells of diphtheria and pseudodiphtheria (1016). The lytic form of antagonism is illustrated by the action of *Ps. aeruginosa*, *Bacillus brevis*, and certain other antagonists upon micrococci and various spore-forming bacteria.

In differentiating between "direct antagonism" and "passive antagonism," attention was directed (627) to the fact that the latter depends not upon the direct action of the antagonist but upon the changed conditions of culture under the influence of the antagonist's growth. This may comprise a change in *p*H and *r*H of medium or an impoverishment of some of the nutrient constituents. "Direct antagonism" was often distinguished (634) from "indirect antagonism," the first being limited to those phenomena in which the antagonistic action is connected with the direct action of the living cell, whereas in the second the metabolic products produced by one organism are injurious to others. Intestinal bacteria were found (365, 367) to repress the anthrax organism only when the former were in an active living state. Other investigators (407) designated the action of the living cell itself as "true antagonism."

Bail (31) suggested that for every bacterium there is a typical constant number of cells capable of living in a given space. When this concentration (M) is reached, multiplication comes to a standstill, independent of exhaustion of the nutrients or formation of toxic substances. The same phenomenon was believed to hold true when two

bacteria live together (983, 984): if the limiting cell-in-space concentrations are different for the two organisms, the one with a higher M value represses the other; however, the weaker species may check the stronger when planted in sufficient excess (244). It has been suggested (368) that certain physiological properties of the individual organisms, designated as "biological activity" and "competitive capacity," must also be taken into consideration in evaluating this relationship (634, 983, 984). Brown (90) explained the fact that the number of yeast cells reaches a maximum independent of the initial number of cells added or the concentration of nutrients in a given volume of medium by the amount of oxygen originally present.

Garré (311) deserves the credit for having first noted that antagonism may be either one-sided or two-sided. In the first case, one organism represses another that is not antagonistic to it; in the second case, both organisms repress each other. A one-sided antagonism may become two-sided under certain conditions of culture. *E. coli* is antagonistic to *E. typhosa;* however, if the latter is inoculated into a medium somewhat earlier than the former, *E. typhosa* becomes antagonistic to *E. coli* (324, 915).

Although the most common antagonisms are between organisms of different species, there are numerous instances where one strain of a species may be antagonistic toward another strain of the same species (52, 368, 611). Certain strains may develop antagonistic properties in the presence of other strains (77). Nonflagellated variants of typhoid bacteria were repressed by a flagellated form, smooth variants of paratyphoid bacteria by rough forms, and so on. The fact that all bacterial cultures stop growing after a certain period of time has been interpreted to be a result of the antagonistic action of some cells upon others. When the filtrates of such cultures are added to fresh nutrient media they may stop the growth of the same species as well as that of other species.

Certain organisms produce pigments in the presence of others; these pigments are believed to be in some way associated with the phenomenon of antagonism. In the presence of *S. lutea*, *V. comma* forms a dark violet pigment that is accompanied by an increase in agglutination and in virulence (627). The destruction of *Dictyostelium mucoroides* by a red-pigment-forming bacterium was accompanied by an in-

crease in intensity of the pigment (690); the blue pigment of *Bacterium violaceum*, however, only delayed the growth of the fungus. According to Doebelt (177), *Penicillium africanum* produces a more intense pigment in contact with other fungi such as *Aspergillus niger;* this pigment accumulates in the mycelium of the latter, which may thereby be killed. Nadson (626) demonstrated that some fungi (*Penicillium luteum* and *Spicaria purpurogenes*) produce a pigment that is used not only for purposes of protection, but also for attack upon other organisms, whereby the latter are killed and stained.

DISTRIBUTION OF ANTAGONISTIC PROPERTIES

Numerous microbes found among the bacteria, fungi, actinomycetes, and protozoa possess the capacity of bringing about injurious or destructive effects upon other microorganisms belonging to their own groups or to others. In some instances, the antagonistic effects are obtained only in the presence of the antagonizing organism; in many other cases, excretion products consisting of definite chemical compounds are produced by the antagonist. A few of these products have been isolated and have been found to be effective against certain few specific organisms or able to act upon a great variety of organisms (920). The wide distribution of antagonistic properties among microorganisms is brought out in subsequent tables.

THEORIES OF THE NATURE OF ANTAGONISTIC ACTION

The various theories proposed to explain the mechanism of antagonistic effects of microorganisms may be summarized under the following processes:

Exhaustion of nutrients
Physicochemical changes in medium
Enzyme action, either directly by the antagonist or as a result of cell autolysis, under the influence of the antagonist
Production and liberation of toxic substances
Pigment action
Action at a distance
Space antagonism

Pasteur (672, 674, 675) ascribed the antagonistic effect of aerobic bacteria upon the anthrax organism to the consumption of the oxygen by the former; the unfavorable influence of normal blood upon the growth of anthrax was believed to be due to competition for the oxygen by the red blood corpuscles. Freudenreich (299) considered the antagonism between *Ps. aeruginosa* and *Bacillus anthracis* as due to exhaustion of nutrients by the former. These studies were soon followed by numerous other investigations in which the exhaustion of nutrients in the media was believed to be responsible for the phenomenon of antagonism; the onset of the stationary phase in bacterial growth was believed (539) to belong here. The change in *p*H of medium and the accumulation of toxic products were also found to become limiting factors. Palevici (667) added fruit juice to a stale medium and brought about improvement in bacterial growth, thus suggesting the exhaustion of growth-promoting substances as the cause of staling. Broom (89) emphasized, however, that the effect was due to addition of nutrients, including glucose.

It thus became apparent, even in the early days of bacteriology, that certain changes are produced by microbes in the medium in which they grow which render it unfit for the growth of other organisms. It also was soon recognized that the problem is more complicated than the mere exhaustion of nutrients. The relationships produced by changes in surface tension, in oxidation-reduction potential, in reaction, and in osmotic pressure were suggested as explanations (24, 627, 827). Among the classical examples of the effect of reaction upon the growth of other organisms is the acidification of milk by lactic acid bacteria. Metchnikov emphasized the fact that *Lactobacillus bulgaricus* acts antagonistically not only by means of the lactic acid that it produces but also by the formation of special substances. The production by bacteria of alkali-reaction products that have an injurious effect upon the further growth of the organisms has also been demonstrated (334). These substances were found to correspond to amino compounds, liberated in the process of cellular disintegration. Numerous other physical and physicochemical factors influence the growth of an organism in an artificial medium. It is to be recalled that the rate of survival of bacterial cells in water or in

salt solution is markedly influenced by the colloids present (991), the concentration of electrolytes (816), the reaction (897), and the temperature (36).

Microbial antagonism was thus looked upon (496) largely as a result of a series of physical factors, including various radiations such as mytogenetic rays (9, 679, 814), *p*H changes, conductivity, electric charge, and surface tension.

Most antagonisms, however, can be explained by the production of toxic substances by the antagonists. Because of their thermolability, sensitivity to chemical reagents, or adsorption on bacterial filters, considerable difficulty has been experienced in isolating the active substances. Many of these substances have been found to be iso-antagonistic (autotoxins [141]), whereas others are able to act upon different bacteria. Most of them have been found to be thermostable.

The first antibiotic substance recognized as such was pyocyanase (235), soluble in alcohol, ether, and chloroform. Somewhat similar substances appear to be produced by *Serratia marcescens* (230), *Ps. fluorescens* (525), *B. mesentericus* (1016), and *Bacillus mycoides*. Whereas Emmerich and Löw (236) considered pyocyanase to be a proteolytic enzyme, others (370, 410, 668, 679) found it to be a lipoid. Since that early work and especially during the last five years, many new agents have been isolated or demonstrated. These will be discussed in detail later. It is sufficient to mention gramicidin and tyrocidine, produced by *B. brevis*, which are polypeptides; citrinin and fumigatin, which are quinone-like in nature; actinomycin, aspergillic acid, and iodinin, which are nitrogenous ring compounds; gliotoxin, which is a sulfur-bearing compound; streptothricin, streptomycin, and proactinomycin, which are nitrogenous bases. Some of the most important compounds (penicillin) have not as yet been sufficiently elucidated. Certain microbial pigments (pyocyanin, hemipyocyanin, prodigiosin) have also received consideration as bacteriostatic and fungistatic agents.

The production of antibiotic substances by various microorganisms is greatly influenced by reaction, temperature, and aeration of substrate, as well as by the presence of other organisms. Evidence is still lacking as to whether these substances may accumulate in the soil and in water

(361), whether the organisms thereby affected are able to overcome their effect, and whether they are destroyed by other members of the soil or water microbiological population (947, 951).

Different organisms possess different degrees as well as different mechanisms of antagonism. Often one organism may completely check the growth of another; later, growth may be resumed, although it will not be quite normal. Antagonism stimulates spore-production and brings about deformed growth of the mycelium in fungi or the formation of gigantic cells in bacteria. Distortions were found to be produced in *Alternaria* (231) and in *Helminthosporium* (695) by a bacterial antagonist. The morphological effects produced by the antagonists comprise changes in form, size, and structure of hyphae, direction of growth, and complete cessation of growth and abbreviation of hyphal segments.

In surveying the phenomena of antagonism among microorganisms, Porter (695) reached the conclusion that, among bacteria, the spore-formers are strong inhibitors. Actinomycetes also exhibit strong inhibitory action against most filamentous fungi. Phycomycetes usually neither cause inhibition nor are inhibited; the Basidiomycetes contain very few organisms possessing antagonistic properties. Ascomycetes and Fungi Imperfecti vary greatly in their ability to produce antibacterial substances; some yeasts are strong inhibitors. Certain algae, notably species of *Chlorella*, produce a substance (chlorellin) that inhibits the growth of various gram-positive and gram-negative bacteria (701).

CHAPTER 4

ISOLATION AND CULTIVATION OF ANTAGONISTIC MICROORGANISMS; METHODS OF MEASURING ANTIBIOTIC ACTION

In nearly all the earlier work and even in many recent investigations on the antagonistic properties of microorganisms and the production of antibiotic substances, two procedures were employed: indiscriminate testing of pure cultures of bacteria and fungi, commonly taken from culture collections, for antagonistic effects against one another or against certain specific or test organisms; and isolation of occasional antagonistic organisms from old plate cultures, as air contaminants, or from mixed infections. These studies were carried out either by medical bacteriologists interested in agents capable of suppressing bacterial pathogens or by plant pathologists interested in organisms capable of inhibiting the growth of fungi, principally those concerned in the causation of plant disease. They resulted in the accumulation of considerable information concerning antagonistic organisms, the nature of the phenomenon of antagonism, and, to a more limited extent, the mechanisms involved. Neither of these methods, however, is suitable for a systematic study of antagonism as a natural process.

The last decade has witnessed a number of systematic attempts to determine the distribution of antagonists in nature, to isolate specific organisms capable of bringing about the desired reactions, and to establish the mechanism involved in these reactions. These studies were undertaken by a group of Russian investigators interested largely in fungi and actinomycetes as agents antagonistic to other microorganisms chiefly causing plant diseases, and by American and British investigators interested in agents active against bacterial pathogens of man.

The early significant, but unrecognized, investigations of Schiller (797, 798) on forced antagonisms and the studies of Gratia and his associates (349, 350) on mycolysates were in direct line of the more recent studies of Dubos (190), who made a systematic attempt to isolate from specially enriched soils bacteria capable of destroying specific

pathogenic organisms. Although it had been previously established that many spore-forming bacteria are capable of producing substances that have antibacterial properties, as shown by the work of Pringsheim (705), Much (621), and others, Dubos was the first to succeed in isolating in crystalline form the active substances involved and in demonstrating their chemical nature. He utilized for the isolation of the organisms the enrichment culture method. This consisted in adding repeatedly various pathogenic bacteria to a soil which, as a result, became enriched with antagonistic organisms capable of destroying the bacteria added; these organisms were then isolated by appropriate procedures. The isolation of the specific substances will be described later (page 156).

These investigations, as well as the work of Fleming (265) and other British investigators (3, 7, 8, 113) on the antibacterial properties of molds belonging to the *Penicillium notatum* group, served as the direct stimulus to numerous studies that followed. The entire series of studies led to the development of simple methods for the systematic isolation of microorganisms capable of inhibiting the growth of fungi and bacteria, both pathogenic and saprophytic (857, 934), and for separating many of the antibiotic substances produced by these organisms.

METHODS OF ISOLATING ANTAGONISTIC MICROORGANISMS

Four, and possibly five, methods are now available for the isolation of antagonistic microorganisms from natural substrates such as soil, stable manure, composts, sewage, water, and food products. These methods are different in nature, but they are all based on the same principle, that of bringing a living culture of a bacterium or fungus into close contact with a mixed natural population, thereby allowing certain members of this population to develop at the expense of the added culture.

Soil Enrichment Method

By this method a soil is enriched with known living pathogenic bacteria. Fresh garden or field soil is placed in glass beakers or pots, and

the moisture of the soil is adjusted to optimum for the growth of aerobic bacteria, which is about 65 per cent of the water-holding capacity of the soil (20 to 50 per cent of the moist soil); the containers are covered with glass plates and placed in an incubator at 28° or 37° C. Washed suspensions of living bacteria are added to the soil at frequent intervals, care being taken to avoid puddling it with an excess of the fluid, so conditions will not be made anaerobic. Samples of the enriched soil are removed at intervals and tested for the presence of organisms antagonistic to the bacteria added. Fresh washed suspensions of the living bacteria are inoculated with the enriched soil as soon as the presence of antagonistic organisms is demonstrated; this results in the development of the antagonistic organisms and the destruction of the bacteria in suspension. Transfers are then made to fresh suspensions of the bacteria, resulting in an enrichment of the antagonist, which can finally be isolated in pure culture (427).

Bacterial Agar Plate Method

This method was first used by Gratia and Dath (350) for the isolation of antagonistic agents, actinomycetes having been found readily by it.

To isolate antagonistic bacteria, agar (1.5 per cent) is washed in distilled water, then dissolved in water supplemented by 1 per cent glucose and 0.2 per cent K_2HPO_4. Ten-milliliter portions of the sugar-phosphate agar are placed in glass tubes and sterilized. The sterile agar is melted, and the tubes are placed in a water bath kept at 42° C. A washed, centrifuged suspension of living bacteria, grown on solid or in liquid media, is then added and thoroughly mixed with the agar. This "bacterial agar" is poured into a series of Petri plates containing one-milliliter portions of fresh or enriched soil, diluted 1:100 to 1:10,000 times with sterile water. The contents of the plates are thoroughly mixed in order to distribute the diluted soil suspension in the bacterial agar. The plates are inverted and incubated at 28° or 37° C.

After 1 to 10 days' incubation, depending on the nature of the organism used for the preparation of the plates, the presence of antagonists is manifested by the formation of clear zones surrounding their colonies (Figure 3). The organisms are isolated from these colonies

and are retested for antagonistic properties, either by transfer to fresh bacterial agar plates or by inoculating solidified agar plates and cross-streaking with test organisms (934, 949).

In the isolation of antagonistic fungi the same method is followed, except that it is preferable to make the bacterial agar acid by using KH_2PO_4 in place of K_2HPO_4. The resulting acidity (*p*H 4.5) inhibits the growth of bacteria and actinomycetes. Since the soil contains fewer fungi than bacteria, lower dilutions of soil are employed for this purpose (1:10 to 1:1,000).

By the use of the soil enrichment and bacterial agar plate methods, it is possible to demonstrate that ordinary soils contain a large population of microorganisms that are antagonistic to bacteria, including both gram-negative and gram-positive forms. The number of antagonists can be greatly increased when the soil is enriched with living cells of bacteria.

Crowded Plate Method

Ordinary field or garden soil is plated out on common nutrient (beef-peptone) agar, very low dilutions (1:10 to 1:1,000) being used to enable a large number of bacterial colonies to grow on the plate. The resultant crowding of these colonies allows the development on the plate of potential antagonists that are normally present in the soil. The production of antibacterial substances by these antagonists inhibits the growth of bacteria in close proximity to them and, in consequence, clear zones are formed around the colonies (Figure 4). It is possible, by means of this method, to demonstrate that many strains of spore-forming bacteria possessing antagonistic properties are present in the soil and can readily be isolated from it (857).

Direct Soil Inoculation Method

Nutrient agar plates are inoculated with the bacteria or fungi for which antagonists are to be found, and the plates are incubated for 24 to 48 hours at 28° or 37° C. Particles of fresh or enriched soil placed on the surface of the bacterial or fungus growth on the plate will give rise to antagonistic organisms. These organisms will bring about the killing or even the lysis of the original culture. By this method, or-

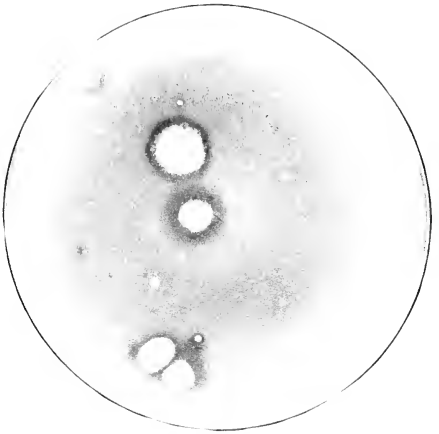


FIGURE 3. Development of antagonistic fungi on bacterial-agar plate. From Waksman and Horning (934).

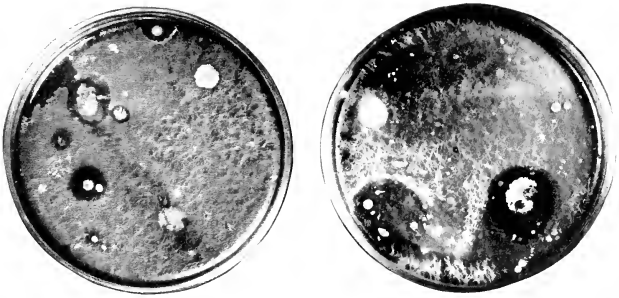
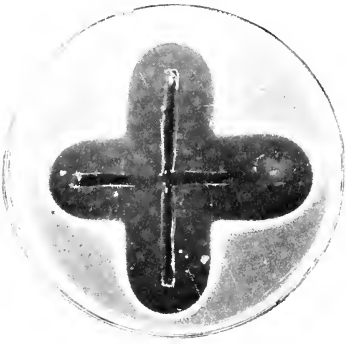


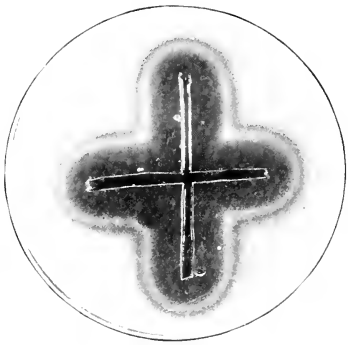
FIGURE 4. Bacterial plates made from soil, showing clear zones surrounding colonies of antagonistic organisms. From Stokes and Woodward (857).

Antagonistic action of *S. antibioticus* upon *S. lutea*

Antagonistic action of *S. antibioticus* upon *B. mycoides*



Bacteriostatic action of actinomycin upon *S. lutea*



Bacteriostatic action of actinomycin upon *B. mycoides*

FIGURE 5. Antagonistic effects of living organisms and their products. From Waksman and Woodruff (945).

ganisms antagonistic to many bacteria and fungi causing plant and animal diseases have been isolated (644, 646).

For the isolation of bacteria antagonistic to fungi, the latter are grown on potato agars until they have spread over the plate; particles of moist soil are then placed on the surface of the mycelium, and the plates are incubated in a moist chamber. Bacteria lysogenic to the fungi grow out of the soil and gradually dissolve the mycelium until the entire surface of the plate becomes free of the hyphae of the fungus. By transferring some of the material from the lysed spots, pure cultures of bacteria have been obtained that are capable of producing destructive effects upon the fungi, similar to the action of the particles of soil.

To these four methods may be added the "forced antagonism" method of Schiller (798), previously referred to, which consists in feeding a culture of an organism with another one, thereby forcing the second to develop the capacity of destroying the first.

Isolation of Antagonistic Microorganisms from Soil

By means of the foregoing methods, as well as various modifications of them, it was possible to demonstrate that soils, composts, and water basins contain an extensive population of microorganisms possessing antibacterial and antifungal properties. When *E. coli* was used as the test organism, it was found that although this organism was capable not only of surviving but actually of multiplying in sterile soil, it died off very rapidly when added to fresh soil. The rate of its destruction was greatly increased with every subsequent addition of washed bacterial cells to the soil. This was accompanied by the development of certain antagonistic microbes capable of destroying *E. coli* in pure culture.

A large number of fungi, actinomycetes, and bacteria possessing antagonistic properties have thus been isolated. The nature of the test organism was found to be of great importance in this connection. When *Staphylococcus aureus, S. lutea,* and *B. subtilis* were used, a large number of antagonists could readily be isolated. With *E. coli,* however, a much smaller number of microbes thus isolated possessed antagonistic properties. Certain other gram-negative bacteria, like *Brucella abortus,* were more sensitive than *E. coli,* whereas certain gram-positive bac-

teria, like *B. mycoides* and *B. cereus*, were less sensitive than *B. subtilis* (934, 936).

Bacteria destructive to fungi, or possessing fungistatic and fungicidal properties, have also been isolated from soils as well as from the surface of plants, such as flax, by transferring small sections of soil or plant stem to plates of fungi growing on potato agar; transfers made from the lytic spots yielded antagonistic bacteria (647). By the use of this method, Chudiakov (130) isolated various bacteria antagonistic to *Fusarium*. The antagonists were found abundantly in cultivated soils, but not in flax-sick soils rich in *Fusarium*. Bamberg (37) demonstrated, in the soil, bacteria capable of bringing about in 10 days complete destruction of *Ustiliago zeae* and other fungi. *Myxobacterium* was also found (457) capable of bringing about the disintegration of fungus mycelium. Nonspore-forming bacteria, similar to the cultures of Chudiakov, were isolated and shown to be able to attack a number of fungi, including species of *Fusarium*, *Sclerotinia*, *Gleosporium*, *Acrostalagmus*, *Alternaria*, and *Zygorhynchus* (695).

METHODS OF TESTING THE ANTAGONISTIC ACTION OF MICROORGANISMS

Once antagonistic organisms have been isolated, it is essential to establish their bacteriostatic spectrum—that is, their ability to inhibit the growth of various specific microorganisms. Usually these antagonists do not affect alike all bacteria and fungi, some acting primarily against gram-positive bacteria and against only a few gram-negative forms (mostly cocci), others acting upon certain bacteria within each of these two groups.

A considerable number of methods have been developed for measuring these antagonistic effects. They measure the selective nature of the antagonistic action and they can also give quantitative information concerning the intensity of this activity. Because of the great differences in the degree of sensitivity of bacteria to the action of the antagonists, the proper selection of one or more test organisms is highly essential. *S. aureus* has been employed most commonly, different strains of this organism having been found to vary greatly in their sensitivity even to

the same substance. *Streptococcus viridans, B. subtilis, Micrococcus lysodeikticus, S. lutea, E. coli,* and *E. typhosa* are other organisms that are frequently employed for testing the activity of antagonists. Although for purposes of concentration and purification of a known substance a single test organism is sufficient, it has been found advisable during the isolation of antagonistic organisms and the study of the nature of the antibiotic substance or substances that they produce to use more than one test bacterium, including one or more gram-positive and one or more gram-negative bacteria.

Most of the methods for testing antagonistic action are based upon the growth of the test organisms in the presence of the living antagonists or of the antibiotic substances produced by them in liquid and on solid nutrient media (302, 627). Only a few of these methods are now utilized, most of them being chiefly of historical interest.

Liquid Media

Several methods using liquid media have been proposed for testing the antagonistic activities of microorganisms:

Simultaneous inoculation of the medium with the antagonist and the test organism.

Inoculation of the medium with the antagonist first, followed after 6 to 48 hours by inoculation with the test organism.

Inoculation of the medium with the test organism first, followed, after a certain interval, by the antagonist.

Effect of the metabolic products of the antagonist upon various microorganisms. In 1888, Freudenreich (299) first filtered the culture through a Chamberland candle and inoculated the filtrate with the test organisms. The culture filtrate is usually added to the fresh medium, either previously inoculated with the test organism for the purpose of establishing the lytic effect of the filtrate, or followed by the test organism, whereby the bacteriostatic action is measured.

Placing a porcelain filter or cellophane membrane between the cultures of the antagonist and of the test organism. Frankland and Ward (297) used a filter of the Pasteur-Chamberland type partly filled with broth and placed in a beaker containing the same kind of broth; the antagonist and test organism were inoculated into the two lots of broth, and the effect of each upon the growth of the other was de-

termined. Frost (302) emphasized, however, that, although theoretically this is an ideal method, it is open to criticism since motile bacteria are usually able to grow through the filter after a certain lapse of time.

Collodion sac method. Collodion sacs, prepared by means of test tubes from which the bottoms have been cut out, are partly filled with broth and placed in a flask containing the same kind of broth. The test organism is inoculated into the medium inside the sac, and the antagonist into the flask (302).

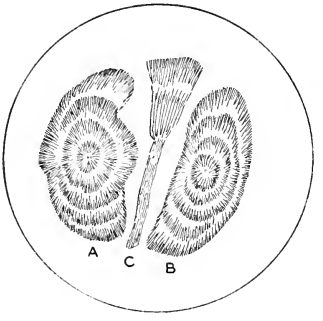
Solid Media

Solid media have also been used extensively for testing the action of antagonists. These media offer certain advantages over liquid media. The following methods are most commonly used:

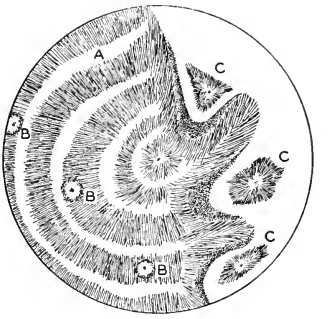
Simultaneous inoculation of antagonist and test organism. This method, introduced by Garré (311) in 1887, consists in streaking the antagonist and the test organism on the surface of a solidified agar or gelatin medium. The streaks are alternate and may be parallel, radiating from a common center, or intersecting at right angles (Figure 5). If the active substance produced by the antagonist does not diffuse for any considerable distance into the medium, the method is not satisfactory. Frost (302) modified this method by inoculating the whole medium with the test organism and, when the medium had hardened, streaking the antagonist across the surface. The first of these came to be known as the anaxogramic method; the second is often spoken of as the implantation method (705). The spotting of the two organisms on the plate is illustrated in Figure 6.

Successive inoculation of the test organism, after the antagonist has already made some growth, so as to enable the active substance to diffuse. This method, suggested by Garré (311), consists in allowing the antagonist to produce a good growth on the surface of the medium; the mass of growth is then removed, and the test organism inoculated into the same medium.

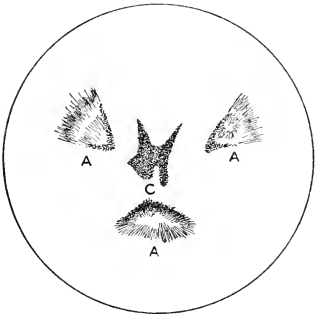
Double plate methods (302). A Petri dish is divided into two parts by means of a small glass tube or rod. After sterilization, one tube of molten agar is heavily inoculated with the antagonist and poured into one half of the plate. When the agar has hardened, another tube of sterile agar is poured into the other half of the plate. Both sides are



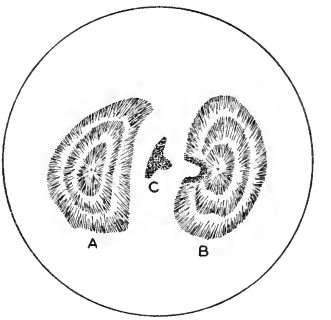
Helminthosporium (A and B) inhibited by *Fusarium* (C)



Pestalozzia (A) inhibited by one species of *Penicillium* (C) but not by another (B)



Helminthosporium (A) inhibited by a bacterium (C)



Helminthosporium (A and B) inhibited by a white yeast (C)

FIGURE 6. Inhibition of fungus development by antagonists. From Porter (695).

then streaked with the test organism, each side being equally inoculated by separate streaking. This can be done by using a loop bent at nearly right angles; the charged loop is moved from the circumference toward the glass rod. The loop is then sterilized, recharged with the test culture, and the streak continued on the other side of the plate. The inoculation with the test organism may be made soon after the plate is poured, or the antagonist may be given an opportunity to develop before the test organism is streaked thus making the antagonistic effect more striking. This method has also been used (261, 267, 270) for testing the antibiotic properties of fungus cultures.

Mixed culture inoculation. The cultures of the antagonist and the test organism are mixed and inoculated upon the surface of the solidified agar or before the molten agar has been added to the plate. The colonies of the antagonist will be surrounded by clear sterile zones, free from any growth of the test organism.

Spot inoculation of the antagonist upon an actively growing culture of a bacterium or fungus on an agar plate (844). This method is particularly convenient for detecting antagonists that possess lytic properties.

A layer of molten sterile agar is used to cover the surface of an antagonist that has made some growth in a plate, and the surface of the agar layer is then inoculated with the test organism. The active substance produced by the antagonist will diffuse through the agar and reduce the growth of the test bacterium (571).

Semisolid media are used for testing the action of antagonists against motility of bacteria (173).

METHODS OF GROWING ANTAGONISTIC ORGANISMS FOR THE PRODUCTION OF ANTIBIOTIC SUBSTANCES

Once the antagonistic action of any organism has been established, the next step is to determine the nature of the antibiotic substance produced by the antagonist and to measure quantitatively this antibiotic action. Before this can be done, however, the organism must be grown upon suitable media and suitable conditions must be established for the favorable production of the antibiotic substance.

The media used for the production of antibiotic substances can be classified into two groups: synthetic media and complex organic media. The first contain a source of carbon, usually glucose (2 to 6 per cent); a source of nitrogen, usually nitrate (0.2 to 0.6 per cent), as well as several salts, namely, K_2HPO_4 or KH_2PO_4 (0.1 to 0.2 per cent), $MgSO_4.7H_2O$ (0.05 per cent), KCl (0.05 per cent), and $FeSO_4.7H_2O$ (0.001 per cent); certain supplementary materials, such as yeast extract, meat extract, or corn steep, and other salts, such as NaCl (0.05 to 0.5 per cent), $ZnSO_4$, $MnSO_4$, or $CuSO_4$ (1 to 2 ppm.) may also be added. The organic media contain a complex form of nitrogen, such as tryptone, peptone, casein digest; either no other source of carbon is used or a carbohydrate is added in the form of glucose, starch, brown sugar, molasses, or similar products as well as several salts similar to those listed above. Some media are supplemented with $CaCO_3$, and others are not, depending upon the extent of acidity produced by the organism.

The medium may be solid or liquid, but the latter type is more common. Agar and bran are used as solid media. Several types of culture vessels are used, depending on the condition of aeration. Since so far as is known all the microorganisms capable of producing antibiotic substances are aerobic, either shallow layers of medium (1.5 to 2 cm. in depth) are placed in stationary vessels (flasks or trays), or deep vessels (tanks) are filled with the medium and properly aerated by forced draft with sterilized and filtered air.

For the production of penicillin, a constant-flow apparatus similar to the quick-vinegar process has been suggested (134), the medium trickling over a column of wood shavings. The establishment and operation of large-scale production of penicillin under submerged conditions have been described in detail by Callaham (103).

The optimum temperature required for the growth of the antagonistic organisms and production of the antibiotic substances ranges between 20° and 30° C. The length of incubation varies from 2 to 6 days for submerged cultures and from 3 to 20 days for stationary cultures.

A knowledge of the preliminary treatment of the inoculum or spore material is essential. For the growth of spore-forming bacteria, the use of a pasteurized spore suspension is advisable in order to avoid the variable factor due to vegetative cells. Actinomycetes and fungi are grown

on agar slants in order to obtain abundant spore material for the inoculation of stationary cultures. For submerged cultures, special spore suspensions are produced by growing the organisms in shaken cultures.

The cultures must be tested carefully in order to establish the optimum activity when the culture filtrate is cooled and extraction of active substance is started.

METHODS OF MEASURING THE ACTIVITY OF ANTIBIOTIC SUBSTANCES

It has long been recognized that the evaluation of bacteriostatic and bactericidal substances is controlled to a considerable extent by the methods employed. These methods are based upon the following factors: (a) proper selection of the test organism, (b) composition of the medium used for testing activity, (c) time of action, (d) conditions of carrying out the test, and (e) nature of the active substance. The results obtained in a comparison of substances containing the same active principle may not be very reliable when different agents are compared, since these vary greatly in their specific action upon different bacteria. This is especially true of antibiotic agents.

In most of the work on chemical disinfectants, which are primarily bactericidal agents, the death rate of the viable cells has been used as a basis for evaluation. Different substances have been compared with a standard, ordinarily phenol. Since antibiotic and chemotherapeutic substances are primarily bacteriostatic in action, the inhibition of the growth and multiplication of the test organism is commonly used as a basis for their evaluation.

In any attempt to select a single standard method for measuring quantitatively the activity or potency of an antibiotic substance, it is essential to recognize several pertinent facts, which may be briefly summarized as follows:

Antibiotic (antibacterial, antimicrobial) substances are primarily bacteriostatic (or fungistatic) in their action; they are bactericidal (or fungicidal) only to a limited extent, although some substances may possess marked bactericidal properties.

Antibiotic substances are selective in their action; they are able to inhibit

the growth of some bacteria in very low concentrations, whereas much larger amounts are required to affect other bacteria and some organisms may not be inhibited at all by the particular substance even in very high concentrations.

Conditions for the bacteriostatic activity of different antibiotic substances vary greatly. Some substances are not active at all, or their activity is greatly reduced in some media because of the neutralizing effect of certain constituents of the media, such as peptone or *p*-amino-benzoic acid. Other agents require the presence in the medium of specific constituents for their activity to become effective. The activity of some is reduced at an acid reaction (287, 1002), whereas that of others is not affected.

The mechanism of the action of different antibiotic agents is different. Some agents interfere with bacterial cell division, others with bacterial respiration, and still others with utilization by the bacteria of essential metabolites.

Many antagonistic organisms produce more than one antibiotic substance. *Ps. aeruginosa* produces pyocyanase and pyocyanin; *B. brevis*, gramicidin and tyrocidine; *Streptomyces antibioticus*, actinomycin A and B; *P. notatum*, penicillin and notatin; *Aspergillus fumigatus*, spinulosin, fumigatin, fumigacin, and gliotoxin; *Aspergillus flavus*, aspergillic acid and flavicin. The culture filtrate of an antagonistic organism often differs, therefore, in its activity from that of the isolated active substance.

The course of production of antibiotic substances by two typical antagonistic organisms is illustrated in Figures 7 and 8.

In view of the bacteriostatic nature of antibiotic substances, few of the methods commonly used for testing the efficiency of antiseptics and germicides can be employed. This is particularly true of the "phenol coefficient test," which measures the germicidal action of phenol upon *E. typhosa*. The limitations of this method, based on the bactericidal action of a single substance on a single organism, even as applied to chemical antiseptics have long been recognized (735, 773).

A number of methods have been developed for determining the activity of antibiotic substances. They vary greatly, each having its limitations and advantages. Because of lack of uniformity in the methods, the results obtained by one are not always comparable with those obtained

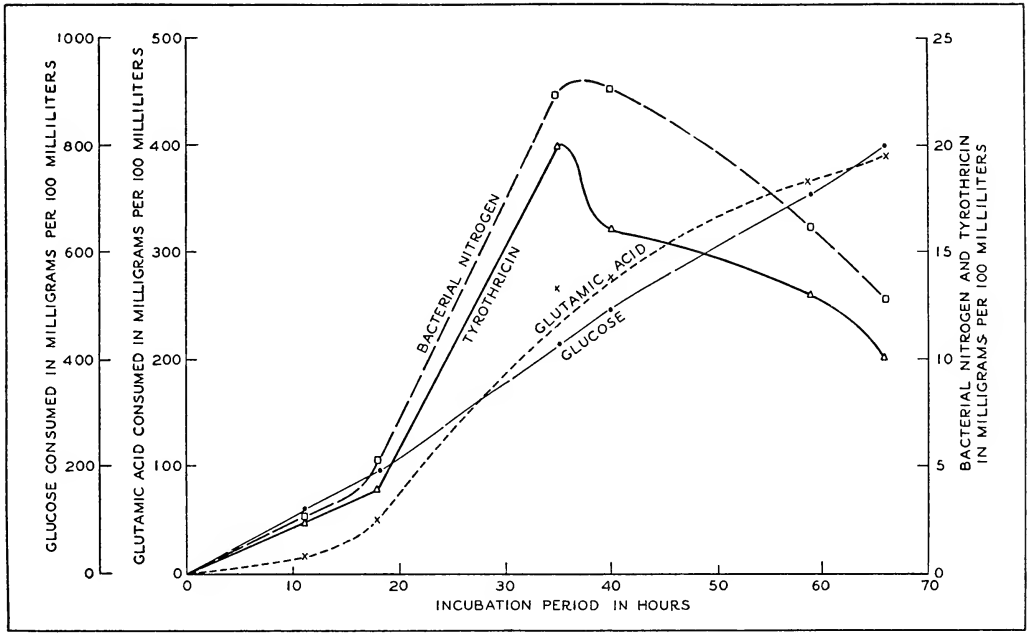


FIGURE 7. Production of tyrothricin in relation to nutrition of *Bacillus brevis*. From Stokes and Woodward (856).

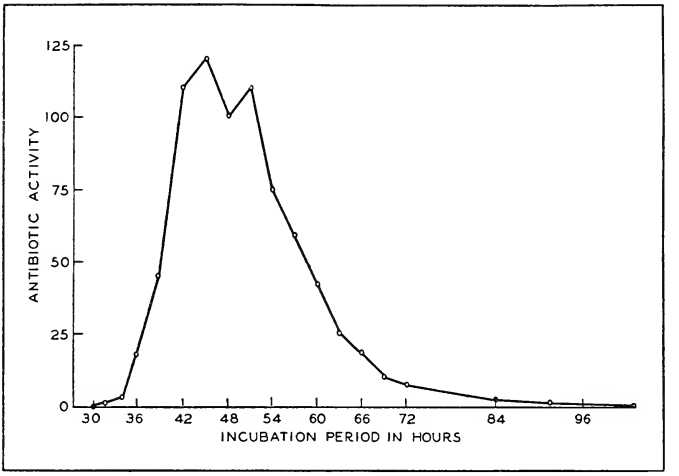


FIGURE 8. Production of gliotoxin by *Trichoderma*. From Weindling (962).

by another. The most important methods at present in use are briefly summarized in the following pages.

The Agar Plate–Dilution Method

If an unknown antibiotic substance is tested, it is essential to employ several test organisms in order to throw light upon the selective activity of the substance on different bacteria. Nutrient agar media have usually been employed. Sterility is not absolutely essential for this method, although it is desirable. The unknown substance is diluted to various concentrations (1, 0.3, 0.1, etc.; or 1, 0.5, 0.25, etc.); these dilutions are added and thoroughly mixed with definite volumes (10 ml.) of sterile agar medium, melted and cooled to 42° to 45° C. The agar is allowed to solidify, and is streaked with three or four test bacteria, among the most common of which are *E. coli*, *E. typhosa*, *Br. abortus*, *B. subtilis*, *S. aureus*, *M. lysodeikticus*, and *S. lutea*. The age of the cultures (16 to 24 hours) is important. The plates are incubated at 28° or 37° C. for

TABLE 8. BACTERIOSTATIC EFFECTS OF AGENTS OF BIOLOGICAL ORIGIN AND OF PURE CHEMICAL SUBSTANCES

ACTIVE SUBSTANCE	*A. aerogenes*		*E. coli*		*B. mycoides*		*B. subtilis*		*M. lysodeikticus*		*S. lutea*	*Actinomyces* sp.
	Nutrient agar	Nutrient broth	Nutrient agar	Nutrient broth	Nutrient agar	Nutrient broth	Nutrient agar	Nutrient broth	Nutrient agar	Nutrient broth	Nutrient agar	Nutrient agar
Actinomycin	<3	3	<3	>3	>10,000	>10,000	30,000	>30,000	30,000	>30,000	>30,000	300
Streptothricin*	100	>30	>300	>300	<3	<3	3,000	1,000	300	>300	>300	>300
Tyrothricin	<.3	<3	<.3	<3	>30	100	>30	>300	3,000	>10,000	100	<30
Gramicidin	<3	<3	<3	<3	<3	<3	<3	<3	1,000	>30,000	300	3
Tyrocidine	<3	<3	<3	<3	>100	>300	>100	>1,000	300	>1,000	300	>100
Pyocyanase	<10	<3	10	<3	>30	>30	>30	>30	100	>30	>30	100
Pyocyanin	10	<3	>30	30	>100	>100	300	300	>100	300	>300	>30
Penicillin	<3	<3	<3	<3	<3	<3	1,000	1,000	>300	>300	1,000	<10
Gliotoxin	10	>3	10	10	100	300	3,000	>300	100,000	30,000	>10,000	>100
Lysozyme†	<10	<10	<10	<10	<10	<10	300	>300	>3,000	>10,000	10,000	>10
Tolu-p-quinone	>3	>3	>3	>3	30	>10	30	>30	30	100	100	10
Phenol	>.3	1	>.3	1	>.3	>.3	>1	>1	>1	>1	1	1
Lauryl sulfate‡	<.1	<1	<.1	<1	>10	>10	30	>300	>10	30	>10	>10
Sulfanilamide	1	1	1	1	<1	<1	10	10	1	<1	1	1

From Waksman and Woodruff (948).

Note. Units of activity per milligram of preparation, by dilution procedure; > indicates that activity is somewhat greater than indicated by figures; < indicates no inhibition by given concentration.

* Crude preparation.

† Fresh egg white, in ml.

‡ Duponal M.E.

16 to 24 hours, and readings are made. The highest dilution at which the test organism fails to grow is taken as the end point. Activity is expressed in units, using the ratio between the volume of the medium and the end point of growth or the dilution at which growth is inhibited (948).

The bacteriostatic spectra of a group of antibiotic substances compared with certain chemical agents are shown in Table 8. Different bacteria show different degrees of sensitivity to the different substances. A comparison with the action of phenol can result in what may become known as the "bacteriostatic phenol coefficient" for each active substance.

Serial Dilution Method

Once a substance is characterized as regards its selective action upon specific bacteria, its activity or concentration can be measured more accurately by the liquid dilution or titration method. One test organism is selected, usually a strain of *S. aureus*. Different strains may vary in their action. In some cases, *Streptococcus hemolyticus*, *B. subtilis*, and others have been used for measuring the activity of a substance against gram-positive bacteria, and *E. coli* for gram-negative bacteria. Definite volumes of the test medium are placed in test tubes and sterilized (sterility is essential in this method), and various dilutions of the active substance are added. The dilutions can range in order of 3:1, 2:1, or even narrower, namely in series of 1.2:1, 1.5:1, etc. The tubes are inoculated with the test organism and incubated for 16 to 24 hours. In some cases the medium is inoculated before it is distributed into the tubes. The highest dilution of the antibiotic substance giving complete inhibition of growth, as expressed by a lack of turbidity of medium, is taken as the end point. Activity is expressed in units as above (804).

The dilution method has two disadvantages (276): first, every assay takes much time; second, during chemical fractionation, the substance may become contaminated with bacteria not sensitive to the active substances.

One modification of the method has been adapted for measuring the activity of penicillin. Several dilutions of the active agent are prepared and 0.5 ml. portions added to 4.5 cm. quantities of liquid medium in

TABLE 9. BACTERIOSTATIC SPECTRUM OF PENICILLIN

ORGANISM AFFECTED	DILUTIONS AT WHICH INHIBITORY EFFECTS WERE OBSERVED		
	Complete	Partial	None
*N. gonorrhaeae**	2,000,000	>2,000,000	>2,000,000
N. meningitidis	1,000,000	2,000,000	4,000,000
S. aureus	1,000,000	2,000,000	4,000,000
S. pyogenes	1,000,000	2,000,000	4,000,000
B. anthracis	1,000,000	2,000,000	4,000,000
A. bovis	1,000,000	2,000,000	4,000,000
Cl. tetani†	1,000,000	.....	.....
Cl. welchii	1,500,000	.....	.....
Cl. septicum	300,000	1,500,000	7,500,000
Cl. oedematiens	300,000	.....	1,500,000
S. viridans‡	625,000	.....	3,125,000
Pneumococcus‡	250,000	500,000	1,000,000
C. diphtheriae (*mitis*)	125,000	.....	625,000
C. diphtheriae (*gravis*)	32,000	64,000	128,000
S. gärtneri	20,000	40,000	80,000
S. typhi	10,000	30,000	90,000
Pneumococcus‡	9,000	.....	27,000
Anaerobic streptococcus‡	4,000	8,000	16,000
P. vulgaris	4,000	32,000	60,000
S. viridans‡	4,000	8,000	16,000
P. pestis	1,000	100,000	500,000
S. typhimurium	<1,000	8,000	16,000
S. paratyphi B	<1,000	5,000	10,000
Sh. dysenteriae	2,000	4,000	8,000
Br. abortus	2,000	4,000	8,000
Br. melitensis	<1,000	2,500	10,000
Anaerobic streptococcus	<4,000	<4,000	4,000
V. comma	<1,000	1,000	2,000
E. coli	<1,000	<1,000	1,000
K. pneumoniae	<1,000	<1,000	1,000
Ps. aeruginosa	<1,000	<1,000	1,000
M. tuberculosis	<1,000	<1,000	1,000
L. icterohoemorrhagioe	<3,600	<3,600	3,600

From Abraham et al. (7).

* Another strain was inhibited only up to 32,000.

† Grown in Lemco broth; in beef broth complete inhibition reached only 100,000.

‡ In *Pneumococcus*, *S. viridans*, and anaerobic streptococci, different strains appear at different levels in the table.

test tubes. These are inoculated with a standard drop (0.04 ml.) of a 24-hour culture of the test organisms. Complete or partial inhibition is shown by the absence of turbidity after 24 hours of incubation at 37° C. Dilutions higher than those required for complete or partial inhibition gave, after 24 hours of incubation, only a retarding effect (2, 7); a microscopic examination (308) indicated defective fission of the bacteria, even though the macroscopic appearance of the culture did not show any inhibition. Pneumococci and *S. viridans* show marked strain differences by this method. In one experiment with *Salmonella typhi,* partial inhibition was obtained in a dilution of 1:10,000; however, elongation of the cells was detected in a dilution of 1:60,000, a concentration which was considered as a therapeutic possibility (Table 9).

The Agar Diffusion or "Agar Cup" Method (7, 284, 285, 385)

This method, first employed by Reddish (735) and by Ruehle (773) largely for qualitative purposes, was later developed (7, 385) for quantitative use. A suitable agar medium is inoculated with the test organisms (*S. aureus* or *B. subtilis*), the active agent being placed upon the agar, within a groove or in a special small glass cup with an open bottom from which the substance diffuses into the medium. The rate of diffusion of the active substance is parallel to its concentration. By measuring the zone of inhibition and comparing it with that of a known standard preparation, the potency of the active substances can be calculated. This method has the advantage of simplicity and convenience, since it does not require sterile material and several preparations or duplicates can be tested on the same plate. The method also possesses certain disadvantages, however, since it cannot be used for comparing different substances but is limited to the measurement of activity of only one type of substance; it cannot be used for the study of unknowns until a standard has been established for each unknown.

Nutrient agar containing 5 gm. NaCl, 3 gm. meat extract, 5 gm. peptone, 15 gm. agar, 1,000 ml. tap water, and adjusted to *p*H 6.8, is poured into plates to a depth of 3 to 5 mm. The plates are seeded thoroughly with the test organism (*S. aureus*) by flooding with 1:10 or 1:50 dilution of 16-to-24-hour-old broth culture in sterile water. The excess fluid may be removed with a pipette. The surface of the agar is

allowed to dry somewhat in the 37° C. incubator for 1 to 2 hours, the lids of the plates being raised about 1 cm. above the bottoms of the dishes. Sterile short glass cylinders (5 mm. inside diameter) are placed on the agar, the lower edge of the cylinder sinking into the agar, and are filled with the test solution. Several cylinders may be placed in one dish.

For measuring the activity of penicillin, the plates are incubated for 12 to 16 hours at 37° C. The diameter of the zone around the cylinder is measured to the nearest 0.5 mm. by means of pointed dividers. The relation of concentration of penicillin in the solution to the zone of inhibition, or the "assay value," is expressed by a curve which is obtained with standard solutions. This curve tends to flatten out above 2 units of penicillin per milliliter. The assay value is not influenced by the *p*H of the test material, the thickness of the agar, or the sterility of the material.

The "Oxford unit," as determined by this method, is the amount of penicillin that will just inhibit completely the growth of the test strain of *S. aureus* in 50 ml. of medium. Thus, a preparation containing one unit of penicillin per milligram of material just inhibits the growth of the test organism in a dilution of 1:50,000 (7, 273, 385).

One of the modifications of this method (285) consists in using a spore suspension of *B. subtilis* as the test organism. It is grown for several days under forced aeration, and the cultures are pasteurized in order to destroy the vegetative cells. The spore suspension is stored in the cold and used as the stock inoculum; it is titrated in order to determine the optimum amount for seeding purposes. The lowest level (usually 0.1 to 0.2 ml. per 100 milliliters of agar) that gives a dense, continuous growth of the organism under the assay conditions is selected as the optimum.

This method is also very convenient for measuring the activity of streptothricin. A standard curve is obtained by filling the cups in quadruplicate with dilutions of the standard containing 10, 20, 40, 60, 80, and 100 streptothricin units per milliliter. The dilution of the unknown contains about 50 units per milliliter. After overnight incubation at 30° C., the inhibition zones around the cups are measured and plotted to give a standard curve. The units of the unknowns are read off this curve by projecting the value of the inhibition zones.

The agar cup method has also been utilized (869) for comparing the disinfectant action of chemical antiseptics. *S. aureus* and *B. subtilis* were found to be most sensitive to the action of aliphatic alcohols. Various modifications of this method have been introduced, including the use of paper discs treated with known dilutions of the active preparation (818, 917, 985).

Turbidimetric Method

End-point methods have long been recognized as having many limitations. Since it is difficult to determine accurately the end point and since it takes a relatively much larger amount of an antibiotic substance to inhibit completely the growth of the test organism as compared with only 50 or 99 per cent inhibition, the suggestion has been made that partial inhibition of growth be measured and, from this, the concentration of the active substance be calculated in a manner similar to the

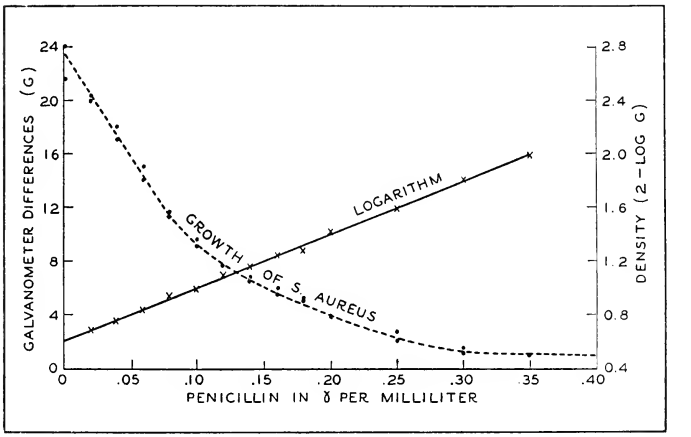


FIGURE 9. Relation between penicillin concentration and inhibition of *Staphylococcus aureus*. The penicillin preparation contained 42 Oxford units per milligram, and the incubation period was sixteen hours at 37° C. From Foster (281).

measurement of the potency of bactericidal agents. Partial inhibition can be determined by plating for the number of viable bacteria, as compared with the control, or it can be measured by a convenient turbidimeter. The results obtained by this method are more nearly quantitative in nature than those obtained by other methods (281, 520), as shown in Figure 9. By proper modifications, the length of time required to obtain a satisfactory reading can be reduced to four hours (465, 572), or even to 90 minutes (281, 520).

Special Methods

Certain methods were found to be specific for measuring the action of certain substances. The ability of tyrothricin to hemolyze red blood cells served as the basis for measuring the potency of this substance (172): the tyrothricin content is calculated from the amount of hemolysis by the unknown and is read from a standard curve. The inhibition of growth of a β-hemolytic streptococcus, group A, as measured by hemolysin production has been used for assaying the potency of penicillin (715, 989). Penicillin can also be estimated by its inhibition of nitrite production by *S. aureus* cultures (343). The antiluminescent test has been utilized (716, 717) not only for measuring the activity of certain substances but also for determining their possible usefulness. The results of a comparative study of a number of antibiotic substances by this and the dilution methods are brought out in Table 10.

Various other methods have been suggested for measuring the activity of antibiotic substances. Some are based upon interference with a given physiological function of the test organism such as dehydrogenase activity and respiration, others upon the prevention of growth of the test organism (pneumococcus) in semi-solid tissue culture medium (387). Although only a single method is usually employed in the concentration and standardization of a given antibiotic substance such as penicillin, it is often advisable to check the results by another method, especially where several test organisms are used, in order to ascertain that one is still dealing with the same type of chemical compound.

Some of the above methods can also be adapted to the determination of the concentration of antibiotic agents in the body fluids and exudates (719).

TABLE 10. ANTILUMINESCENT AND ANTIBACTERIAL ACTIVITIES OF VARIOUS ANTIBIOTIC SUBSTANCES

SMALLEST AMOUNT SHOWING ACTIVITY, IN MICROGRAMS				AL/AB RATIO	
Antiluminescent test		Antibacterial test*			
Tolu-p-quinone	0.11	Gramicidin	.002	Tolu-p-quinone	.002
Pyocyanase	3	Tyrothricin	.008	Pyocyanase	.07
Clavacin I	11	Penicillin II	.0156	Clavacin I	.18
		Penicillin I	.06		
		Flavatin	.256		
Aspergillic acid	15	Gramidinic acid	.23	Sodium clavacinate	.18
Gliotoxin	17	AP21†	.31	Clavacin II	.19
Clavacin II	22	Actinomycin A	.54	Sulfanilamide	<.56
				Phenol	.5
Pyocyanin	47	Aspergillic acid	2.0	Pyocyanin	1.7
Actinomycin A	54	Gliotoxin	2.1	Lauryl sulfate	4.6
Streptothricin	56	Streptothricin	2.8	Aspergillic acid	7.5
Sodium clavacinate	94	Fumigacin	13.0	Gliotoxin	8.0
Flavatin	256				
Fumigacin	273	Pyocyanin	27.0	Streptothricin	20.0
Lauryl sulfate	273	Pyocyanase	42.0	Fumigacin	21.0
Phenol	1170	Tolu-p-quinone	55.0	Actinomycin A	100.0
				Flavatin	1000.0
Penicillin I	1650	Lauryl sulfate	59.0	AP21†	>1630
Sulfanilamide	3940	Clavacin I	63.0	Gramidinic acid	>2175
Gramicidin	>500	Clavacin II	113.0	Penicillin I	27,500
Gramidinic acid	>500	Sodium clavacinate	500.0	Tyrothricin	>62,500
Tyrothricin	>500	Phenol	2300.0	Gramicidin	>250,000
AP21†	>500	Sulfanilamide	>7000.0	Penicillin II	>325,000
Penicillin II	>5000				

From Rake, Jones, and McKee (716).
* *Streptococcus pyogenes* used as test organism.
† A tyrothricin-like preparation.

METHODS OF MEASURING BACTERICIDAL ACTION OF ANTIBIOTIC SUBSTANCES

Several methods are commonly employed for measuring bactericidal action of antibiotic substances. A suspension of washed bacterial cells in saline or other suitable solution, or a 5-to-12-hour-old broth culture of the test organism, is treated with various dilutions or concentrations of

the active substance. After incubation at 37° C. for 1 to 24 hours, the number of living cells is determined. If the active substance has lytic properties or if the test organism undergoes lysis readily, the readings are simplified. If no lysis occurs, the treated bacterial suspension or culture is streaked or plated out. The streaking procedure gives only a relative idea of the extent of bactericidal action. If 50 to 90 per cent killing of cells is to be taken as a unit of measurement, the culture is plated on a suitable medium and the actual number of surviving cells determined.

Various modifications of this method have been developed. In one such modification (607), the bacterial cells are suspended for 15 to 30 minutes in various dilutions of the active substance; the cells are then centrifuged off, washed, and cultured. This procedure can be utilized for substances that have a rapid bactericidal action. Its value is limited, however, by the fact that most antibiotic substances do not kill bacteria so rapidly as do chemical antiseptics (p. 189).

Some of the foregoing methods can also be utilized for measuring the fungistatic and fungicidal properties of antibiotic substances. Protective fungicides may first function as fungistatic agents, others function better either as fungicidal or as fungistatic agents, and still others show either a high or a low for both (550). The growth of *Ceratostomella ulmi* was inhibited by several substances, comprising actinomycin, clavacin, and hemipyocyanin in concentration of 1:100,000 (803).

METHODS OF TESTING THE IN VIVO ACTIVITIES OF ANTIBIOTIC SUBSTANCES

Ordinary pharmacological, bacteriological, and pathological procedures are used for testing the toxicity and activity of antibiotic substances in the animal body. Some of the results obtained are presented in subsequent chapters.

ISOLATION AND UTILIZATION OF ANTIBIOTIC SUBSTANCES

The isolation of antagonistic microorganisms from natural substrates, the determination of the nature of the antibiotic substances pro-

duced by them, and the utilization of such substances for chemotherapeutic purposes involve ten distinct steps which may be briefly summarized as follows:

1. Enrichment of soil or water with specific organisms against which antagonists are to be obtained.
2. Plating of the enriched or unenriched soil or water upon special bacterial agar.
3. Isolation of the antagonistic organisms from the plates or other culture media.
4. Testing of the isolated culture for bacteriostatic and fungistatic properties against a variety of bacteria or fungi.
5. Growing the antagonist in suitable media and testing the filtrate or extract of the culture for the presence of the antibiotic substance.
6. Separation and concentration of the antibiotic substance from the culture medium, and determinations of its bacteriostatic spectrum.
7. Chemical isolation of the antibiotic substance.
8. Determination of the chemical nature of the active substance.
9. Testing of the bacteriostatic and bactericidal properties of the substance *in vitro*.
10. Animal experimentation and practical application; study of toxicity, *in vivo* activity, and therapeutic action.

CHAPTER 5

BACTERIA AS ANTAGONISTS

FOLLOWING the early work of Pasteur (674) on the antagonistic effects of bacteria against the anthrax organism, considerable attention has been centered upon bacteria as agents possessing antibacterial properties. A systematic study of this phenomenon was first made by Babes in 1885 (150), followed by Garré in 1887 and Freudenreich in 1888. Freudenreich (299) demonstrated that when certain bacteria were grown in a liquid medium, the filtrate obtained by passing the culture through a porcelain candle supported the growth of the typhoid organism not at all or only very feebly. Garré (311) observed that *Ps. putida* inhibited the growth of *S. aureus*, *E. typhosa*, and *Bacillus mucosus-capsulatus* but not of *B. anthracis* and other bacteria. It was soon reported (524), however, that *B. anthracis* was also killed by the *Pseudomonas* antagonist, whereas the growth of *S. aureus* and *V. comma* was only retarded; no effect at all was exerted upon *E. typhosa* and *E. coli*. In consequence, the antagonist was claimed to be active against *B. anthracis* but not against other bacteria. Olitsky (655) concluded that *Ps. fluorescens* inhibited the growth not only of *E. typhosa* but also of *B. anthracis*, *V. comma*, *S. marcescens*, and *S. aureus*. These and other apparently contradictory results were undoubtedly due to differences in the specific nature of the strains of the organisms used by the various investigators and to different methods of cultivation.

The presence of *Ps. fluorescens* in sewage was found (517) to reduce greatly the period of survival of the typhoid organism. The latter did not develop even in gelatin upon which *Ps. fluorescens* had previously grown (326, 430, 431, 432), and it could not be detected in sterile sewage in which the antagonist was present for seven days. According to Frost (302), *E. typhosa* can be antagonized by a number of different soil bacteria, of which *Ps. fluorescens* exhibits the strongest effect. He observed that although *P. vulgaris* acted more rapidly, the active substance did not diffuse to so great a distance in the medium, thus point-

ing to a different inhibition mechanism. Mixed cultures showed greater activity than pure cultures, either because the latter lose their antibiotic property when grown for a long time on artificial media or because mixed cultures comprise two or more species with a greater combined action. The antagonistic substances produced by these bacteria were active at 37° C., whereas at ice-chest temperature the action was delayed so that the pathogen had an opportunity to develop. This was believed to offer a possible explanation for the fact that when water supplies become contaminated in cold weather, their power of producing infection is retained for a longer time than when the contamination takes place in warm weather.

Frost concluded that the phenomenon of antagonism results in checking the growth of *E. typhosa* as well as in killing the pathogen. Evidence that antagonistic substances exist in an active state in the soil or in water appeared to be lacking; rather, the results suggested that formation of such substances depends on the actual development of specific antagonistic organisms. Changes in environment, such as temperature, oxygen supply and reaction of the medium, and nature and concentration of nutrients, were believed to have little or no influence on the production of the antibiotic substances; these were produced under conditions favoring growth of the antagonists.

The activity of the influenza organism was found (993) to be largely dependent on the presence of accompanying bacteria. Some of these, especially micrococci, are favorable to the growth of this organism whereas others, such as *Ps. aeruginosa* and *B. subtilis*, are injurious.

According to Lewis (525), luxuriant growth of *Ps. fluorescens* in manured soil and in protein solution containing *B. cereus* is due to antagonistic action of the former organism against the latter. The former also inhibits the growth of *B. anthracis*, *Bacillus megatherium*, *V. comma*, *Chromobacterium violaceum*, and *Rhodococcus*. Other species of the genera *Bacillus*, *Eberthella*, *Sarcina*, *Neisseria*, and *Phytomonas* are somewhat more resistant to the action of *Ps. fluorescens*. *Salmonella* species are less sensitive, whereas *E. coli*, *A. aerogenes*, and *S. marcescens* are highly resistant. *Ps. fluorescens* produces a thermostable substance which is toxic to all bacteria except the green fluorescent forms

and which is active against actinomycetes but not against fungi. This substance is water-soluble and dialyzable through collodion and other membranes.

In addition to the aforementioned bacteria, numerous other groups were found to contain strains which had strong antagonistic properties toward bacteria as well as fungi. Some of the antagonists were highly specific, as in the case of those acting upon the various types of pneumococci (819, 821, 822); others were less selective, as in the case of certain soil bacteria that can bring about the lysis of living staphylococci and inhibit the growth of various gram-positive and gram-negative bacteria (820). *S. marcescens* was shown (61) to be antagonistic to *B. subtilis*, *B. mycoides*, and *B. megatherium*. These spore-formers, in turn, were antagonistic to sarcinae, bringing about their lysis, to *V. comma*, and to various other bacteria. It was further found that the antagonists modified the physiology of the antagonized organism. When two bacteria were planted, for example, in the same medium, metabolic products were formed that were not produced in the culture of either organism alone, whereas certain decomposition processes were either hastened or retarded (632).

The various antagonistic bacteria can be divided into several groups, on the basis of their morphological properties.

SPORE-FORMING BACTERIA

Many strains of aerobic spore-forming bacteria possessing antagonistic properties and differing in morphological, cultural, and physiological characteristics have been isolated from a great variety of sources, such as soil, sewage, manure, and cheese. Among these bacteria, *B. subtilis*, *B. mycoides*, *B. mesentericus*, and *B. brevis* occupy a prominent place, as shown in Table 11. It was established (205) that some of these bacteria produce in peptone media an alcohol-soluble, water-insoluble substance endowed with bactericidal properties.

Duclaux (208) was among the first to isolate and describe antagonistic spore-forming bacteria. Cantal cheese was their origin, and the organisms were designated as *Tyrothrix*. Nicolle (640) isolated from the dust in Constantinople a strain of *B. subtilis* that had decided bacterio-

lytic properties, especially against members of the pneumococcus group as well as against various other bacteria such as the typhoid, anthrax, and Shiga organisms. *E. coli*, *V. comma*, and staphylococci were less affected, and *Bacillus suipestifer* was least acted upon. The filtrate of the organism grown in peptone broth had strong antibiotic properties;

TABLE II. SPORE-FORMING BACTERIA ANTAGONISTIC TO OTHER BACTERIA

ANTAGONIST	ORGANISM AFFECTED	KNOWN PROPERTY	REFERENCES
B. anthracis	Anthrax, typhoid, and lactic acid bacteria		299, 781, 827
B. brevis	Gram-positive bacteria	Produces tyrothricin	190, 191, 203
B. mesentericus	Many bacteria	Bacteriolytic	408
B. mesentericus	Diphtheria bacteria	Bactericidal	28, 956
B. mesentericus vulgatus	*C. diphtheriae*	Substance thermolabile, nonfilterable	705
B. mycoides	7 to 20 species of bacteria	Lytic	61, 620, 621
B. mycoides	*M. tuberculosis*	Thermostable substance produced, precipitated by tungstic acid	482, 483
B. mycoides, var. *cytolyticus*	Most pathogens and many nonpathogens		294
B. subtilis	Various bacteria	Bacteriolytic	61, 640
B. subtilis	Various bacteria, especially certain plant pathogens		442
B. subtilis	*M. tuberculosis*, *E. typhosa*, etc.		903
B. subtilis-mesentericus	Mostly living gram-positive bacteria and dead gram-negative bacteria	Lytic	768, 770
B. thermophilus	*S. lutea*	Suppression of growth	835

it liquefied gelatin and hemolyzed red blood corpuscles. When various bacteria cultivated on a solid medium were suspended in physiological salt solution and seeded with the antagonist, the latter developed abundantly and the bacterial suspensions became clarified. The lysed solutions of pneumococcus prepared by the use of the filtrate of *B. subtilis* could be used for purposes of vaccination. In this connection, Nicolle spoke of the work of Metchnikoff who had proved, in 1897, that organisms belonging to the *B. subtilis* group are capable of destroying various bacterial toxins. Humfeld and Feustel (442) recently demonstrated that an acid extract of *B. subtilis* cultures has a very high activity against certain plant pathogens. This substance was designated as subtilin.

Rosenthal (768) isolated, from soil and from fecal matter, facultative thermophilic antagonistic bacteria belonging to the *B. mesentericus* group capable of dissolving both living and dead bacteria. The simultaneous growth of the antagonist with *V. comma* and other bacteria brought about the clarification of the culture of the latter in about 5 or 6 days. These bacteriolytic organisms were designated as "lysobacteria." It was recognized that the action of antagonists is different from that of phage in several respects: (a) the filtrate of the antagonist is active against other bacteria; (b) both living and dead cultures of bacteria are dissolved; (c) antagonistic action is not so specific as that of phage; (d) races of *E. coli* resistant to phage are dissolved by the filtrate of the antagonist. The active substance was believed to be of the nature of an enzyme. Friedländer's bacillus was not acted upon, possibly because of the formation of a pellicle by the bacillus. The active substance was formed in 4 to 5 days but increased in activity after 2 to 3 weeks. It was essential that a surface pellicle of the organism be maintained. Submerged growth was less favorable. Fresh filtrates had the greatest activity, the property being lost after storage for 3 months. The substance was thermolabile, activity being destroyed at 70° C. The filtrate of an organism dissolved by the action of the antagonist proved to be as active as the filtrate of the culture of the antagonist. It acted injuriously upon intestinal bacteria not only *in vitro* but also *in vivo*.

Much and associates (620) isolated several strains of *B. mycoides* that possessed strong antagonistic properties. The active strains were

said to be found only rarely in nature. They gave a mesentericus-like growth, producing a pellicle and no turbidity in bouillon. One strain was able to lyse 20 species of bacteria, another acted upon 18, a third on 12, and a fourth on only 7. Marked differences were shown (621) to exist in the degree of antagonistic activity of the different strains. *P. vulgaris, E. typhosa,* and *V. comma* were lysed in 24-hour bouillon cultures as a result of adding pieces of agar containing colonies of the antagonist. A lytic effect was also exerted upon staphylococci (786) and *M. tuberculosis* (482). The active substance (483) was precipitated by 10 per cent tungstic acid and lead acetate and was thermostable.

Much and Sartorius (621) came to the conclusion that *B. mycoides* Flügge comprises two groups of organisms. One produces branching colonies on agar and forms no pellicle in meat broth, the flaky growth dropping to the bottom and the medium remaining more or less clear. The second group forms flat surface growth similar to that of *B. mesentericus* on agar and a pellicle on the surface of liquid media. Many of the pellicle-forming strains have the capacity, in varying degrees, of dissolving various cultures of bacteria. This is not due to their proteolytic activity, since members of the first group may be more actively proteolytic. The culture filtrate of the antagonist dissolves the bacteria but does not destroy their antigenic properties. The lytic substance, designated as Much-lysin, was said to have a double effect: one, bound to the living cells of the organism, had nothing to do with phage, and the other, found in the bacteria-free filtrate, had an apparent similarity to phage but was distinct from it.

The idea that in the case of bacterial antagonists one is dealing with specific strains rather than with distinct species was further emphasized by Franke and Ismet (294). Various strains of *B. mycoides,* designated as *cytoliticus,* were shown to be able to lyse many pathogenic and nonpathogenic bacteria but not their own cells; the same action was exerted by the culture filtrate (Table 12). The lytic action of strains of *B. subtilis* upon different bacteria, including *M. tuberculosis* (903), pneumococci, typhoid, diphtheria (62), and other organisms has also been definitely established.

Pringsheim (705) isolated a strain of *B. mesentericus-vulgatus* that had a decided inhibiting effect upon a variety of bacteria, particularly

Corynebacterium diphtheriae. On agar plates the antagonist produced a circular zone of inhibition, just beyond which was a ring of larger colonies, indicating a stimulating effect. It was suggested that the antagonist produced a toxin that was stimulating in small doses and injurious in larger concentrations. The active substance was thermolabile and nonfilterable. The antagonistic properties appeared to be inherent in the particular strain of an organism and were not increased by serial passage (1016). The action of filtrates of *B. mesentericus* against diphtheria organisms was considered (956) as highly specific. Other strains of this organism were reported to be active against *Pasteurella pestis* (246). Living gram-positive bacteria were found (768) to be more susceptible than gram-negative organisms to the antagonistic action of spore-forming aerobes; in the case of dead organisms, the reverse was true. Plates were heavily seeded with the test bacteria and the centers

TABLE 12. LYSIS OF PATHOGENIC BACTERIA BY VARIOUS STRAINS OF A SPORE-FORMING ANTAGONIST (CYTOLYTICUS)

ORGANISM LYSED	STRAIN NUMBER OF CYTOLYTICUS						
	I	II	III	VI	VII	VIII	IV
E. typhosa	+++	+++	+++	++	+++	o	o
Paratyphoid A	+	+++	+++	+	++	+++	+++
Paratyphoid B	o	+	+++	+	++	++	o
Shigella	++	++	++	+	++	++	o
Y bacillus	+	+++	+++	+	++	o	+
E. coli	+++	+++	+++	++	+++	o	o
C. diphtheriae	+++	++	+++	+	++	+++	+
Ps. pyocyaneus	o	++	+++	+	+++	+++	o
S. aureus	+	o	+++	o	++	+++	++
S. albus	+	+	+++	o	+++	+++	+
S. citreus	+++	+++	+++	o	+++	+++	+++
S. viridis	+	+++	+++	+	+++	+++	+++
S. haemolyticus	++	+++	+++	+	++	+++	+++
S. mucosus	+++	+++	+++	+	++	+++	+++
P. vulgaris (Weil-Felix)	++	+++	+++	+	+	+	++
Pneumococcus	+++	+++	+++	+++	+++	o	o

From Franke and Ismet (294).
o no clearing.
+ trace but no true clearing.
++ clearing, slight sediment.
+++ clearing without sediment.

of the plates inoculated with the antagonist. Inhibition of growth and lysis were used as measures of antagonistic action.

Hettche and Weber (408) isolated 41 strains of *B. mesentericus* from 25 samples of soil. These were streaked on blood agar, and the diphtheria organism was used for testing their effect. In 18 strains the antagonistic action was detected in 24 hours; there was no parallelism between inhibition and hemolysis. Of the 18 active strains, 11 lost the property after two transfers and 2 were exceedingly active.

Dubos (190) isolated from a soil enriched with various living bacteria a gram-negative, spore-bearing bacillus (*B. brevis*) that had a marked lytic effect against gram-positive bacteria, including staphylococci and pneumococci. The antagonist was grown for 3 to 4 days in shallow layers of peptone media at 37° C. The bacterial cells were removed by centrifuging, and the filtrate was acidified, giving a precipitate from which a highly active substance (tyrothricin) was isolated. Dubos and Hotchkiss (205) soon demonstrated the presence in natural substrates, such as soil, sewage, manure, and cheese, of various spore-forming bacteria that have marked antagonistic properties against various gram-positive and gram-negative bacteria.

Hoogerheide (427) isolated from the soil an aerobic, spore-forming bacterium that produced a highly active bactericidal substance; it also prevented the formation of capsules by Friedländer's bacterium. This substance appeared to be similar to gramicidin (885).

It has thus been definitely established (857) that strains of spore-forming bacteria possessing antagonistic properties are widely distributed in the soil and possess certain physiological characteristics that differentiate them from the inactive strains. This is brought out in Table 13.

Spore-forming bacteria are also able to produce substances antagonistic to fungi (231, 695, 734, 738). Cordon and Haenseler (149) isolated an organism (*B. simplex*) that was antagonistic to *Rhizoctonia solani*, an important plant pathogen. The antagonist produced a thermostable substance that inhibited the growth and even caused the death of the fungus. When the substance was added to the soil it controlled to some extent seed decay and damping-off disease of cucumbers and peas. Christensen and Davies (128) found that a strain of *B. mesentericus*

produced on artificial media an active substance that suppressed the growth of *Helminthosporium sativum*. It increased sporulation of the fungus, inhibited or retarded spore germination, caused abnormal hyphal development, and induced mutations in certain strains of the fungus. The substance was thermostable and diffusible. It passed through a Berkfeld filter, was absorbed by infusorial earth, withstood freezing and desiccation, and did not deteriorate readily. It was destroyed by alkalies but not by acids. It was inactivated or destroyed, however, by certain fungi and bacteria.

TABLE 13. BIOCHEMICAL CHARACTERISTICS OF ACTIVE AND INACTIVE STRAINS OF SPORE-FORMING SOIL BACTERIA

STRAIN	ACID PRODUCTION FROM			PRODUCTION OF H_2S	LIQUEFACTION OF GELATIN	HYDROLYSIS OF STARCH	GRAM STAIN
	Dextrose	Lactose	Sucrose				
ACTIVE STRAINS							
A-2	−	−	−	+	+	−	−
A-5	−	−	−	+	+	−	−
A-10	−	−	−	+	+	−	−
A-21	−	−	−	+	+	−	−
A-23	−	−	−	+	+	−	−
A-27	−	−	+	+	−	−	−
A-34	−	−	−	+	+	−	−
INACTIVE STRAINS							
A-15	−	−	+	−	−	+	+
A-31	−	+	+	−	+	+	+
A-32	+	+	+	−	+	+	+

From Stokes and Woodward (857).
− reaction becoming alkaline. + acid produced.

NONSPORE-FORMING BACTERIA: PS. AERUGINOSA, PS. FLUORESCENS, AND S. MARCESCENS

Among the bacteria, those belonging to the fluorescent, green-pigment and red-pigment producing groups have probably received the greatest attention as antagonists. Bouchard (81) first reported, in 1888, that *Ps. aeruginosa* was antagonistic to *B. anthracis;* the presence

of the antagonist was shown (62, 120) to reduce considerably the action of the pathogen. When grown on artificial media the pyocyaneus organism was found (299) to be strongly antagonistic to a number of bacteria, including *E. typhosa*, *Pfeifferella mallei*, *V. comma*, and *Bacterium tyrogenes*. The growth of staphylococci, micrococci, diplococci, and spore-forming rods was also reduced. The antagonist inhibited its own growth as well.

These early observations were amply substantiated (Table 14). *Ps. aeruginosa* was shown to be active against *E. coli*, *M. tuberculosis* (62, 81, 120, 760, 999), and a variety of other bacteria (522). The addition of top minnows (*Gambusia*) to water polluted with *E. coli* caused the disappearance of the bacteria; this was shown to be due to the inhibiting effect of the pyocyaneus organism present in the intestinal flora of *Gambusia*. The presence of this antagonist in water renders the colon index of the water an unreliable guide to pollution (384). When a mixture of the antagonist and the colon organism was incubated, the former tended to outgrow the latter after 24 hours (356). Even after steriliza-

TABLE 14. NONSPORE-FORMING BACTERIA AS ANTAGONISTS TO BACTERIA

ANTAGONIST	ORGANISMS AFFECTED	KNOWN PROPERTY	REFERENCES
Ps. aeruginosa	*B. anthracis*, *E. typhosa*, *V. comma*, etc.	Thermostable, filterable substance	62, 81, 120, 235, 236, 299
Ps. aeruginosa	Gram-negative bacteria, *M. tuberculosis*, and yeasts	Depresses growth	62, 81, 760, 762, 763, 999
Ps. fluorescens	*E. coli*, *S. marcescens*, *C. diphtheriae*, *B. anthracis*, etc.	Thermostable, filterable substance	246, 302, 311, 326, 406, 407, 409, 410, 430–432, 524, 525, 656, 827
Ps. fluorescens	Actinomycetes	Lytic action	552
S. marcescens	*Cl. chauvoei*, *B. anthracis*, staphylococci, micrococci	Colorless, thermostable, lytic substance	50, 209, 230, 743, 764
S. marcescens	Gram-positive but not gram-negative bacteria	Alcohol-soluble pigment	409

TABLE 14 (*continued*)

ANTAGONIST	ORGANISMS AFFECTED	KNOWN PROPERTY	REFERENCES
E. coli	Typhoid, paratyphoid, diphtheria, staphylococci, and proteolytic bacteria	Growth-inhibiting	52, 54, 61, 93, 121, 366, 491, 643, 736, 759, 876, 886, 954
E. coli	Other *E. coli* strains		643
E. coli	*M. tuberculosis* and spore-forming bacteria		105, 336, 365, 367, 448, 469, 781, 823, 898
A. aerogenes	*B. anthracis*, *P. pestis*		246, 365, 367
E. typhosa	*E. typhosa*, *Ps. fluorescens*, *E. coli*, *B. anthracis*		224, 311, 347, 898, 915
S. paratyphi	*E. coli*, *B. anthracis*, *P. pestis*		246, 453, 823, 898
Streptococci	*B. anthracis*, *C. diphtheriae*	Activity not associated with hemolysis or virulence	52, 105, 131, 178, 233, 302, 627, 676, 799, 800
Streptococci	*B. anthracis*, *Ph. tumefaciens*, *S. lactis*, *P. pestis*, *L. bulgaricus*	Thermostable, non-filterable substance	69, 246, 765, 979
Staphylococci	Dead cells of various bacteria		351, 994, 996
Staphylococci	Gram-positive bacteria, *C. diphtheriae*, *P. pestis*		52, 150, 213, 214, 246, 248, 861
Micrococci	*V. comma*, *M. tuberculosis*, *E. typhosa*, *Br. melitensis*		211, 212, 541, 627
Diplococci and pneumococci	Various bacteria	Thermolabile substance	211, 212, 244, 246, 368, 540, 541, 634, 669, 733
K. pneumoniae	*B. anthracis*, *C. diphtheriae*, *P. pestis*	Active filtrate	52, 54, 246, 302, 634, 676, 823
P. vulgaris	*B. anthracis*, *P. pestis*, *Cl. sporogenes*		40, 246, 426, 649, 898, 958
P. avicida	*B. anthracis*, *E. typhosa*		299, 672
Myxobacteria	Plant-disease-producing bacteria	Thermostable lytic substance	841
Anaerobic bacteria	*M. tuberculosis*, *B. anthracis*		425, 671

tion, media in which *Ps. aeruginosa* had grown depressed the growth of other microorganisms including *S. marcescens*, *Ps. fluorescens*, and *Saccharomyces cereviseae;* spore formation by the last was favored (762).

The specific antagonistic action of *Ps. aeruginosa* upon various bacteria was found by early investigators to be due to the production of an active heat-resistant substance (120, 299). By filtering the culture through a Berkfeld, evaporating to a small volume, dialyzing through a parchment membrane, precipitating with alcohol, and drying over sulfuric acid, Emmerich and Löw (236) obtained a preparation which was designated as pyocyanase. It was soluble in water and highly bacteriolytic.

Pyocyanase was at first looked upon as an enzyme belonging to the class of nucleases. It was found to have, even in very low concentrations, a marked destructive effect upon diphtheria, cholera, typhus, and plague organisms, as well as on pyogenic streptococci and staphylococci. It rapidly dissolved *V. comma* cells and in a few seconds rendered inactive such bacterial toxins as that of diphtheria. Since the bacteriolytic action of pyocyanase was in direct proportion to the time of its action and concentration, and in inverse proportion to the numbers of bacteria acted upon, its enzymatic nature was believed to be substantiated. The preparation withstood heating in flowing steam for 2 hours. Other proteolytic systems of bacteria are known to be resistant to high temperatures and to remain active even when kept in a moist state for 15 to 30 minutes at 100° C. (1). Pyocyanase was believed to be transformed in the bodies of animals into high molecular proteins which still retained the bacteriolytic action of the free enzyme.

Since the early work of Emmerich and Löw, an extensive literature has accumulated on the nature of pyocyanase. Its lytic effect has been established against diphtheria (52), streptococci (237), meningococci, the typhoid organism, pneumococci (66), *P. pestis* (246), *Vibrio metchnikovi* (501), *V. comma* (692), and many others (794). There has been considerable disagreement, however, concerning the chemical nature and therapeutic action of pyocyanase (234, 322), due largely to the variation in the nature of the preparations obtained (540). Kramer (501), for example, has shown that the activity of the substance depends on three factors: nature of strain, not all strains being equally

effective; composition of medium, glycerol-containing media being most favorable; and method of extraction of active substance from culture media.

The enzymatic nature of pyocyanase was not universally accepted, largely because of the thermostability of the substance (489). Dietrich (171) ascribed the action of pyocyanase to a change in osmotic pressure. Raubitchek and Russ (733) emphasized that the solubility of the substance in ether, chloroform, or benzol is not indicative of an enzyme, nor is the fact that temperatures of 0 to 37° C. fail to influence its activity (55, 185, 409, 669, 919).

Ps. aeruginosa was found to produce (409), in addition to pyocyanase, a blue pigment, pyocyanin. Both substances possess lytic properties, 1:1,000 dilution of the pigment being able to lyse *E. coli* in 6 hours (366). The pigment was believed (501) to act only on gram-positive bacteria. Pyocyanin was said (407) to be more effective in younger cultures, and pyocyanase in older.

In order to test the action of *Ps. aeruginosa* upon other bacteria, Kramer (501) placed a drop of a suspension of this organism upon a plate inoculated with *M. tuberculosis* or with *V. metchnikovi.* In 24 hours, a sterile zone surrounded the colony of the antagonist, the width of the zone depending upon the moisture content of the medium, the degree of diffusion of the active substance, its concentration, and the resistance of the test bacteria. When the two pathogens were inoculated into liquid media and the antagonist was introduced simultaneously or within 24 hours, the latter had a decided bactericidal effect. No bactericidal filtrate could be obtained. These results were confirmed (634, 919), the conclusion being reached that the active molecules do not pass through the ultrafilter (949). Pyocyanin had a bactericidal action also upon *S. hemolyticus, S. albus, S. aureus, C. diphtheriae, M. tuberculosis, V. metchnikovi,* and the Y-Ruhr bacillus, but not upon *P. vulgaris, E. coli,* or the typhoid organism. In general, gram-positive bacteria were largely affected.

More recently, Schoental (809) succeeded in isolating three antibacterial substances from the chloroform extracts of cultures of *Ps. aeruginosa:* (a) a blue pigment, pyocyanin; (b) a yellow pigment, *a*-oxyphenazine, a derivative of pyocyanin; and (c) an almost colorless

bacteriolytic substance most readily found in old culture media. None of these substances was enzymatic in nature. Schoental found that pyocyanin had a strong bactericidal activity, but its high toxicity and instability made it unpromising for therapeutic purposes. The action of *a*-oxyphenazine against many bacteria made it comparable to the flavines; however, it was less toxic and non-irritant. The third antibacterial substance had a marked lytic action on vibrios, being bactericidal in a concentration of 1:10,000 and bacteriostatic in 1:100,000.

No less extensive is the literature on the antagonistic action of the fluorescent group of bacteria, first established by Garré (311) in 1887 and later by others (50, 246, 302, 326, 430–432, 462, 524, 525, 655, 827). Hettche and Vogel (407) described the inhibiting effect of strains of this organism on the growth of various gram-negative and gram-positive bacteria (Table 15). The active substance is thermostable, dialyzes through a membrane, and passes through Seitz and Berkfeld filters (525). It is said (407) to be soluble in chloroform. Aerobic culture conditions are favorable to its accumulation. Members of this chromogenic group of bacteria were also found to be able to bring about the lysis of infusoria (123). Rahn (711) observed the phenomenon of iso-antagonism, which is associated with the formation of a thermolabile substance that does not pass through a filter. Certain bacteria were found (228, 711) to be favored by their own metabolic products, whereas others had an adverse effect; the products of the first group were usually thermostable and nonfilterable, and the second were thermolabile (at 60° to 100° C.) and were destroyed by light.

S. marcescens is known to exert antagonistic effects against a number of bacteria, including diphtheria, gonococci, anthrax (743, 764), and *Cl. chauvoei* (209), as well as fungi causing insect diseases (587). The formation of antibiotic substances by this organism has been demonstrated by various investigators. These substances are active not only *in vitro* but also *in vivo* (743). Their activity increases with the age of the culture. Their formation was believed not to be associated with the production of the pigment by the organism. They were also of a nonlipoid nature. Hettche (409), however, asserted that the bactericidal action of *Serratia* is closely related to pigment production. The pigment was extracted with alcohol and was found capable of dissolving dead gram-

TABLE 15. ANTAGONISTIC ACTION OF PS. FLUORESCENS UPON VARIOUS MICROORGANISMS

ORGANISM	PERCENTAGE OF AGED MEDIUM IN THE AGAR									
	0.5	1.0	2.5	5.0	10	15	20	30	40	50
B. cereus	–	–	+							
B. mycoides	–	–	+							
B. anthracis	–	+								
B. vulgatus	–	–	+							
B. subtilis	–	–	+							
B. megatherium	–	+								
R. cinnebareus	–	+								
R. roseus	–	–	+							
M. flavus	–	–	–	+						
N. catarrhalis	–	–	–	+						
Ps. aeruginosa	–	–	–	–	–	–	–	–	–	–
Ps. fluorescens	–	–	–	–	–	–	–	–	–	–
S. lutea	–	–	–	+						
S. marcescens	–	–	–	–	–	+				
S. albus	–	–	+							
S. aureus	–	–	–	+						
S. citreus	–	–	+							
K. pneumoniae	–	–	–	+						
V. comma	–	+								
Ch. violaceum	–	+								
E. typhi	–	–	+							
Sh. paradysenteriae	–	–	+							
S. enteritidis	–	–	–	+						
S. suispestifer	–	–	–	+						
S. pullorum	–	–	–	+						
E. coli	–	–	–	–	–	–	+			
A. acrogenes	–	–	–	–	–	–	+			
Ph. boxolesii	–	–	+							
Sac. marianus	–	–	–	–	–	–	–	–	–	–
Sac. ellipsoideus	–	–	–	–	–	–	–	+		
Sac. pastorianus	–	–	–	–	–	–	–	–	+	
Zygosac. priorianus	–	–	–	–	–	–	–	+		
Torula sphaerica	–	–	–	–	–	–	–	–	–	–
A. niger	–	–	–	–	–	–	–	–	–	–

From Lewis (525).
+ denotes complete inhibition.

positive bacteria but not gram-negative organisms. Eisler and Jacobsohn (230) ascribed the antagonistic action of *Serratia* not to the pigment but to certain water-soluble, thermostable (70° C. for 30 minutes) lytic substances.

THE COLON-TYPHOID BACTERIA

Members of the colon-typhoid group are not typical soil inhabitants, although they find their way continuously into the soil and into water basins. Various organisms belonging to this group have been said to possess antagonistic properties (425). Bienstock (54) reported, in 1899, that proteolytic bacteria are repressed by the presence of *E. coli* and *A. aerogenes*. Tissier and Martelly (886) emphasized that this phenomenon occurs only in the presence of sugar, the effect being due to the fermentation of the sugar by *E. coli*, resulting in the production of acid.

Wathelet (954) observed in 1895 that in mixed culture the colon bacterium gradually replaces the typhoid organism and this was later fully confirmed (141, 383, 491, 619, 643, 799, 800, 876). The occurrence of slowly growing lactose-fermenting strains of *E. coli* in stools has been ascribed to the phenomenon of antagonism (453), and the inhibitory action upon *E. typhosa* added to certain stools was also ascribed to the antagonistic action of *E. coli* (643). Different strains of *E. coli* repress the typhoid organism to a different extent. The ratio of the two organisms developing on agar was designated as the antagonistic index; an index of 100:20 means that for every 100 colonies of the colon organism, 20 colonies of typhoid developed. Manteufel (581), however, ascribed this antagonistic action of *E. coli* to the exhaustion of nutrients in the medium.

Active colon strains were found to be inhibitive to other strains of the same organism. The existence of strong and weak antagonistic strains has been questioned frequently (1005). Nissle (642) ascribed to many of these strains a strong antagonistic action against the pathogenic intestinal flora. These results were contested, however (98, 512). The action of *E. coli* of different origin varies (750), freshly isolated strains being more active than stock cultures (837). It has also been

reported that fresh, actively growing cultures of *E. typhosa* inhibited the growth of *E. coli*, older cultures not being antagonistic (915). The antagonistic properties of *E. coli* were often believed to be associated with the formation of unstable, thermolabile lytic substances, that would not pass through a filter (365, 367, 580).

A bacteriophage was found (532) to develop as a result of the antagonistic action of *E. coli* against the Shiga bacillus and was said to occur in the intestines where antagonistic conditions are always present. A similar effect was observed by Fabry (245), due to the antagonistic stimulus of *E. coli* by a strain of *S. albus*. Gratia (348) found that the filtrates of one race of *E. coli* inhibited another race and caused an agglutination of the latter in fluid media. According to Hashimoto (383), the weakest antagonists belong to the paracolon group, the strains of medium activity to the colon group, and the strongest antagonists to the colon-immobilis type. Whenever the feces were found to contain large numbers of *E. coli*, no typhoid organisms were present. The resistance of certain persons to intestinal diseases was, therefore, ascribed to the high antagonistic colon index. By utilizing the principle of antagonism of some strains of *E. coli* against others, two types of *E. coli* resistant to the antagonistic substance were isolated (168): one produced giant colonies, the other small punctiform, translucent colonies.

E. coli exerts an antagonistic action also upon *Salmonella schottmülleri* (305), *C. diphtheriae* (52, 905), staphylococci (366, 491), *M. tuberculosis* (680, 681), *B. anthracis* (105, 365–367, 781, 823, 898), various spore-forming soil bacteria (469), and putrefactive water bacteria (759). The action against anthrax was said to be only temporary (336). It was also suggested (448) that only living cultures of *E. coli* are active. The simultaneous inoculation of *S. aureus* and *E. coli* was found (736) to be injurious to the first and not to the second organism; this effect was increased by an increase in the number of *E. coli* cells in the inoculum. Gundel and Himstedt (366) have shown that *E. coli*, but not *A. aerogenes*, is antagonistic to *S. aureus* and *S. albus*.

The term autophage has been used (334) to designate the process of clearing a water emulsion of dead cells by a culture of an antagonist such as *E. coli*. This clearing effect was said to be due to the fact that the

dead cells are used as nutrients by the living organism. The mechanism of the action was variously explained by a change in the *p*H value of the medium or in the oxidation-reduction potential or by a direct enzymatic effect. In some cases thermolabile, filterable substances were demonstrated (141, 365, 367, 580). These substances have been considered either as autotoxins (141) or as proteolytic enzymes (683). According to Schilling and Califano (799, 800), the filtrate of *E. coli* depressed only the dysentery organism of Shiga. From a bouillon culture of *E. coli,* Gundel (370) isolated thermostable lipoids capable of bringing about the lysis of the colon bacteria and other bacteria. The antagonistic relations between *E. coli* and *V. comma* have been well established, the cholera organism also possessing antagonistic properties (307, 480).

The typhoid organism is also capable of exerting an antagonistic action against itself, as well as against *Ps. fluorescens* (311) and *E. coli* (323). Similar antagonistic effects (224) have been obtained against various other bacteria, including *B. anthracis* (823, 898). The nature of the action is not clearly understood. *Salmonella paratyphi* possesses antagonistic properties against *E. coli* (453), *B. anthracis* (898), *P. pestis* (246), and various other bacteria.

It may be of interest to record here that *E. coli* isolated from persons affected by rectal cancer are able to convert substances related to bile acids to carcenogenic bodies. The possibility of synthesis of such substances has also been suggested (186).

COCCI

Numerous cocci have been found to possess antagonistic properties against other bacteria. Doehle (178) first demonstrated in 1889 that streptococci are able to antagonize *B. anthracis,* especially on solid media. Similar action was exerted against diphtheria bacteria (52, 71, 131, 799, 800), a phenomenon apparently not correlated with the hemolytic properties or the virulence of the antagonist. Further studies established the effect of various streptococci against anthrax (233, 366, 676). According to Cantani (105) this effect is more pronounced in liquid than in solid media, and is highly specific as regards the strain. *S. pyogenes* was shown to be antagonistic, *in vivo,* to *B. anthracis* and to

Phytomonas tumefaciens, even to the extent of suppressing vegetative malformations brought about by the latter (69). *Streptococcus cremoris* was active against *Streptococcus lactis* (979), *Streptococcus mastidis* against *S. lactis* and *Lactobacillus acidophilus*, and *Streptococcus mucosus* against *P. pestis* (246). Rogers (765) reported an antagonistic effect of *S. lactis* against *L. bulgaricus*; the active substance was thermostable and was unable to pass through a bacterial filter (150, 178, 669, 676).

Freudenreich (299) first emphasized the antagonistic action of staphylococci against various bacteria. The list was later enlarged to include gram-positive acid-resisting forms (211), corynebacteria (52, 213, 214), and the plague organism (246). Some of these antagonists were found to be able to lyse the dead cells of their own kind (351, 994–996) as well as those of various other organisms. Gundel (370) isolated from staphylococci an active lipoid which had bactericidal properties. A water-soluble, alcohol-insoluble substance, said to be an enzyme capable of bringing about the lysis of corynebacteria, was isolated from a strain of staphylococcus (213, 214).

Various micrococci possess strong antagonistic properties. Löde (541) isolated a micrococcus which affected a variety of microorganisms three or more centimeters away, the active substances being dialyzable. An organism related to *Micrococcus tetragenus* and described as *Micrococcus antibioticus* (211) was found to possess a strong antagonistic action against *V. comma*, *M. tuberculosis*, *E. typhosa*, *Ph. tumefaciens*, *Br. melitensis*, various spore-forming bacteria, numerous cocci, and others (627). Diplococci exerted an antagonistic action against various bacteria (541), including pyogenic staphylococci and streptococci in the sputum (363), spore-formers, and gram-negative bacteria (447). They produced, under aerobic conditions only, a filterable substance that was heat resistant.

The antagonistic action of pneumococci has definitely been established (212, 244, 246, 368, 571, 634, 669, 733). The active substance of these organisms was said (541) to be thermolabile, since it was destroyed at 80° to 85° C.; it was produced only under aerobic conditions. In reviewing the literature on the longevity of streptococci in symbiosis, Holman (425) observed that many chances of error are inherent in mixed cultures, particularly with closely similar organisms; pneumo-

cocci, for example, were found to be able to live for long periods together with nonhemolytic streptococci. Peculiar antagonistic relations between pneumococci and staphylococci were also reported (15). Adaptive alterations could be expected in the growth of bacteria in mixed cultures (31). Which of the two organisms antagonizes the other was believed to depend frequently upon the numerical abundance of one or the other (244).

OTHER AEROBIC AND ANAEROBIC BACTERIA

The antagonistic action of *Klebsiella pneumoniae* against *B. anthracis* has been reported (216, 676, 823). Freudenreich (299) found that the filtrate of this antagonist repressed the growth of a number of bacteria including the diphtheria (52, 634) and plague (246) organisms.

Other aerobic bacteria were found capable of exerting antagonistic effects against one or more organisms, these effects varying considerably in nature and intensity. It is sufficient to mention the action of *P. vulgaris* against *B. anthracis* and *P. pestis* (246, 898); of *Ps. aviseptica* against *B. anthracis* (672) and *E. typhosa* (299); of *Bacterium lactis aerogenes* against *B. anthracis* (365, 367) and *P. pestis* (246). *B. anthracis* is capable of iso-antagonism (781, 827) and of antagonizing certain other organisms, including *E. typhosa* and *Bacterium acidi lactici* (299, 827). Certain Myxobacteriales have been shown (841) to be capable of bringing about the lysis of various plant-disease-producing bacteria; a thermostable lytic substance, passing through cellophane but not through a Seitz filter, was obtained. Although certain bacteria like *Achromobacter lipolyticum* were found capable of reducing the pathogenicity of *M. tuberculosis*, no active cell-free extract could be obtained (82).

The morphology of one bacterium may be considerably modified by the presence of another. Living cultures of *L. bulgaricus* influenced the variation of *E. coli* from the "S" to the "R" phase, inhibited development of the organism, and even brought about its lysis. No active substance could be demonstrated; the lactic acid itself had only a limited effect (11). Korolev (499) has shown that when a yellow sarcina was added to solid media a stimulating effect was exerted on the growth of

species of *Brucella* (*Br. melitensis, Br. abortus, Br. suis*); in liquid media, however, the activities of these species were repressed, the sarcina thus acting as an antagonist. A white staphylococcus exerted an antagonistic action on *Brucella* species both in liquid and on solid media.

Certain acid-producing aerobes were found capable of inhibiting toxin production by *Clostridium botulinum* in glucose but not in noncarbohydrate media (372). Since acid itself cannot bring about this effect, Holman (426) suggested that the acid must be active in a nascent state. A mixture of a *Clostridium sporogenes* and *Cl. botulinum* also interfered with the development of the toxin; it was even thought possible that the first anaerobe might cause the disappearance of toxin already produced (158, 159, 463). *S. aureus, E. coli, P. vulgaris,* and other bacteria permitted the growth of *Cl. botulinum* in aerobic cultures, accompanied by toxin production (291). However, *Streptococcus thermophilus* inhibited the growth of *Cl. botulinum,* the toxin of the latter being gradually destroyed (478).

Passini (671) claimed that *Bacillus putrificus verrucosus* destroyed *M. tuberculosis* in nine days. The effect of other anaerobes on the survival of anthrax spores in dead animals has been extensively studied (425). Novy (649) reported that the injection into guinea pigs of *P. vulgaris* and *Clostridium oedematiens* resulted in rapid death of the animals and extensive growth of the anaerobe in the animal bodies; however, the simultaneous inoculation of *Cl. sporogenes* and *P. vulgaris* did not result in putrid lesions (426). According to Barrieu (40), the presence of *P. vulgaris* and certain nonpathogenic spore-bearing aerobes in wounds favors, through their proteolytic activity, the virulence of pathogenic bacteria. Pringsheim (705) grew *Cl. welchii* with *Alkaligenes fecalis* for ten generations on agar slants and could easily detect in the growth of the latter the opaque colonies of the anaerobe. A liquefying sarcina allowed *Cl. welchii* and *Clostridium butyricum* to grow in open tubes. Weinberg and Otelesco (958) believed that many war-wound infections are due to an association of *P. vulgaris* with anaerobes, since the former increased the virulence of *Clostridium perfringens* and others.

The antagonistic effects of lactic acid bacteria of the *L. bulgaricus* and *L. acidophilus* groups have received considerable attention (76), espe-

cially in regard to their action against intestinal bacteria. This was believed to be due to the production of acid by the bacteria rather than to the formation of specific antagonistic substances (590). This phenomenon aroused particular interest because of the function of some of these organisms in replacing bacterial inhabitants of the human digestive system (497).

CHAPTER 6

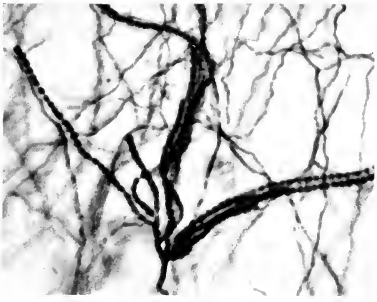
ACTINOMYCETES AS ANTAGONISTS

ACTINOMYCETES are found in large numbers in many natural substrates. They occur abundantly in soils, composts, river and lake bottoms, in dust particles, and upon plant surfaces. Certain species are capable of causing serious animal and plant diseases.

Actinomycetes, like fungi, produce a mycelium, but they are largely unicellular organisms of dimensions similar to those of bacteria. Some of the constituent groups are closely related to the bacteria, others to the fungi. On the basis of their morphology, the order *Actinomycetales* has been divided into three families, *Mycobacteriaceae*, *Actinomycetaceae*, and *Streptomycetaceae*, comprising the genera *Mycobacterium*, *Actinomyces*, *Nocardia*, *Streptomyces*, and *Micromonospora*. These genera are represented in nature by many thousands of species, of which several hundreds have been described. A few are shown in Figure 10.

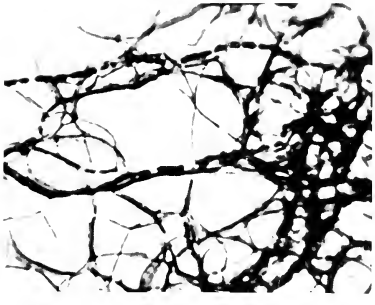
Comparatively little is known of the physiology of actinomycetes. Some produce certain organic acids from carbohydrates (287, 1002); others prefer proteins and amino acids as sources of energy, many species being strongly proteolytic. Some are able to attack starch, with the production of dextrins and sugar, accompanied by the formation of diastatic enzymes. Many reduce nitrates to nitrites. Some attack sucrose and form the enzyme invertase; others, however, do not. Certain species are able to utilize such resistant compounds as rubber and lignin. Synthetic media are favorable for the production of a characteristic growth and pigmentation. Among the pigments, the melanins have received particular attention. They range from the characteristic brown to various shades of black and deep green and are formed in protein-containing and in some cases also in protein-free media. The other pigments range from blue, yellow, and orange to various shades of grey.

According to Beijerinck (43), the process of pigment production by actinomycetes in gelatin media is associated with the formation of a quinone, which turns brown at an alkaline reaction and in the presence of oxygen. The action of quinone in the presence of iron was found to

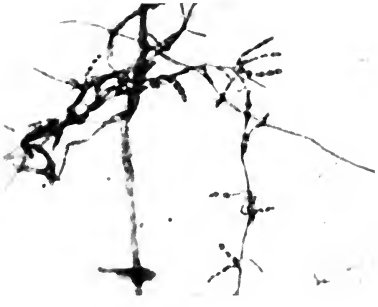


S. antibioticus, important antagonist. From Waksman and Woodruff (945)

S. lavendulae, important antagonist



Submerged growth of *S. lavendulae*. From Woodruff and Foster (1002)



Thermophilic *Streptomyces*. From Waksman, Umbreit, and Cordon (944a)

M. vulgaris. From Waksman, Cordon, and Hulpoi (931)

Streptomyces 3042, showing close spiral type of branching. Prepared by Starkey

FIGURE 10. Types of actinomycetes.

be similar to that of the enzyme tyrosinase. Since an excess of oxygen is required for the formation of quinone, only limited amounts are found in deep cultures. The quinone is believed to be formed from the peptone in the medium; although good growth was produced on media containing asparagine, KNO_3, and ammonium sulfate as sources of nitrogen, only traces of quinone, if any, were found. The tyrosinase reaction is not involved in the production of all black pigments by actinomycetes (945); some species produce such pigments in purely synthetic media, in the complete absence of peptone.

Actinomycetes grow in liquid media in the form of flakes or small colonies, usually distributed either on the bottom and walls of the container or throughout the liquid; often a ring is formed on the surface of the medium around the wall of the vessel. In many cases, a full surface pellicle is produced, which may be covered with aerial mycelium. As a rule, the liquid medium does not become turbid, even in the presence of abundant growth. When grown on solid media, actinomycetes form small, compact, soft to leathery colonies; a heavy lichen-shaped mat is produced that may become covered by an aerial mycelium. The addition of a small amount of agar (0.25 per cent) to a liquid medium is highly favorable to growth, especially in large stationary containers.

Actinomycetes can also be grown in liquid media in a submerged condition, with suitable agitation and aeration in order to supply oxygen; the medium may also be kept in shaken state (287, 926, 1001, 1002). Growth occurs in the form of a homogeneous suspension of discrete colonies and mycelial fragments throughout the liquid. Responses in growth and biochemical activities as a result of treatments may thus be obtained under more homogeneous physiological conditions.

Although most actinomycetes are aerobic, some are anaerobic, and many can grow at a reduced oxygen tension. The aerobic actinomycetes commonly found on grasses and in soil are said (490) never to have been isolated from animal infections. Mixed infections consisting of anaerobes growing at body temperature together with aerobes have often been demonstrated. Certain aerobic species also are capable of causing infections in man and other animals, and certain plant diseases (potato scab, sweet potato pox) are caused by aerobic species of actinomycetes.

ANTAGONISTIC PROPERTIES

Many actinomycetes have the ability to antagonize the growth of other microorganisms, notably bacteria, fungi, and other actinomycetes; this is brought out in Tables 16 and 17. The antagonistic species are not limited to any one genus but are found among three genera, *Nocardia*, *Streptomyces*, and *Micromonospora*.

Gasperini (317) first demonstrated, in 1890, that certain species of the genus *Streptomyces* had a marked lytic effect upon other microorganisms. He emphasized that "*Streptothrix* develops habitually in a spontaneous manner upon the surface of bacteria and fungi, upon which it lives to a limited extent in the form of a parasite, due to the faculty that its mycelium possesses to digest the membrane from these lower fungi." Greig-Smith (360, 361) found that soil actinomycetes are able to antagonize not only bacteria but also certain fungi; since actinomycetes grow abundantly in normal soils, it was suggested that they may become an important factor limiting bacterial development. Lieske (527) demonstrated that specific actinomycetes are able to bring about the lysis of many dead and living bacterial cells; they are selective in their action, affecting only certain bacteria such as *S. aureus* and *S. pyogenes*, but not *S. lutea*, *S. marcescens*, or *Ps. aeruginosa*.

Rosenthal (767) isolated from the air an actinomyces species which he designated as the true biological antagonist of the diphtheria organism. He inoculated the surface of an agar plate with an emulsion of the bacteria and inoculated the actinomyces into several spots. At the end of two days, the plate was covered with the diphtheria organisms, but the colonies of the actinomyces were surrounded by large transparent zones. In another method utilized, agar was mixed with an emulsion of the diphtheria bacteria killed by heat, and the mixture was poured into plates. After solidification of the medium, the antagonist was inoculated in several spots on the plates. Its colonies gradually became surrounded by clear zones, thus proving that it produced a lytic substance that diffused through the agar and dissolved the diphtheria cells.

Gratia and Dath (350) suspended dead cells of staphylococci and other bacteria in 2 per cent agar and exposed the plates to the air. A cul-

TABLE 16. ANTAGONISTIC PROPERTIES OF VARIOUS ACTINOMYCETES

ANTAGONIST	ORGANISMS AFFECTED	KNOWN PROPERTY	REFERENCES
S. albus	Pneumococci, streptococci, staphylococci, *Ps. aeruginosa*, etc.	Thermolabile substance, causes lysis of dead cells	347, 350, 971–973
S. albus	Various fungi	Protein, enzyme, causes lysis of dead and certain living bacteria	12–14
S. antibioticus	All bacteria and fungi, especially gram-positive types	Thermostable substance, bacteriostatic	947
S. lavendulae	Various gram-positive and gram-negative bacteria	Organic base, water-soluble	950
S. praecox	*S. scabies*		604, 605
Streptomyces sp.	Bacteria and fungi	Lytic action	317
Streptomyces sp.	Diphtheria	Growth inhibition	767
Streptomyces sp.	*B. mycoides*, proactinomycetes, mycobacteria	Bactericidal action, with or without lysis	80, 504
Streptomyces sp.	*Fusarium*	Lytic action	595
N. gardneri	Gram-positive bacteria	Bacteriostatic action	309, 936
Micromonospora	Gram-positive bacteria	Thermostable active substance produced	936
Actinomycetes	Dead and living bacteria	Lysis	527
Actinomycetes	Spore-forming bacteria	Repression of growth	360, 970
Actinomycetes	Gram-positive bacteria	Thermostable substance, produced on synthetic media, resembles lysozyme	507, 628
Actinomycetes	*Pythium*	Thermostable substance	884

ture of a white actinomyces developed on the plates, each colony being surrounded by a clear zone of dissolved bacterial cells. By transferring this culture to a suspension of dead staphylococci in sterile saline, a characteristic flaky growth was produced, the bacterial suspension be-

TABLE 17. ANTIBACTERIAL SPECTRUM OF CERTAIN ANTAGONISTIC ACTINOMYCETES

TEST ORGANISM	ZONE OF INHIBITION, IN MILLIMETERS			
	S. violaceus	*S. aurantiacus*	*S. griseus*	*S. globisporus*
N. rubra	35	32	0	0
N. corallina	40	45	22	10
N. alba	40	25	0	0
M. rubrum	40	33	10	0
M. citreum	38	37	0	0
M. tuberculosis	8	10	0	0
M. smegmae	10	8	0	0
M. phlei	20	25	0	0
Corynebacterium sp.	12	10	0	0
E. coli	0	0	0	0
S. aureus	25	19	0	0
M. ruber	35	28	0	0
B. mycoides	30	10	0	0
B. megatherium	33	5	0	0
B. mesentericus	30	2	0	0
B. subtilis	23	1	0	0
B. tumescens	22	0	0	0
Ps. fluorescens	0	0	0	0
Ps. aeruginosa	0	0	0	0
P. vulgaris	0	0	0	0
S. marcescens	0	0	0	0
M. luteus	30	25	0	0
M. candicans	37	22	0	0
M. roseus	42	27	0	0
M. lysodeikticus	38	33	0	0
S. lutea	30	27	0	0
Az. vinelandii	3	0	0	0
Az. chroococcum	5	0	0	0
Rh. leguminosarum	0	0	0	0
Radiobacter	0	0	0	0

From Krassilnikov and Koreniako (504).

coming clarified in 36 hours. When the lysed emulsion was filtered, the filtrate could again dissolve a fresh suspension of dead staphylococci. This culture was found able to attack all staphylococci tested as well as certain other gram-negative bacteria, such as *Ps. aeruginosa;* however, it was inactive toward *M. tuberculosis* and *E. coli.* Some antagonistic strains could also attack *E. coli,* though this property was readily lost.

This type of antagonism was believed to be widely distributed in nature and to be directed against many bacteria, pathogenic and saprophytic. The culture of the antagonist in bouillon gave a very active agent, whereas the lysed bacterial suspension was weaker in its action. The active substance was present extensively in old cultures and was fairly stable. The material obtained by lysing the suspension of bacteria by means of an antagonist was designated as "mycolysate." It did not possess the toxicity of the nonlysed suspension but it preserved its antigenic properties (349). Gratia (347) also reported that actinomycetes were able to attack living cells of bacteria, except *E. coli* and *E. typhosa* which had to be first killed by heat before they could be dissolved.

Welsch (972, 973) made a detailed study of the lytic activity of an actinomyces culture, presumably identical with the one employed by Gratia and later described as *Actinomyces albus.* The culture was grown in different media, the best results being obtained in very shallow layers of ordinary bouillon. The active substance present in the filtrate was designated as "actinomycetin." It was able to dissolve, at least partly, all dead bacteria, whether killed by heat or by chemicals, gram-positive or gram-negative, though gram-negative bacteria were, as a rule, more susceptible. The growing culture of the antagonist brought about better clarification (lysis) of the bacterial suspension than the filtrate. The solubilizing properties of the active agent, its susceptibility to heat and to ultraviolet rays, its size as measured by ultrafiltration, suggested its protein nature. The kinetics of its action pointed to its being an enzyme (971). It was precipitated by acetone, alcohol, and ammonium sulfate. Most of the gram-negative bacteria were not attacked either by actinomycetin or by the living culture of the antagonist. Only a few of the gram-positive bacteria, including certain pneumococci and streptococci, could be dissolved by sterile actinomycetin. A definite parallelism in the activity of the preparation against dead bacteria and of the living

culture against living bacteria suggested that the same substance is concerned in both cases. The bacteria were therefore divided (970), on the basis of their relation to actinomycetin, into three groups:

Bacteria that were lysed by the culture filtrate; these included pneumococci and hemolytic streptococci

Bacteria that were not dissolved even by the most active soluble substance, but which were depressed by the mycelium of the living actinomyces; these comprised various sarcinae and fluorescens types

Bacteria that were not acted upon by either the living culture or the actinomycetin preparation; these included the colon-typhoid and the pyocyaneus groups, though when the latter were killed by heat or inactivated by radium emanations, as in the case of *E. coli*, or were placed under conditions unfavorable to multiplication, they were dissolved by the lytic substance.

The first detailed survey of the distribution of antagonistic organisms among actinomycetes was made by a group of Russian investigators. According to Borodulina (80), actinomycetes are able to antagonize various spore-forming bacteria and to bring about the lysis of their living cells. A thermostable substance was produced on agar media. The activity of this substance was greatly reduced at an alkaline reaction, whereas an acid reaction favored it. When *B. mycoides* and an antagonist were inoculated simultaneously into peptone media, no inhibitive effect was obtained, because the bacterium changed the reaction of the medium to alkaline, thereby making conditions unfavorable for the production of the antibiotic substance by the antagonist. When the antagonist was first allowed to develop in the medium, before the bacterium was inoculated, a strong antagonistic effect resulted, which led to the elongation of the vegetative cells of *B. mycoides;* this was due to a delay in fission and was accompanied by the suppression of spore formation.

Krassilnikov and Koreniako (504) found that many species of actinomycetes belonging to the genus *Streptomyces* but not *Nocardia* produced a substance that possessed a strong bactericidal action against a large number of microorganisms. This substance was particularly active against nocardias, mycobacteria, and micrococci; it was less active upon

spore-bearing bacteria and had no action at all on nonspore-forming bacteria, as illustrated in Table 17. Under the influence of the antibiotic factor, the microbial cells were either entirely lysed or killed without subsequent lysis. The action upon spore-bearing bacteria was bacteriostatic but not bactericidal. The nonspore-forming bacteria, including species of *Rhizobium* and *Azotobacter*, not only were not inhibited but were actually able to develop in filtrates of the antagonists.

Nakhimovskaia (628) found that antagonistic actinomycetes are widely distributed in nature. Of 80 cultures isolated from different soils, 47 possessed antagonistic properties, but only 27 secreted antibiotic substances into the medium (Table 18). These agents were capable of inhibiting the growth of gram-positive but not of gram-negative bacteria or fungi. The nature of the action of the various antagonists

TABLE 18. OCCURRENCE OF ANTAGONISTIC ACTINOMYCETES IN DIFFERENT SOILS

NATURE OF SOIL	TOTAL STRAINS OF ACTINOMYCETES TESTED	NUMBER OF ANTAGONISTIC STRAINS	STRAINS WHICH LIBERATED TOXIC SUBSTANCES
Chernozem	24	10	9
Podzol	11	7	3
Solonets	4	4	4
High altitude soil	9	6	5
Sandy soil	6	5	1
Dry desert soil	5	4	1
River bank meadow	14	7	2
Cultivated soil	7	4	2
Total	80	47	27

From Nakhimovskaia (628).

was found not to be identical. Some secreted water-soluble substances into the medium, others did not. All the antibiotic agents were thermostable, since heating for 30 minutes at 1.5 atmospheres only reduced somewhat their activity. For those antagonists which did not excrete any substance into the medium, the presence of the growing antagonist was essential in order to bring about an inhibition of bacterial develop-

ment. On the basis of their sensitivity to the antibiotic substance of actinomycetes, mycobacteria could be differentiated from nonspore-forming, especially nodule-forming, bacteria. The production of the antibiotic substance was highest in synthetic media and was rather weak or even totally absent in media that contained proteins. The substance was filterable and was able to resist the effect of radiation.

It was further reported (628) that the antagonistic effects of actinomycetes were manifested not only in artificial media, but also in soil, the interrelations here being much more complex. Some of those strains that produced antagonistic effects in artificial nutrient media were ineffective under soil conditions. The antagonistic action was more intense in light podzol soils and was greatly reduced in heavy or chernozem soils. One of the factors that resulted in a decrease in the antagonistic properties of actinomycetes in the heavy soils was apparently the high content of organic matter. By adding peptone to a light soil, the antagonistic action of the actinomycetes was greatly weakened. When actinomycetes were allowed to multiply in the soil before inoculation with *B. mycoides*, the antagonistic effect was highly pronounced even in the presence of high concentrations of peptone.

An attempt to isolate an antibiotic substance from some of the soil actinomycetes was made by Kriss (507). On the basis of its properties, he was led to conclude that this substance could be classified definitely with lysozyme. It was insoluble in ether, petroleum ether, benzol, and chloroform, and was resistant to the effects of light, air, and high temperatures. The optimum reaction for the production of this substance by *Streptomyces violaceus* was found to be *p*H 7.1 to 7.8, the activity not being increased by selective cultivation. On the basis of its properties, this substance could hardly be classified with egg-white lysozyme. It must be concluded also that the differences in the antibiotic properties of the various antagonistic actinomycetes isolated by the Russian investigators definitely point to the fact that more than one antibiotic substance was involved.

In a more recent survey (936) of the distribution of antagonistic actinomycetes in soils and in composts, it was found that of 244 cultures isolated at random from different soils, 49, or 20 per cent, of the cultures were actively antagonistic; 57, or 23 per cent, showed some antagonistic

properties; and 138, or 57 per cent, possessed no antagonistic action at all (Table 19). A somewhat similar distribution of antagonistic properties was observed among a group of well-identified species taken from a type culture collection, embracing 161 pure strains. Only one of the

TABLE 19. ISOLATION OF ANTAGONISTIC ACTINOMYCETES FROM VARIOUS SUBSTRATES

SOURCE OF ORGANISMS	TOTAL CULTURES ISOLATED	GROUP I		GROUP II		GROUP III		GROUP IV	
		Cultures	Percentage of total	Cultures	Percentage of total	Cultures	Percentage of total	Cultures	Percentage of total
Fertile, manured, and limed soil	74	20	27.0	5	6.8	1	1.3	48	64.9
Infertile, unmanured, limed soil	75	11	14.7	8	10.7	4	5.2	52	69.3
Potted soil	13	1	7.7	1	7.7	0	0	11	84.6
Potted soil, enriched with *E. coli*	21	1	4.8	4	19.0	4	19.0	12	57.2
Potted soil, enriched with mixtures of bacteria	15	12	80.0	2	13.3	0	0	1	6.7
Lake mud	9	3	33.3	4	44.4	0	0	2	22.2
Stable-manure compost	37	1	2.7	20	54.0	4	10.8	12	32.4
Total	244	49	20.1	44	18.0	13	5.3	138	56.6

From Waksman, Horning, Welsch, and Woodruff (936).
Note. The organisms in group I were the most active antagonists, those in groups II and III had more limited antagonistic properties, and those in group IV showed no antibacterial effects with the methods used.

members of the genus *Nocardia* proved to be antagonistic; only one of the *Micromonospora* forms was active. Most of the antagonists were found among the members of the genus *Streptomyces*. These cultures were also examined for bacteriolytic properties, living *S. aureus* being used as the test organism. On this basis, 87 cultures (53.1 per cent)

were found to be inactive, 53 cultures (32.3 per cent) were moderately active, and 24 cultures (14.6 per cent) were highly active. The conclusion was reached (970) that bacteriolytic activities against killed bacteria and living gram-positive bacteria are widely distributed among the actinomycetes. Growth-inhibiting properties of actinomycetes were found to be significantly associated with bacteriolytic action upon living gram-positive bacteria (Table 20).

TABLE 20. BACTERIOLYTIC AND BACTERIOSTATIC PROPERTIES OF VARIOUS SPECIES OF ACTINOMYCETES

ORGANISMS	BACTERIOLYSIS BY LIVING ORGANISMS*	BACTERIOLYSIS BY BROTH FILTRATE†	BACTERIOSTASIS OF B. SUBTILIS BY AQUEOUS EXTRACTS
GROUP I. ACTINOMYCETES HIGHLY BACTERIOSTATIC TO B. SUBTILIS			
S. antibioticus	o	–	++
S. californicus	+	c	o
S. candidus	++	C, S	++
S. cellulosae	+	c	o
S. griseus (3326b)	+	c	o
S. lavendulae	+	c	++
S. reticuli	+	c	o
S. roseus	+	C	+
S. ruber	+	–	o
S. saprophyticus	++	C, S	o
S. scabies (3031)	+	c	o
Streptomyces sp. (3069)	++	c	o
S. albus (G)	++	C, S	o
Streptomyces sp. (3387)	++	C, S	o
N. gardneri	o	c	++
Micromonospora sp.	o	–	++
GROUP II. ACTINOMYCETES MODERATELY BACTERIOSTATIC TO B. SUBTILIS			
S. albus (3361)	++	C, S	o
S. cretaceus	+	c	o
S. albus, var. *ochraleuceus*	++	C, S	o
S. annulatus	+	–	o
S. aureus	+	c	o
S. bovis	+	c	o
S. fradii	++	C, S	o

TABLE 20 (*continued*)

ORGANISMS	BACTERIOLYSIS BY LIVING ORGANISMS*	BACTERIOLYSIS BY BROTH FILTRATE†	BACTERIOSTASIS OF B. SUBTILIS BY AQUEOUS EXTRACTS
GROUP II (*continued*)			
S. griseus	++	C, S	o
S. halstedii	+	c	o
S. hominis	++	C, S	o
S. lipmanii	+	c	o
S. microflavus	+	c	o
S. odorifer	++	–	o
S. praecox	+	c	o
S. rutgersensis	++	C, S	o
S. sampsonii	++	C, S	o
S. scabies (3352)	+	–	o
S. scabies (3021)	++	C	o
S. setonii	++	C, S	o
S. tetanusemus	++	C, S	o
S. coelicolor (3033)	+	Not tested	Not tested
Streptomyces sp. (Lieske, No. 23)	++	C, S	o
Streptomyces sp. (Lieske, No. 25a)	++	C	o

From Waksman, Horning, Welsch, and Woodruff (936).
* No activity against *S. aureus* is indicated by o, moderate activity by +, high activity by ++.
† Lysis of heat-killed *E. coli* is indicated by C (high activity) and c (moderate activity); lysis of living *S. aureus* is indicated by S; – indicates no activity.

Actinomycetes also show antagonistic activities against fungi (12–14, 844). *S. albus*, for example, was capable of inhibiting the growth of all species of fungi tested, an effect shown to be due to the production of an active substance. By the use of a culture of *Colletotrichum gloeosporioides*, the antagonistic activities of 80 type cultures of actinomycetes were measured. The antagonist was allowed to grow for 5 days on maltose agar, at *p*H 7.4, and the fungus was then inoculated. The cultures of actinomycetes were divided, on this basis, into three groups: strong, weak, and noninhibitors. The first group comprised 17.5 per cent of the cultures; the second, 38.8 per cent; and the third, 43.7 per cent. These results are surprisingly similar to those reported for the distribution of actinomycetes possessing antibacterial properties, includ-

ing those that were isolated at random from the soil and those taken from a culture collection.

Meredith (595) made a survey of the distribution of organisms antagonistic to *Fusarium oxysporum cubense* in Jamaica soils; most of these antagonists belong to the actinomycetes. The antagonists were not evenly distributed in the various soil samples, 10 of the 66 samples giving 44 per cent of the antagonistic organisms. Those actinomycetes that were antagonistic to *Fusarium* when grown in their own soil-solution agar were not always antagonistic when tested in soil-solution agar prepared from other soil. A culture of actinomyces isolated from a compost produced lysis of the *Fusarium*. When spores of both organisms were mixed in an agar medium, the fungus developed normally for two days but began to undergo lysis on the fifth day, large sections of the mycelium disappearing. On the seventh day only chlamydospores were observed. In 9 days the fungus completely disappeared, the actinomyces making a normal growth.

NATURE OF ANTIBIOTIC SUBSTANCES

It has already been established that antagonistic actinomycetes readily produce a variety of different types of antibiotic substances. Some of these have been isolated and even crystallized and information has been gained concerning their chemical nature. Others have been obtained in the form of crude but highly active preparations. Still others are known but they have not been isolated as yet and have, therefore, been rather insufficiently studied. So far, six substances have been definitely recognized; namely, actinomycetin, actinomycin, streptothricin, streptomycin, proactinomycin, and micromonosporin.

Among the various antagonistic actinomycetes, five species have been studied in detail and, therefore, deserve particular attention, namely, *S. antibioticus* (945), *Streptomyces lavendulae* (973), *Streptomyces griseus* (795), *Nocardia gardneri* (309), and *S. albus* (970).

S. antibioticus produces two highly active antibiotic substances that have been isolated and described as actinomycin A and B. The first of these has been studied in greater detail. It was shown to be antagonistic to all species of bacteria tested as well as to many fungi, as brought out

in Table 21. The organism produces dark-brown to black pigments on media containing protein and peptone. It is not affected by heat. It is soluble in ether and in alcohol as well as in other solvents, but in water only in very high dilutions. It is highly toxic to animals. Actinomycin

TABLE 21. BACTERIOSTATIC SPECTRUM OF ACTINOMYCIN

ORGANISM	GRAM STAIN	ACTINOMYCIN ADDED, MILLIGRAMS PER LITER OF MEDIUM			
		0.1	1.0	10	100
S. marcescens	−	3	3	3	3
A. aerogenes	−	3	3	3	3*
E. coli (intermediate)	−	3	3	3	3*
E. coli	−	3	3	3	1*
Ps. aeruginosa	−	3	3	3	0
Ps. fluorescens	−	3	3	3	0
Br. abortus	−	3	3	3	0
N. catarrhalis	−	3	3	2	0
E. carotovora	−	3	3	2	0
Sh. gallinarum	−	3	2	2	0
A. stutzeri	−	3	2	1	0
H. pertussis	−	3	3	0	0
Az. vinelandii	−	3	0	0	0
S. cellulosae	+	3	2	1	0
S. californicus	+	3	3	2	0
M. tuberculosis	+	3	3	0	0
Cl. welchii	+	3	0	0	0
B. macerans	+	3	3	0	0
B. megatherium	+	3	0	0	0
B. polymyxa	+	3	0	0	0
B. mycoides	+	1	0	0	0
B. mesentericus	+	1	0	0	0
B. cereus	+	1	0	0	0
B. subtilis I	+	0	0	0	0
B. subtilis II	+	0	0	0	0
G. tetragena	+	0	0	0	0
S. lutea	+	0	0	0	0
Streptococci and staphylococci	+	0	0	0	0

From Waksman and Woodruff (946).

Note. 0 indicates no growth; 1, trace of growth; 2, fair growth; 3, good growth.

* If 200 mg. per liter were added the results were usually as follows: for *A. aerogenes*, fair; for *E. coli* (intermediate), trace; for *E. coli*, no growth.

is produced in both organic and synthetic media, the addition of a small amount of agar increasing considerably the growth of the organism and the production of the active substance. The addition to the medium of a small amount of starch, as well as of phosphate and sodium chloride, was also found to be favorable. *S. antibioticus* is strictly aerobic, and is able to produce the active substance only under aerobic conditions that can be brought about by growing it either in very shallow layers or in aerated or agitated submerged growth. Actinomycin is extracted directly from the medium by means of ether; the ether is then evaporated and the substance taken up in alcohol. Further purification is accomplished by means of petrol ether and passage through a chromatographic column, as shown later (p. 171).

Streptomyces lavendulae is capable of inhibiting the growth of many gram-negative bacteria as well as of various gram-positive forms. The antibiotic substance produced by this organism was designated as streptothricin. The organism is grown in a tap-water medium containing 1.0 per cent glucose, 0.5 per cent tryptone, 0.2 per cent K_2HPO_4, 0.2 per cent NaCl, 0.001 per cent $FeSO_4$, and 0.25 per cent agar. The glucose

TABLE 22. COMPARATIVE ACTIVITY OF TWO STRAINS OF S. LAVENDULAE

STRAIN NUMBER	SOURCE OF NITROGEN	TREATMENT OF CULTURE	DAYS OF INCUBATION	GROWTH IN MG. PER 100 ML. OF MEDIUM	ACTIVITY IN UNITS *E. coli*	ACTIVITY IN UNITS *B. subtilis*
8	Tryptone	Shaken	2	346	150	1,000
14	Tryptone	Shaken	2	361	150	750
8	Tryptone	Shaken	5	253	100	1,000
14	Tryptone	Shaken	5	296	100	500
8	Glycine	Shaken	2	162	30	30
14	Glycine	Shaken	2	146	30	30
8	Glycine	Shaken	5	266	100	500
14	Glycine	Shaken	5	271	30	150
8	Tryptone	Stationary	8	245	20	200
14	Tryptone	Stationary	8	–	75	300
8	Glycine	Stationary	8	239	25	150
14	Glycine	Stationary	8	–	75	200

From Waksman (926).
Note. The organism was grown in 1 per cent starch medium.

can be replaced by starch, in which case the presence of agar is unnecessary. When grown in submerged or agitated cultures, the agar is left out. The tryptone can be replaced by a variety of simple nitrogenous compounds, such as glycine, alanine, aspartic acid, asparagine, and glutamic acid (Table 22); the carbohydrate may be left out completely, with only limited reduction in activity. No growth of the organism is obtained on tryptophane, phenyl alanine, and certain other forms of nitrogen. With ammonium sulfate or sodium nitrate good growth may be obtained but the production of the active substance is limited unless the organism is grown under submerged conditions. Iron appears to play an essential role in the production of the active substance. An increase in growth as a result of an increase in carbohydrate concentration does not result in an increase in streptothricin content, but an increase in growth as a result of an increase in the amino-acid concentration, with the same amount of carbohydrate, causes an increase in the production of streptothricin. When the medium contains one amino acid as the only source of carbon and nitrogen, there is a gradual increase in the alkalinity of the medium, resulting in the destruction of the streptothricin. Neither the growth of the organism nor the production of the streptothricin, however, is influenced by the reaction of the medium, within certain limits, even between *p*H 4.4 and 8.0 (1001). The metabolism of *S. lavendulae* and the course of production of streptothricin under stationary and submerged conditions are illustrated in Figure 11. Methods of isolation of streptothricin and its chemical properties are described later (p. 173). Its bacteriostatic spectrum or action against various bacteria is shown in Table 23. It has only limited toxicity to animals and is active *in vivo* against both gram-positive and gram-negative bacteria (755).

Certain strains of *Streptomyces griseus* produce an antibiotic substance, designated as streptomycin, that is also active against both gram-positive and gram-negative bacteria. It is similar in its solubility and various chemical properties to streptothricin; however, it acts readily against *B. mycoides* and is more active than the latter against certain gram-negative bacteria, such as *Ps. aeruginosa*. The organism grows well in stationary liquid media containing meat extract or corn steep. Streptomycin is active *in vivo* against a variety of bacteria, some of

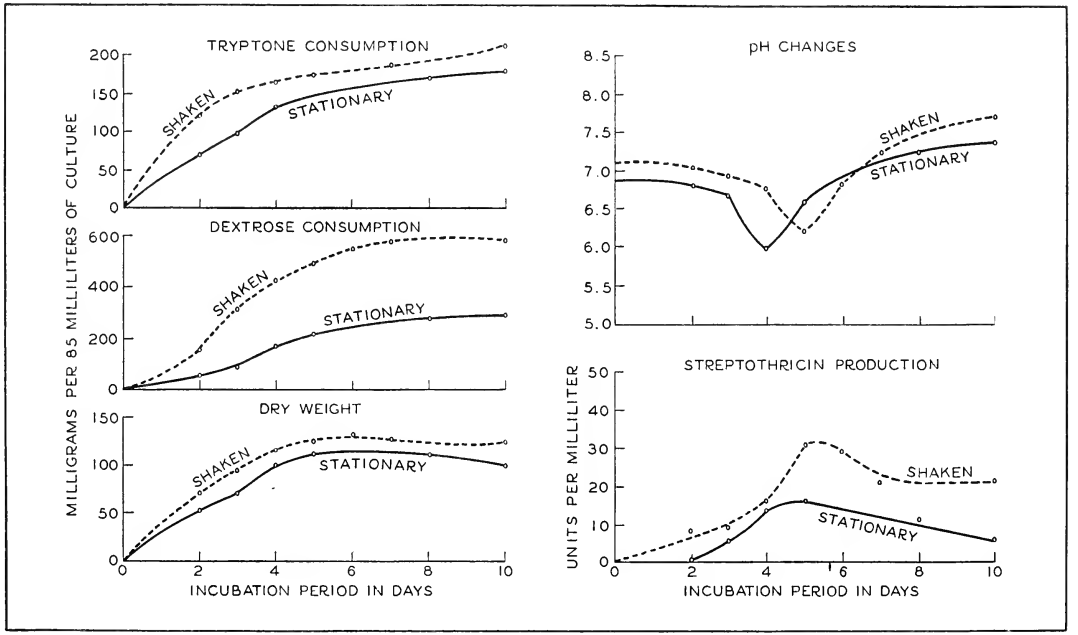


FIGURE 11. Metabolism of *S. lavendulae* and production of streptothricin. From Woodruff and Foster (1002).

TABLE 23. INHIBITORY EFFECT OF STREPTOTHRICIN UPON GROWTH OF VARIOUS BACTERIA

ORGANISM	CRUDE STREPTOTHRICIN ADDED, MILLIGRAMS PER 10 CUBIC CENTIMETERS AGAR					
	3	1	0.3	0.1	0.03	0.01
B. subtilis	0	0	0	0	0	1
B. mycoides	2	2	2	2	2	2
B. macerans	2	2	2	2	2	2
B. megatherium	0	0	0	0	1	2
B. polymyxa	0	0	2	2	2	2
B. cereus	2	2	2	2	2	2
M. lysodeikticus	0	0	0	1	2	2
S. muscae	0	0	0	1	2	2
S. lutea	0	0	0	0	1	2
*A. aerogenes**	0	0	1	2	2	2
A. aerogenes	0	0	0	Tr	2	2
E. coli†	0	0	0	0	2	2
E. coli (4348)	0	0	Tr	1	2	2
S. marcescens	0	1	2	2	2	2
S. marcescens	1	1	2	2	2	2
Ps. fluorescens‡	2	2	2	2	2	2
Sh. gallinarum	0	0	0	0	1	2
P. pseudotuberculosis	0	0	0	Tr	2	2
Br. abortus	0	0	0	0	2	2
S. cholerasuis	0	0	0	Tr	2	2
S. schottmülleri	0	0	0	1	2	2
S. abortivoequina	0	0	0	Tr	2	2
S. typhimurium	0	0	0	2	2	2
H. suis	0	0	0	2	2	2
H. influenzae	0	0	0	0	0	1
Br. abortus	0	0	0	0	2	2
Az. agile	0	0	0	0	0	2
Az. vinelandii	0	0	0	0	0	2
Az. chroococcum	0	0	0	Tr	2	2
Az. indicum	0	0	0	2	2	2
M. phlei	0	0	0	1	2	2
Cl. butyricum§	2	2	2	2	2	2
L. casei§	0	0	0	2	2	2
S. albus	0	0	0	1	2	2
S. violaceus-ruber	0	0				
S. lavendulae	0	1	2	2	2	2

From Waksman and Woodruff (950).
Note. 0 indicates no growth; 1, limited growth; 2, good growth; Tr, trace of growth.
* Representing 3 distinct strains.
† Representing 5 strains of *E. coli* obtained from different sources.
‡ Representing 4 strains.
§ Cultured anaerobically.

which, like *Ps. aeruginosa*, are rather resistant to streptothricin (460a, 795).

N. gardneri produces an active bacteriostatic substance which has been designated as proactinomycin (309). Its bacteriostatic spectrum is shown in Table 24. It is produced both on synthetic and organic media. Its action is largely directed against gram-positive bacteria, although to a more limited extent than that of actinomycin. Its isolation and chemical nature are brought out later (p. 175).

TABLE 24. BACTERIOSTATIC EFFECT OF PROACTINOMYCIN

ORGANISM	APPROXIMATE DILUTION OF MATERIAL IN MILLILITERS GIVING HIGHEST EFFECT
D. pneumoniae	1,500,000
S. pyogenes	500,000
S. aureus	500,000
N. meningitidis	500,000
B. anthracis	500,000
V. cholerae	6,000
S. typhi, S. paratyphi B, Shigella, E. coli	2,000

From Gardner and Chain (309).

S. albus produces a bacteriolytic substance designated as actinomycetin; it has been described on page 107. This substance is a protein and is enzymatic in nature. It has not yet been isolated in a pure state. Its lytic action was visualized by Welsch (971) as a two-step reaction: first, the susceptible cells are killed by the selectively bactericidal lipoid; second, those dead cells are dissolved by the bacteriolytic enzyme, which alone is responsible for the lysis of heat-killed bacteria. The phenomenon does not take place in complex culture-media, since the bactericidal action of the lipoid is greatly impaired under those conditions; the presence of living actinomyces is generally necessary, since free lipoid should be secreted in the susceptible suspension.

Wieringa and Wiebols (981) observed that certain actinomycetes can produce lytic agents that are capable of exerting a lytic effect not only upon the actinomycetes themselves but also upon other organisms.

The formation of an autolytic substance by a thermophilic actinomyces was also demonstrated (477, 502). The filtrates of such lysed cultures were said to offer promise in the treatment of actinomycosis caused by *Actinomyces bovis* (175).

Despite a seeming similarity in their growth characteristics, and despite the fact that some investigators (349, 973) assumed that all actinomycetes are able to act as antagonists, it is now definitely established (504, 945) that one is dealing here with highly specific types or even strains. For example, an examination of many species for an active substance similar to actinomycin brought out the fact that only *S. antibioticus* was capable of producing this substance. Although many other forms yielded an ether-soluble substance that had some bacteriostatic activity, it could not be compared in chemical nature and in biological action with actinomycin (944, 946).

ANTAGONISTIC EFFECTS OF ACTINOMYCETES AGAINST AGENTS PRODUCING PLANT DISEASES

Various species of *Streptomyces* are also strongly antagonistic against bacteria causing plant diseases, such as *Bacterium solanacearum* (414). According to McCormack (552), aerobic conditions are necessary for the development of the antagonistic properties of actinomycetes; those requiring less oxidized conditions are themselves antagonized. *B. megatherium*, for example, was said to be antagonistic to certain species but was antagonized by others. *Ps. fluorescens*, however, was antagonistic to actinomycetes as a whole, causing their lysis.

Actinomycetes possess antagonistic properties not only against bacteria but also against other actinomycetes (552). The more aerobic species are antagonistic to the less aerobic types. Millard (604) believed that he succeeded in controlling potato scab caused by *Streptomyces scabies* by the use of green manures such as grass cuttings. The development of scab on potatoes grown in sterilized soil and inoculated with *S. scabies* was reduced by the simultaneous inoculation of the soil with *Streptomyces praecox*, an obligate saprophyte (605). By increasing the proportion of the latter organism to the pathogen, the degree of scab-

bing on the test potatoes was reduced from 100 per cent to nil. The sterilized soil provided sufficient nutrients for the development of the antagonist and only a small increase in the control was obtained when grass cuttings were added and sterilized along with the soil.

Sanford (782) was unable, however, to control potato scab by the inoculation, with *S. scabies* and *S. praecox*, of both steam-sterilized and natural soil containing different amounts of green plant materials. These organisms were perfectly compatible on potato dextrose agar, as well as in a steam-sterilized soil medium. The control of scab (605), therefore, was said to have been due not to the direct action of *S. praecox* but to certain other undetermined microorganisms favored by the presence of the green manure. *S. scabies* was found (782) to be very sensitive to various products of fungi and bacteria. When grown in close proximity to various bacteria, the acid production of the latter inhibited *S. scabies* to a considerable degree. Its complete inhibition was not due to the acid reaction alone, however, since a certain bacterium was isolated from the soil which definitely inhibited the growth of this plant pathogen.

Goss (342) observed that the severity of scab is dependent on the amount of *S. scabies* present in the soil, which was believed to be controlled by the soil microflora. No evidence was obtained as to whether the effect of the soil flora on *S. scabies* was due to specific organisms. Kieszling (481) isolated two cultures of bacteria which were antagonistic to *S. scabies;* when added to the soil, these bacteria prevented the development of scab on potatoes.

The ability of antibiotic substances produced by actinomycetes to exert a marked inhibiting effect upon plant pathogenic bacteria has been established (930).

IN VIVO ACTIVITY OF SUBSTANCES PRODUCED BY ACTINOMYCETES

Just as the chemical nature of the antibiotic agents produced by actinomycetes varies, so does the action of these agents in the animal body. Some, like actinomycin, are very toxic, whereas others, like streptothricin and streptomycin, have low toxicity and give great promise of prac-

tical application. Because of the activity of streptothricin and streptomycin against gram-negative bacteria (460a, 597, 752) and because of the lack of reliable chemotherapeutic agents active against these bacteria, the utilization of these substances in the treatment of certain diseases caused by such bacteria becomes very significant (p. 243). Some preparations, like actinomycetin, have been utilized in the preparation of a bacterial hydrolysate (mycolysate) for vaccination purposes.

CHAPTER 7

FUNGI AS ANTAGONISTS

THE antagonistic interrelationships in which fungi are involved comprise the following reactions: (a) the antibacterial activities of fungi; (b) the antagonistic effects of fungi upon fungi; (c) the effects of bacteria and actinomycetes upon fungi; (d) the action of fungi upon insects and other animal forms. From the point of view of practical utilization, two aspects deserve special consideration: (a) the utilization of fungi for combating human and animal diseases; (b) the antagonistic interrelationships of fungi with other organisms, since fungi comprise the most important group of microorganisms that cause plant diseases.

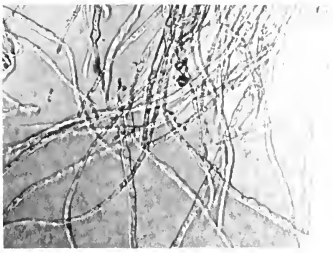
ANTIBACTERIAL EFFECTS OF FUNGI

Duchesne (207) was the first to report, in 1897, that certain green Penicillia are capable of repressing the growth of various bacteria or of bringing about their attenuation. Vaudremer (912) demonstrated in 1913 that the presence of *A. fumigatus* results in the attenuation of the cells of *M. tuberculosis*.

Since these early studies a number of fungi have been found to possess antibacterial properties; this phenomenon has sometimes been spoken of as mycophagy (914). Several fungi have been studied in detail, and in some cases one or more antibiotic substances have been isolated (Figure 12). The property of inhibiting the growth of bacteria is not characteristic of any one genus or even species, but of certain strains within a given species. Some organisms produce more than one active substance. Two genera, *Penicillium* and *Aspergillus*, have been found to comprise a large number of antagonistic forms. Several other genera are also known to contain organisms that possess antibacterial properties; very few of these, however, were ever found among the Phycomycetes and Basidiomycetes. The known fungi capable of producing antibiotic substances may be divided (934) into the following ten groups:

Aspergillus clavatus
A. flavus-oryzae
A. fumigatus
Penicillium cyclopium-claviforme

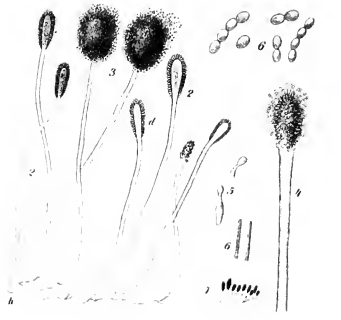
P. notatum, sporulating bodies (x530). Prepared by Foster



P. notatum, submerged growth (x530). Prepared by Foster

P. citrinum. From Biourge (54a)

P. chrysogenum. From Biourge (54a)



A. clavatus. From Wehmer (955)

A. fumigatus. From Wehmer (955)

FIGURE 12. Some typical fungi producing antibiotic substances.

TABLE 25. ANTAGONISTIC EFFECTS OF SOME REPRESENTATIVE FUNGI AGAINST BACTERIA

ANTAGONIST	ORGANISMS AFFECTED	ACTIVE SUBSTANCE	REFERENCES
A. clavatus	Gram-negative and gram-positive bacteria	Clavacin, highly bactericidal	935, 942, 982
A. flavus	Streptococci, staphylococci, and certain gram-positive bacteria	Aspergillic acid	461, 708, 978
A. flavus	Mostly gram-positive bacteria	Flavicin, similar to, if not identical with, penicillin	100, 565, 929
A. fumigatus	Gram-positive bacteria	Fumigacin, gliotoxin	95, 933, 935
A. fumigatus	Various bacteria	Fumigatin, spinulosin	663
A. fumigatus and *A. albus*	*M. tuberculosis*	Active filtrate	1015
Chaetomium sp.	Various gram-positive bacteria	Chaetomin	934
Gliocladium and *Trichoderma*	Various gram-positive and gram-negative bacteria	Gliotoxin, highly bacteriostatic	948
P. citrinum	Various bacteria	Citrinin	714
P. claviforme	Gram-positive and gram-negative bacteria	Claviformin	114, 115
P. notatum and *P. chrysogenum*	Mostly gram-positive and also certain gram-negative (*Neisseria, Gonococcus*) bacteria	Penicillin, active *in vivo*, low toxicity	3, 7, 79, 135, 266, 424, 737, 934
P. notatum	All bacteria tested, in presence of glucose	Notatin, penatin, penicillin B, *E. coli* factor	59, 151, 493, 494, 751, 934
P. puberulum and *P. cyclopium*	Various bacteria	Penicillic acid	56, 57, 661, 664
P. resticulosum	Various bacteria	Crude metabolic product	58
Penicillium sp.	Gram-negative as well as gram-positive bacteria	Penicidin	26

Penicillium luteum-purpurogenum	*Fusarium-Cephalosporium*
Penicillium notatum-chrysogenum	*Chaetomium* and other Ascomycetes
Trichoderma-Gliocladium	Miscellaneous other fungi

A comparative study of a number of fungi taken from a culture collection brought out (986) the fact that about 40 per cent of the Aspergilli (*Aspergillus fumaricus, A. fumigatus, Aspergillus schiemannii, Aspergillus terreus*) and 25 per cent of the Penicillia (*Penicillium chrysogenum, Penicillium claviforme, Penicillium funiculosum, Penicillium expansum*) possessed antagonistic properties. Out of many Phycomycetes tested, only *Phythophthora erythroseptica* showed some activity. A few Ascomycetes were also found to be active. A summary of the antibacterial properties of various fungi and of the antibiotic substances produced by them is given in Table 25. In addition to the specific strain of the organism, the composition of the medium and the conditions of growth, especially aeration, are most important in controlling the amount and nature of the antibiotic substance produced by the organism, as shown in Table 26. The fact that different strains of

TABLE 26. EFFECT OF AERATION, AS ILLUSTRATED BY DEPTH OF MEDIUM, ON ANTIBACTERIAL ACTIVITY OF SEVERAL FUNGI

CULTURE	VOLUME OF MEDIUM PER ONE-LITER FLASK, IN MILLILITERS	ACTIVITY IN UNITS *E. coli*	*B. mycoides*	*B. subtilis*	*S. lutea*
Chaetomium sp.	100	0	20	20	600
A. fumigatus 20	100	0	300	150	800
A. fumigatus 20	300	0	300	60	800
A. fumigatus 84	100	0	600	300	>1,000
A. fumigatus 84	300	0	300	100	>1,000
P. luteum 108a	100	0	0	0	0
P. luteum 108a	300	0	0	20	10
P. notatum F	100	0	3	15	
P. notatum F	700	10	10	>100	
P. notatum W	100	0	45	70	70
P. notatum W	700	100	80	450	150

From Waksman and Horning (934).
Note. Cultures were incubated 5 to 6 days at 28° C.

the same organism when grown under identical conditions vary greatly in the production of the antibiotic substance is brought out in Table 27.

Penicillium notatum-chrysogenum Group

Because of the production by these organisms of penicillin, which has already found a wide practical application, this group of fungi deserves first consideration. Fleming (265) first observed that a fungus culture growing on a staphylococcus plate brought about destruction of the bacteria, as shown by the fact that the colonies became transparent and were undergoing lysis. The fungus was isolated in pure culture and was later identified as *P. notatum*. It was found to possess marked bacteriostatic and bactericidal properties for some of the common pathogenic bacteria, largely the gram-positive cocci and the staphylococci, the streptococci, the diphtheria organism, and the gonococci and meningococci;

TABLE 27. PRODUCTION OF CLAVACIN BY FIFTEEN STRAINS OF ASPERGILLUS CLAVATUS

STRAIN NUMBER	*p*H OF MEDIUM		ACTIVITY OF 5-DAY-OLD CULTURE IN E. COLI UNITS	CLAVACIN ISOLATED	
	5 days	14 days		Yield in grams per liter	Activity, *E. coli* units per gram
120	6.5	8.4	0	0.016	25,000
121	4.2	6.7	75	1.442	1,000,000
122	4.5	8.0	0	0.035	4,000
123	4.6	4.5	20	0.467	120,000
124	6.2	8.4	0	0.016	8,000
125	3.2	3.9	0	0.248	600
126	6.3	8.2	0	0.039	20,000
127	7.4	8.1	0	0.007	8,000
128	6.7	8.0	0	0.017	7,000
129	3.6	6.8	100	0.950	400,000
129T	6.6	5.9	20	0.512	80,000
130	4.8	4.7	10	0.323	500,000
130T	6.9	7.9	10	0.050	4,000
131	6.9	7.8	10	0.035	5,000
164	4.3	4.6	30	0.430	1,000,000

From Waksman and Schatz (942).
Note. Eight-day-old culture was used for extraction of the clavacin.

bacteria belonging to the colon-typhoid–dysentery group were not affected. The culture filtrate of the fungus was found to contain an active substance, which was designated as penicillin.

A glucose-nitrate solution was used as the basic medium for the production of penicillin. It was supplemented with yeast-extract or corn steep liquor, or brown sugar was employed in place of glucose; the growth of the organism and the production of the antibacterial substances were thus greatly facilitated (7, 282, 804). The reaction of the medium was found to change from slight acidity initially (*p*H 6 to 7) to distinct acidity (*p*H 3.0), followed later by alkalinity, finally reaching a *p*H of 8.0 or even 8.8. A faint to deep yellow color is produced in the medium. Penicillin production is usually at its maximum at about *p*H 7 and may remain constant for several days or may fall again rapidly. Aerobic conditions are essential for the formation of penicillin. Once a fungus pellicle has been produced, the medium can be replaced several times, giving fresh lots of penicillin in about half the time required during the initial growth period. Crude penicillin cultures are capable of inhibiting the growth of staphylococci in dilutions of 1:800; recently, even more active preparations were obtained.

There is considerable variation in sensitivity to penicillin among bacteria belonging to the same group: 27 strains of enterococci and 6 strains of *S. lactis* were shown to be resistant to the action of this agent, whereas 13 strains of *S. viridans* were susceptible (79). The ability of a strain to resist the action of penicillin can be greatly increased by successive transfers of the culture to media containing this substance (564, 566).

Chain et al. (113) were the first to succeed in isolating from the culture medium of *P. notatum* a water-soluble, stable, brown powder which had marked antibacterial activity. This preparation inhibited, in dilutions of 1 to several hundred thousand, the growth of many aerobic and anaerobic bacteria. The active material was relatively nontoxic to laboratory animals. Intravenous and subcutaneous injections of 10 mg. or more to mice had little or no effect. The material was active *in vivo*, subcutaneous injections saving the lives of mice injected intraperitoneally with *S. pyogenes* or *S. aureus*. Intramuscular infections of mice with *Cl. septicum* were also successfully treated by repeated subcutaneous injections of penicillin.

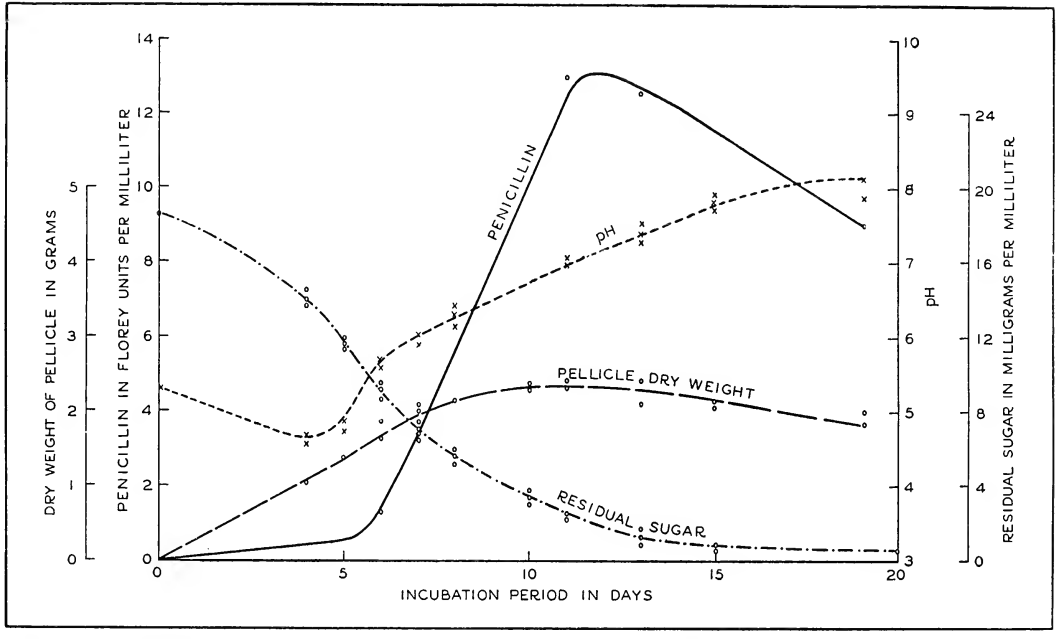


FIGURE 13. Metabolism of *P. notatum* and production of penicillin. From Foster, Woodruff, and McDaniel (289).

An extensive literature soon began to accumulate on the production (5, 117, 118, 164, 282, 332, 422), isolation (113, 469), and identification (6, 8) of penicillin. The course of its formation in the culture of the organism is illustrated in Figure 13. Conditions of nutrition were found to be particularly important. Preparations having an activity of 2,000 Oxford units or 100,000,000 dilution units have been obtained. The importance of the dual nature of *P. notatum* (the culture being composed of two distinct cell constituents) must be recognized for maximum penicillin production (34, 376). The low toxicity of penicillin, its solubility in water, and its *in vivo* activity make it an ideal agent for combating disease caused by gram-positive bacteria (p. 232).

In addition to true penicillin, *P. notatum* was found to produce another substance, which in glucose-containing media is active against not only gram-positive but also gram-negative bacteria. It was designated as the *E. coli* factor, penatin, notatin, and penicillin B (p. 179).

P. notatum represents an extremely variable group of organisms, some of the strains producing considerable penicillin, others producing little penicillin but large amounts of notatin. Some strains of a closely related fungus, *P. chrysogenum*, are also capable of producing penicillin that is apparently the same as the penicillin of *P. notatum*. The *P. notatum-chrysogenum* group of fungi is widely distributed in nature, having been isolated from different soils (919) and from various moldy food products; however, only a few strains produce enough penicillin to justify their use for the commercial production of this substance (732). Members of the *A. flavus* group of fungi, as well as strains of *A. niger*, *Aspergillus nidulans*, *A. oryzae*, *Penicillium citreo-roseum* (282), *A. giganteus* (688), *A. parasiticus* (142), and others, are also capable of producing penicillin or closely related compounds.

Among the other fungi that produce antibiotic substances largely active against gram-positive bacteria may be listed *Aspergillus flavipes* (976), *Chaetomium cochliodes*, and others.

Certain species of *Penicillium* are also capable of producing other antibacterial substances, namely, citrinin, penicillic acid, and claviformin (p. 181), the first of which is also produced by certain species of *Aspergillus* belonging to the *candidus* group (883).

Atkinson (26) tested 68 cultures of *Penicillium* and found that 18 possessed antibacterial properties. These cultures were divided into two groups: first, those largely active against gram-positive bacteria and producing substances like penicillin and citrinin; second, those active also against gram-negative bacteria and producing substances of the penicillic acid and penicidin types.

Aspergillus flavus-oryzae Group

The *A. oryzae* members of this group possess only limited antagonistic properties. Many of the *A. flavus* strains, however, have apparently the property of producing at least two antibacterial substances when grown on suitable media and under suitable conditions.

White and Hill (978) isolated from cultures of a strain of this organism grown on tryptone media a crystalline substance, aspergillic acid, that showed antibacterial activity against certain gram-negative as well as gram-positive bacteria. The substance was produced when the organism was grown on organic media, but not on synthetic. It was soluble in ether, alcohol, acetone, or acetic acid, but not in petroleum ether; it was soluble in dilute acid or alkaline aqueous solutions, and was precipitated by phosphotungstic acid. Aspergillic acid proved to have relatively high toxicity, and showed no protective action against hemolytic streptococci or pneumococci infections in mice.

Glister, in England, isolated a strain of *A. flavus* (330) that also produced an antibacterial agent with a wide range of activity, both gram-positive and gram-negative bacteria being inhibited by the culture filtrate. An extract was obtained that inhibited the growth of these bacteria in a dilution of approximately 1:200,000.

Jones, Rake, and Hamre (461) demonstrated that *A. flavus* of White produces frequent variants; two strains were isolated and were found to give consistently far higher yields of the antibiotic substance, aspergillic acid, than those reported by White. The substance was found to have wide activity, being very active against gram-positive cocci and less active against the anaerobes of gas gangrene and the gram-negative bacilli. No significant differences were found in the spectrum of activity as shown by filtrates or by solutions of purified aspergillic acid.

Bush and Goth (100) isolated from *A. flavus* a second substance designated as flavicin. They grew the organism for 6 to 8 days on a nitrate-glucose medium containing 2 per cent corn steep. The filtrate was acidified to *p*H 2.5 to 3.0 with 5 μ phosphoric acid and extracted with purified isopropyl ether. The ether was treated with a slight excess of 0.2*N* $NaHCO_3$ (5 to 10 cc. per liter of culture), giving a yield of 75 to 100 per cent of active material obtained. Purification was obtained by acidification of the $NaHCO_3$ extract with 5 μ H_3PO_4 to *p*H 2 to 3 and removal of the precipitate, the latter containing most of the toxicity (due no doubt to aspergillic acid) and the filtrate most of the activity. The filtrate was treated with ice-cold isopropyl ether, saturated with CO_2, washed with cold distilled water, and reextracted. The combined extracts were distilled at 0° C. to dryness under CO_2. A yellow-orange glassy residue was obtained. It had a low toxicity and was active *in vivo*. In its properties it resembled penicillin.

McKee and MacPhillamy (565, 567) further established, by chemical isolation and composition, solubility and stability, biological behavior, low toxicity to animals, and therapeutic activity, that the second antibiotic substance produced by *A. flavus* is similar to penicillin. A sodium salt, assaying 240 O.U./mg. was obtained chromatographically and gave the following composition: 45.36 per cent C, 4.16 per cent H, 3.02 per cent N, and 13.36 per cent Na, $[\alpha]_D = +108°$ C. (in water).

Waksman and Bugie (929) have shown by means of the bacteriostatic spectrum that, under submerged conditions, different strains of *A. flavus* produced two substances, one comparable to aspergillic acid and the other to penicillin. Some strains produced little or no activity in submerged cultures, and most strains produced very little activity in stationary cultures. No activity was produced in synthetic media. The production of gigantic acid by a species of *A. giganteus* and parasiticin by *A. parasiticus* (142) appears to be comparable to that of flavicin.

Aspergillus fumigatus Group

Four antibacterial substances were isolated from strains of *A. fumigatus:* the two pigments, spinulosin and fumigatin (663), which are not selective in their action against bacteria, the colorless fumigacin

that is active largely against gram-positive organisms (935) and gliotoxin (331, 593). Helvolic acid, isolated from a strain of *A. fumigatus* (116, 155) was found (593, 933) to be identical with purified fumigacin.

Fumigacin is active against *S. aureus* in dilutions of 1:200,000 to 1:750,000 and is very stable. The pigment fumigatin, however, was said to deteriorate on standing, inhibition of *S. aureus* being reduced from 1:50,000 to 1:25,000 in 7 days. Fumigacin has a certain degree of resistance to high temperatures. Boiling in aqueous solution for 5 to 10 minutes reduced but did not destroy completely its activity. Heating at 80° C. for 15 minutes reduced the activity only slightly. When fumigacin was dissolved in alcohol and precipitated by addition of nine volumes of water, the alcohol-water solution was found to contain 0.25 mg. per ml. A comparison of the antibacterial activity of fumigacin with that of the other substances produced by *A. fumigatus* is given in Table 28.

TABLE 28. CHEMICAL PROPERTIES AND BACTERIOSTATIC ACTIVITY OF FOUR ANTIBIOTIC SUBSTANCES PRODUCED BY ASPERGILLUS FUMIGATUS

SUBSTANCE	CRYSTALLIZATION	MELTING POINT ° C.	FORMULA	BACTERIOSTATIC ACTIVITY IN DILUTION UNITS		
				E. coli	*S. aureus*	*B. subtilis*
Spinulosin	Purplish-bronze plates	201	$C_8H_8O_5$	–	–	–
Fumigatin	Maroon-colored needles	116	$C_8H_8O_4$	1,200	200,000	40,000
Fumigacin	Very fine white needles	215–220	$C_{32}H_{44}O_8$	1,200	2,000,000	100,000
Gliotoxin	Elongated plates	195	$C_{13}H_{14}O_4N_2S_2$	6,000	1,500,000	750,000

Vaudremer (912) reported that a group of patients suffering from tuberculosis were treated with extracts of *A. fumigatus*, with varying degrees of success. The disease-producing organism (*M. tuberculosis*) was rendered nonpathogenic by such treatment.

Aspergillus clavatus Group

This comprises a number of strains that produce highly active antibiotic substances. By treating the culture filtrate with charcoal and eluting the active substance with ether, Wiesner (982) obtained a preparation having a bactericidal potency in dilutions of 1:100,000. This activity was not inhibited by serum, pus, or urine; strains of bacteria that proved to be resistant to sulfonamides or mandelic acid were inhibited by this material.

The active substance was designated (935) as clavacin. A detailed study was made of its production by a variety of strains of *A. clavatus*. The substance was found to be active against *E. coli* and other gram-negative bacteria, as well as against gram-positive bacteria. It is distinct, in this respect, from fumigacin. Whereas the latter acts much more readily upon *B. mycoides* than *B. subtilis*, clavacin shows the opposite effect—greater activity against *B. subtilis* than against *B. mycoides*. Clavacin possesses a high bactericidal action, as compared with other antibiotic substances. It has been suggested (942) that the marked differences in the physiology of the different strains of *A. clavatus* explain the differences in the production of clavacin by different strains. Those that change the reaction of the medium to alkaline, for instance, tend to inactivate the clavacin (Table 27).

Since clavacin is produced by several fungi, this substance has received a number of designations, including patulin produced by *Penicillium patulum* (713), claviformin by *P. claviforme* (114, 115), and clavatin (47). It is also produced by strains of *P. expansum*, *A. giganteus*, *Gymnoascus*, and other fungi (470).

Trichoderma and Gliocladium Group

Certain strains of fungi of the genera *Trichoderma* and *Gliocladium* were found to exert a marked antagonistic action against various fungi and bacteria. An antibiotic substance designated as gliotoxin was isolated and found (962) to be highly bactericidal. In order to produce this substance, the fungus is grown in a submerged condition in shake-cultures. An abundant supply of oxygen and a high acidity (*p*H 5.0 or lower) are essential. Ammonium salts as nitrogen sources give better

results than peptone or nitrates. Glucose and sucrose were found to be good carbon sources. It is of particular interest to note that whereas penicillin and flavicin are produced in media containing complex organic materials as sources of nitrogen, fumigacin, clavacin, and gliotoxin are produced in synthetic media, the presence of complex nitrogen sources often being deleterious.

Gliotoxin was isolated from the culture filtrate by the use of lipoid solvents, chloroform being most effective. Nonsterilized media adjusted to *p*H 2.5 to 3.0 could be used for large-scale production, the high acidity reducing the effect of contaminants (966). Gliotoxin is stable in neutral and acid solutions at room temperature; at alkaline reactions, it is very unstable, the rate of decomposition increasing with increasing alkalinity and temperature. At *p*H 2.4, heating to 122° C. for 30 minutes did not affect the active substance. With decreasing acidity, especially at *p*H 5.0, it became less thermostable. As pointed out above, gliotoxin is also produced by *A. fumigatus* (593).

Other Groups

Various other fungi, including *A. albus*, *A. niger*, and *Monilia albicans*, were found (1015) to exert a marked antibacterial action against human and bovine tubercle bacteria; active filtrates were obtained, but the specific agents were not isolated. The nature of the antibiotic substances produced by the other groups listed above has not been sufficiently studied.

ANTAGONISTIC ACTION OF FUNGI AGAINST FUNGI

Numerous fungi were found to exert antagonistic effects either against fungi belonging to the same species (64, 87, 152, 304, 547, 578, 801, 851) or against other fungi (Table 29). This phenomenon is particularly important in connection with the study of plant diseases (160, 225, 644, 695, 738, 815, 851, 852, 959, 1011). The effects are selective in nature. The hyphae of *Peziza* will kill various Mucorales, whereas different species of *Aspergillus* and *Penicillium* are able to kill *Peziza*

(738). A single spore of *P. luteum* was found (955) capable of germinating in cultures of *Citromyces*, and of bringing about their destruc-

TABLE 29. ANTAGONISTIC INTERRELATIONSHIPS AMONG DIFFERENT FUNGI

ANTAGONIST	ORGANISMS AFFECTED	REFERENCES
Acrostalagmus sp.	*Rhizoctonia*	964
Alternaria tenuis	*Ophiobolus*	88
A. clavatus	Various fungi	928
A. flavus	*Peziza*	738
A. niger	*Peziza, Rhizoctonia*	738, 911, 964
Botrytis allii	*Monilia, Botrytis*, etc.	911
Botrytis cinerea	*Rhizoctonia*	964
Cephalothecium roseum	*Helminthosporium*	353
Cunninghamella elegans	*Monilia*	911
Fusarium lateritium	*Rhizoctonia*	964
Fusarium sp.	*Deuterophoma*	790
Gliocladium sp.	*Helminthosporium, Mucor*, etc.	695
Helminthosporium sp.	*Colletotrichum, Fusarium, Botrytis*, etc.	695
H. teres	*Fusarium, Ustilago, Helminthosporium*, etc.	695
H. sativum	*Ophiobolus*	88
Mucor sp.	*Ophiobolus, Mucor*	88, 801
Penicillium sp.	*Peziza, Rhizoctonia*, etc.	738
Penicillium sp.	*Ophiobolus, Fusarium*, etc.	88, 955
Peziza sclerotiorum	*Mucor, Trichothecium, Dematium*, etc.	738
Peziza trifoliorum	*Peziza*	738
Sclerotium rolfsii	*Helminthosporium*	695
Sterigmatocystis sp.	*Alternaria*	695
Thamnidium elegans	*Mucor*	801
Torula suganii	*Aspergillus, Monascus*, etc.	654
Torulopsis sp.	Blue-staining fungi	592
Trichoderma lignorum	*Rhizoctonia, Armillaria, Phytophthora*, etc.	962–964
T. lignorum	*Rhizoctonia, Pythium*, etc.	17, 60, 91, 911
Verticillium sp.	*Rhizoctonia*	964

From Novogrudsky (644).

tion. *P. luteum-purpurogenum* produces a thermostable substance, soluble in ether and in chloroform, that is antagonistic to the growth and acid production of *A. niger* (668). *Coniophora cerebella* was inhibited by a species of *Penicillium*, its mycelium being considerably modified; however, in time the former organism adapted itself to the latter and overgrew it, its rate of growth being eventually more rapid than that of a pure culture (377). Certain fungi are able to parasitize other fungi: *Piptocephalis*, for instance, attacks various species of *Penicillium* and *Aspergillus* (176). The germination of the spores of one fungus may be reduced by the presence of spores of another (519).

Different fungi produce different types of fungistatic and fungicidal substances, which may be stable or unstable in nature. These are formed particularly by the lower fungi or the molds, with the exception of the Phycomycetes that have so far not been found to produce any antibiotic substances. Their action consists in modifying or killing the mycelium of the other fungus, or merely in preventing spore germination. Brömmelhues (88), studying the effects of *H. sativum* and *Penicillium* sp. against *Ophiobolus graminis*, emphasized that the inhibitory action was due to a toxic substance, thermostable in nature and diffusible in agar. In some cases, no relation could be observed between the acidity produced by one organism and its ability to influence the growth of another (1011); in other cases, as in the mutualistic effects of *Sclerotium rolfsii* and *Fusarium vasinfectum*, the first overgrew completely the second at *p*H 6.9, whereas in alkaline ranges the reverse took place (766).

Random isolations of *Penicillium* cultures and of other soil-inhabiting fungi were tested for their effects on the virulence of *H. sativum* on wheat seedlings grown in steam-sterilized soil (785). Some forms exerted a marked degree of suppression, some had no effect, and others increased the virulence of the pathogen; marked variations in activity were observed among the different species of *Penicillium*. Because Hyphomycetes were found to be capable of parasitizing the oospores of *Pythium* (184), Hyphomycetes were believed to serve as effective agents in promoting soil sanitation. Various species of *Torulopsis*, in addition to certain bacteria, are capable of inhibiting the growth of *Dematiaceae*, fungi that cause the blue staining of wood pulp (592).

Certain fungi may affect the reproduction of others. *Melanospora*

pampeana, for example, normally does not form any perithecia in culture but is able to do so in the presence of *Basisporium gallarum* or *Fusarium moniliforme*. This effect was ascribed to a special substance that resists heating at 110° C. Different fungi have a special influence on the germination of spores of various ascomycetes and of other fungi [25, 721], these effects being characteristic of the antagonists.

The edible mushroom, *Psalliota campestris*, exerts a definite antagonism against the parasitic fungus *Mycogone* [122]. This phenomenon has been looked upon as a case of antibody formation. Species of *Fusarium* are able to antagonize the mushroom fungus; however, an actively growing culture of the latter may become antagonistic to the former [608]. In the destruction of paper pulp by fungi, a marked antagonism was shown [333] to take place between different organisms, especially by *Trichoderma lignorum* against various species of *Fusarium* and other fungi, as illustrated in Figures 12 and 13.

Certain species of *Trichoderma* and *Gliocladium* are able to inhibit the growth of various plant pathogenic fungi, especially *R. solani*, as well as of *Blastomycoides dermatitis*, a causative agent of human skin diseases [261–262]. The active substance, gliotoxin, is liberated during the early stages of growth. The mycelium of older cultures contains another substance that is soluble in acetone; this has only an inhibiting effect and is not fungicidal as is gliotoxin. The fungicidal effect of gliotoxin upon the germinating spores of *Sclerotinia americana* and hyphae of *R. solani* was found to be greater than that of $CuSO_4$ and less than that of $HgCl_2$.

Various other fungi are able to exert antagonistic effects against plant pathogens. *T. lignorum* and *A. niger* restricted the growth of the fungi *Marasmius phaseoli* and *R. solani*, which produce cotton root rot, and reduced the activity of the filtrates of the pathogens causing wilting of the plants [911].

Satoh [738] has shown that *Ophiobolus miyabeanus* produces both growth-promoting and growth-retarding substances, the first of which is heat stable and passes through a Chamberland filter; the second is inactivated at 120° C. and does not pass through a filter. The formation of two substances by *Torula sugarii*, both of which were thermostable, however, was also demonstrated [654].

FIGURE 14. Antagonistic effect of one fungus, *P*. [illegible] upon another, *T. lignorum*. From G[illegible] et al. [illegible]

FIGURE 15. Attack of an antagonistic fungus, *T. lignorum*, upon another fungus, *F*. [illegible] (in center). From G[illegible] et al. (1933).

ANTAGONISTIC EFFECTS OF BACTERIA AND ACTINOMYCETES AGAINST FUNGI

Various bacteria and actinomycetes have marked selective fungistatic and fungicidal effects (Table 30). Bacteria active against *U. zeae* were isolated (37) from corn, these bacteria being capable of destroying the colonies of the smut fungi. The widespread distribution of such bacteria in the soil was believed to check the multiplication of the pathogenic fungi. Four types of bacteria antagonistic to smuts and to certain other fungi have been described (456). Some of these bacteria produce enzymes that are able to dissolve the chemical constituents of the cell walls of the fungus sporidia; they were also found to be active in the

TABLE 30. ANTAGONISTIC EFFECTS OF BACTERIA AGAINST FUNGI

ANTAGONIST	ORGANISMS AFFECTED	REFERENCES
Achromobacter sp.	*Fusarium, Sclerotinia*	130
Al. faecalis	*Helminthosporium*	695
Bacillus "D"	*Ustilago, Penicillium*	37
B. anthracis	*S. cerevisiae*	496
B. mesentericus	*Helminthosporium*	128, 695
B. mycoides	*Helminthosporium*	695
B. simplex	*Rhizoctonia*	149
B. subtilis	*Cephalothecium roseum*	13
Bacterium sp.	*Fusarium, Sclerotinia*, etc.	695
Bacterium sp.	*Ustilago*	456
Bacterium sp.	*Alternaria*	231
Myxobacterium	*Ustilago*	248, 456
P. vulgaris	*Basisporum, Phytophthora*, etc.	485, 695
Ps. aeruginosa	*Saccharomyces*	496
Ps. juglandis	*Dothiorella*	248
Ps. phaseoli	*Fusarium*	60, 248
Ps. translucens	*Ophiobolus*	86
Ps. vulgaris	*Ophiobolus*	86
S. marcescens	*Beauveria*, etc.	12, 13, 14, 587
M. tuberculosis	*Pythium*	910
Spore-forming bacteria	Fungi	37, 231, 695, 734, 738

From Novogrudsky (644).

soil against the specific fungi. Brown (92) observed that *H. sativum* and a certain bacterium produced thermostable mutually inhibiting substances. The bacterium as well as its metabolic products inhibited the growth not only of the particular fungus but also of other members of the same genus, but not of *Fusarium conglutinans*. These bacteria produced a diffusible agent that inhibited the growth of *H. sativum* (108). The active substance was not destroyed by autoclaving; it diffused into fresh agar and water, producing "stale water" that was inhibitory to the fungus.

Chudiakov (130) isolated from the soil two bacteria that were capable of bringing about the lysis of different species of *Fusarium* as well as other fungi. These bacteria were found to be widely distributed in most soils; they were absent, however, in flax-sick soils, in spite of the abundance of *Fusarium*. When this fungus was added to the soil containing antagonistic bacteria, it did not develop, and the plants did not become diseased. The antagonistic action of a variety of other bacteria against plant pathogenic fungi has been definitely established, as in the case of *B. simplex* against *Rhizoctonia*, *P. vulgaris* against *Phytophthora* (472), and *B. mesentericus* against *Helminthosporium* (128). *B. simplex* was grown (475) for 7 days at 28° C. in potato-dextrose medium containing 1 per cent peptone, and the active substance was removed by charcoal and dissolved in alcohol. Different fungi differed in the degree of tolerance to this substance. The majority were repressed by 10 per cent concentration of the stale medium added to fresh medium.

The ability to produce a thermostable substance toxic to the plant-disease-producing fungus *Rhizoctonia* is widespread among spore-forming bacteria. The toxic substance is insoluble in ether, chloroform, and benzol, but is soluble in ethyl alcohol. It passes through collodion, cellophane, and parchment membranes. It is readily destroyed on boiling in alkaline media but is more resistant in acid media.

Nakhimovskaia (629) found that various bacteria are able to inhibit the germination of rust spores. Nonspore-forming bacteria, such as *Ps. fluorescens* and *S. marcescens*, prevented the germination of the spores of *Ustilaga avenae*, *Ustilaga hordei*, *Ustilaga nuda*, and *Ustilaga reae*. Spore-forming bacteria, including *B. mycoides* and *B. mesentericus*, as well as sarcinae (*S. ureae*, *S. lutea*), exerted no antagonistic action on

the rust spores. The presence of these bacteria, however, influenced the nature of the germination of the spores, which gave rise to mycelium-like forms with great numbers of copulating filaments, whereas in the control cultures yeast-like forms prevailed and copulating cells were rarely encountered. The presence of a certain concentration of bacterial cell substance was essential to this antagonistic effect. With a more limited amount of cell material, the bacteria ceased to inhibit the germination of the spores but influenced the germination process in the same manner as do nonantagonistic bacteria, that is, they stimulated the sexual process. An increase in concentration of cell substance, even of nonantagonistic organisms, would inhibit spore germination.

The common occurrence of the fungus *Pyronema confluens* in freshly burned-over soils, but not in natural soils, was explained (645) as due to the destruction of the bacterial antagonists by heating of the soil. *Ps. fluorescens* was particularly effective as an antagonizing agent. A comparative study of the fungistatic action of substances of bacterial origin (855) has shown these to be more active than common disinfectants. Tyrothricin inhibited the growth of animal pathogens in dilutions of 1:5,000 to 1:20,000, pyocyanin in 1:2,000 to 1:5,000, and hemipyocyanin in 1:20,000 to 1:60,000.

Actinomycetes may also exert a marked depressive effect upon the

TABLE 31. FUNGISTATIC AND FUNGICIDAL ACTION OF ANTIBIOTIC SUBSTANCES UPON CERATOSTOMELLA ULMI

SUBSTANCE	MILLIGRAMS OF SUBSTANCE PER 6 CC. OF NUTRIENT BROTH		
	Complete fungistatic action	Partial fungistatic action	Fungicidal action in 48 hours
Penicillin	0	0	0
Actinomycin	0.1	0.03	0.1
Streptothricin	0	0	0
Clavacin	0.15	0.045	<0.15
Fumigacin	0	5.0	0
Hemipyocyanin	0.5	0.1	0.1
Gliotoxin	0.5	0.1	

From Waksman and Bugie (928).

growth of fungi. The active substances produced by these organisms show considerable selective action just as in the case of the bacteria. Actinomycin was found (945) to inhibit the growth of *Penicillium*, *Aspergillis*, *Ceratostomella*, and yeasts in concentrations of 1:50,000; larger amounts (1:10,000) were required to inhibit other fungi, including *Rhizopus* and *Trichoderma*. Streptothricin is less effective against fungi, although it inhibits the growth of certain yeasts (1002). A comparison of the fungistatic activity of several antibiotic substances upon the causative agent of Dutch elm disease is brought out in Table 31.

ACTIVITY OF FUNGI AGAINST INSECTS AND OTHER ANIMAL FORMS

A number of fungi are capable of parasitizing insects and other animal forms. Comparatively little is known concerning the production of antibiotic substances by these animal parasites.

CHAPTER 8

MICROSCOPIC ANIMAL FORMS AS ANTAGONISTS

THE microscopic animal world inhabiting the soil and water basins comprises protozoa, insects and insect larvae, nematodes and other worms. Their relationships to the microbiological flora of soils and waters are varied. Many, if not most, of these animals feed upon the bacteria and fungi, as well as upon the smaller animal forms. Some carry a bacterial population in their digestive tract and appear to depend upon these bacteria for some of the digestion processes. Many of the animal forms are parasitized by bacteria and fungi. Some of these forms are subject to the action of specific substances produced by microbial antagonists. No detailed discussion will be presented of these varied relationships, but attention will be directed to a few specific phenomena which have a bearing on the subject under consideration. The ability of higher animals to produce antibacterial substances has been amply established. Some of these substances are well characterized, as in the case of lysozyme found in mammalian tissues and secretions (262, 264) and inhibins found in fresh human urine (180).

INTERRELATIONSHIPS BETWEEN PROTOZOA AND BACTERIA

The lower animal forms inhabiting the soil, manure piles, and water basins often utilize bacteria in the synthesis of their foodstuffs. Although many of the smallest organisms, namely the protozoa, are able to obtain their nutrients from simple organic compounds and mineral salts, they frequently depend upon the bacteria to concentrate the nutrients present in dilute forms in the natural substrate. It has been shown (102), for example, that when carbohydrates are present in water in very low concentration, the protozoa may not be able to use them in that dilute form; however, the bacteria can assimilate these carbohydrates and can build up extensive cell substance, and the pro-

tozoa are then able to multiply by consuming the bacteria. Protozoa are apparently also able to destroy pathogenic bacteria (747).

The fact that some of the protozoa feed upon bacteria served as the basis for a theory designated as the "protozoan theory of soil fertility" (776). According to this theory, the capacity of protozoa to consume bacteria is responsible for the limited fertility of certain soils. The bacteria were viewed as the sole agents responsible for the liberation of nutrients in the decomposition of soil organic matter and for the transformation of these nutrients into forms available to higher plants. The protozoa, because of their capacity to digest bacteria, were looked upon, therefore, as the agents injurious to soil fertility. The increased fertility which results from the treatment of soil with heat and with certain chemicals was believed to be due to the destruction of the protozoa, considered as the "natural enemies of the bacteria."

Subsequent investigations did not support this theory. When protozoa were added to cultures of bacteria responsible for certain specific processes they did not exert any detrimental effect upon the particular reactions brought about by the bacteria, despite the fact that they fed upon and thereby considerably reduced the numbers of these bacteria. In many cases, the effect of protozoa upon bacterial activities may actually be considered beneficial (156, 591, 630). This was found true for such processes as the fixation of atmospheric nitrogen, the liberation of ammonia from proteins, and the formation of carbon dioxide from carbohydrates. It has been suggested that the presence of protozoa in the soil may keep the bacteria at a level of maximum efficiency (157).

Failure to confirm the protozoan theory of soil fertility was due primarily to the fact that several assumptions were made that were not fully justified, namely, (a) that bacteria are the only important soil organisms responsible for the decomposition of the soil organic matter; (b) that protozoa, by consuming some of these bacteria, are capable of restricting bacterial development and, *ipso facto*, organic matter decomposition. The fact was overlooked that the soil harbors, in addition to the bacteria, many fungi and actinomycetes capable of bringing about the decomposition of plant and animal residues, resulting in the liberation of ammonia, and that this could take place even if all the bacteria were completely eliminated from the soil.

The favorable effect of partial sterilization of soil upon fertility still remains to be explained. Various other theories have been proposed, the most logical of which is one based upon a soil condition designated as "microbiological equilibrium" (943). It has also been suggested (498) that the phenomenon is due to the disappearance of the bacterial antagonists in the soil as a result of partial sterilization.

In many cases, however, protozoa are responsible for bringing about extensive destruction of bacteria. This may find a practical application in the purification of water and sewage. The action of the protozoa is due in this case to the actual ingestion of the bacteria (440, 595, 743).

The idea (157) that protozoa may favor soil processes because of the stimulation of bacterial development and hence the accelerated transformation of soil materials is not always justified. The assumption is usually made that these processes take place in the soil in a manner similar to those brought about in artificial culture media, a generalization that may be justified only in very special cases. No consideration is given to the fact that the presence of numerous other organisms in the soil may modify considerably the activities of the protozoa. The use of artificial media gives only a one-sided conception of the significance of protozoa in soil processes. Although the more recent claim concerning the function of protozoa in the soil (157) is based upon more direct experimental evidence, it is still inadequate, because it gives insufficient consideration to the numerous elements involved in the complex soil population.

The protozoa make up only a small portion of the soil population, both in numbers and in the actual amount of cell substance synthesized. Their ability to reduce bacterial numbers in normal soil is not very significant. The indirect method of studying protozoa in solution media, whereby the types observed and the activities obtained are quite different from those occurring in the natural soil, has been largely responsible for the exaggerated importance attached to these organisms.

One may conclude that the protozoa, by consuming some of the bacteria, keep these organisms at a high state of efficiency, thus assisting in the breakdown of the plant and animal residues in the soil. In other words, the rate of energy transformation brought about by bacteria and even the total amount of change produced in the substrate are increased

by the presence of protozoa. Thus, an interrelationship among microorganisms which was at first thought to be antagonistic actually has proved to be associative (943). The protozoan *Oikomonas termo* was found capable of living at the expense of a large number of bacteria, namely 83 per cent of those tested. The fact that *Oikomonas* causes many species of bacteria to flocculate was suggested as explanation for the ability of the protozoa to digest these bacteria (378).

The ability of protozoa to destroy bacteria was said (414) to be responsible for the protection of certain plants against attack by plant pathogenic bacteria and fungi. This was said to hold true of attack of potatoes by *Bacterium aroideae* and of other plants by *Pseudomonas hyacinthi* and *Pseudomonas citri*, as well as by species of *Fusarium* and *Penicillium*.

Various bacteria may exert a toxic action upon protozoa, thus limiting the development or bringing about the destruction of the latter (122, 545, 687). Certain plant pathogenic bacteria inedible by amebae were found to produce a toxin that was harmful to these amebae. The toxin, however, appeared to be without effect on the flagellate *Cercomonas*, which could eat all these bacteria partly or completely (826). In some cases, the protozoa were capable of developing a certain resistance to specific bacterial products (687).

Certain factors in the medium seem to affect the encystment of protozoa (874); it remains to be determined to what extent these factors can be classified with antibiotic substances.

RELATIONS OF PROTOZOA TO FUNGI

The presence of *Colpoda* and other infusoria in an active form was found to repress the growth of *Verticillium dahliae* in culture media and to prevent infection of tomato plants by this pathogen; *Colpoda* was also active in soils and reduced the incidence of wilting (87).

Myxamoebae of the slime mold *Dictyostileum discoideum* also live upon bacteria. They are able to utilize the gram-negative somewhat better than the gram-positive types, with certain few exceptions. Bacterial spores are also ingested by these organisms, but they are not digested. The ability of various fungi to destroy protozoa and nematodes has been studied in detail by Drechsler (183).

MALARIAL AND TRYPANOSOME PARASITES

In connection with the recent interest in antibiotic substances, considerable work has also been done on the effect of these substances upon different strains of *Plasmodium* causing malaria and upon different trypanosomes causing various tropical diseases. Because of the war, however, the results thus obtained have not yet been published. They are highly interesting and offer promise of added application of these substances.

Weinman found (967) that the general correlation between the gram-stain of bacteria and their sensitivity to gramicidin also extends to protozoa (*Leishmania, Trypanosoma*) and to the *Leptospira* tested. Tyrocidine had a marked effect, in concentration of 5 γ per ml., upon the flagellates; they remained active for many hours, gradually losing their motility; a few escaped giving rise to delayed growth.

INSECT DISEASES AND MICROBIAL CONTROL

Insects are subject to attack by various groups of microorganisms, including bacteria, fungi, protozoa, nematodes, and other insects. Many attempts have been made to control insect pests by the use of pure or mixed cultures of microorganisms. In this connection the following relationships must be considered: the receptivity of the insect to microbial attack during its various stages of development; the environmental conditions favoring the attack on the insect by the disease-producing organism; the influence of environment upon the virulence of the attacking microbe; the manner in which the parasite attacks the host; the coordination of the optimum activity of the disease-producing agent with the abundance of the host and the proper stage of its development (867).

The microbial agents that keep in check the spread of insects, some of which are highly injurious to plants and animals, are far more important than any other methods of control. These microbial agents can be classified into three groups, depending upon the nature of the host: (a) microbes that attack economically useful insects and that must be controlled in order to avoid important losses from disease; (b) microbes that attack injurious insects and that must therefore be favored and en-

couraged; (c) microbial agents infectious to plants, animals, and man that are spread by insects.

Various bacterial diseases that formerly caused considerable destruction of silkworms and bees have been controlled, once the nature of the organisms concerned was established. One of Pasteur's important contributions to microbiology was the control of Flacheria among silkworms. However, most of the problems of control of injurious insects have been difficult to solve. A great number of bacterial, fungus, and virus diseases of insects are now known, but the many attempts to employ these pathogens in combating the insect hosts have not always been successful. The investigations so far carried out in this important field may be considered as at a very primitive stage.

Metalnikoff (596) compared the bacterial treatment of caterpillars of *Pectinophora gossypiella* with the action of arsenical poisoning. The dry spores of *Bacterium ephestiae, Bacterium gelechiae, Bacterium* 5, and *Bacterium cazaubon,* in powder form, were mixed with water at the rate of 1 to 4 ounces to 2½ gallons of water, with the addition of 4 per cent of molasses; this preparation was sprayed on the plants two to four times, at regular intervals, at the rate of 196 gallons or less per acre. The best results were obtained for plants treated with *B. ephestiae,* the infestation being reduced by about 50 per cent as compared with the controls. A slightly smaller reduction occurred on plots sprayed with *B. cazaubon,* while *B. gelechiae* reduced the infestation by less than 40 per cent. Those plants that were treated with the arsenical spray showed a reduction of only 18 per cent.

Recently microorganisms have been used for the control of the larvae of Japanese and other beetles in the soil. A variety of bacteria, fungi, and nematodes were found capable of destroying these larvae. Once the attacking microorganisms have become established in the soil, the larvae and the beetles themselves tend to disappear. Glaser (327) utilized for this purpose *Neoaplectana glaseri.* This parasite possesses great reproductive capacity and is capable of destroying large numbers of grubs. Glaser demonstrated the presence of this nematode also in localities where the grub was not present.

Fungi have also been utilized for the control of insects. Sweetman (867) emphasized the importance of entomogenous fungi as destructive

enemies of insects. A limitation to their practical importance in the fight against insects is that the fungi require special conditions for development, especially high humidity and favorable temperature, which are not always found under natural conditions.

Dutky (219) described two spore-forming bacteria (*Bacillus popilliae* and *Bacillus lentimorbus*) which cause the milky disease of the larvae of the Japanese beetle. These bacteria are grown in the larvae and then inoculated into soil. They are capable of infecting the grub, and are said to be responsible for the reduction in the beetle population. Bacteria pathogenic to the citrus red scale have also been isolated from the soil (840).

Glasgow (328) established that some of the caecal bacteria of Heteroptera show a marked antagonism toward other bacteria and protozoan parasites that occur in the intestines of these insects. The caecal system of the insects was removed and dropped into nutrient bouillon, where it remained for a month or more without showing any bacterial growth. This was believed to be proof of the fact that the caecal bacteria are antagonistic to ordinary saprophytic and parasitic bacteria and prevent their development; also they apparently kill these bacteria when they invade the alimentary canal of the insect.

According to Duncan (215), the bactericidal principle found in different insects and ticks shows differences in regard to the types of bacteria affected and the degree of their susceptibility. The gut-contents of *Argas* and *Stomoxys* show the widest range of action; that of bugs, the least. Spore-forming bacteria are especially affected by material from *Stomoxys,* whereas staphylococci appear to be more susceptible to the action of *Argas* material. The gut-contents of ticks was found to have a weak activity upon *P. pestis,* whereas the contents of certain insects favored the growth of the latter. This phenomenon may have a bearing upon the function of the plague flea. The action of the lethal principle is greater and more rapid at 37° C. than at room temperature. The lethal principle has been found to be active for at least six months when kept in a dry state. It is thermostable, resisting temperatures as high as 120° C., and is not destroyed by proteolytic enzymes. It appears to be bound to proteins, since it is precipitated from solution by alcohol and acetone, but it is not affected by these reagents. It is insoluble in the

common fat solvents. It becomes inactivated when allowed to act upon bacteria and appears to be adsorbed by killed bacteria, even by species that are not destroyed by it. This substance does not have the properties of either bacteriophage or lysozyme.

The presence in certain insects of a variety of other substances, such as allantoin, which affect bacterial activities has also been established. These observations give rise to the hope that man may in time succeed in developing and utilizing microorganisms for the biological control of injurious insects (849).

RELATION OF NEMATODES TO SOIL MICROORGANISMS

Nematode worms are represented in the soil by a number of saprophytes as well as by many plant and animal parasites. The latter vary greatly in their relation to the host. The larvae of the cereal parasite *Tylenchus tritici* penetrate the wheat seedlings between the leaf sheaths, near the growing or apical points. When the head is formed, the larvae enter the flowering parts and form galls. They become sexually mature, mate, and lay eggs which hatch in the galls, and then become dormant. When the galls fall to the ground and decompose, the larvae are liberated and proceed to find and attack new wheat plants and cereal plants.

Some nematodes attack plants by feeding upon the roots. The methods of control require, therefore, a knowledge of their life history. Some species produce resistant forms or cysts that may survive in the soil for many years, even in the absence of the host plant. Soil sterilization by steam or by chemicals is frequently employed as a measure of nematode extermination.

Antagonistic relationships may be utilized for the control of nematodes. Linford et al. (528) found that the root-knot nematode of pineapple (*Heterodera marioni*) may be controlled by heavy applications of organic material. The decomposition of this material results in a greatly increased population of saprophytic nematodes in the soil. The decomposed organic residues also support large numbers of such other soil microorganisms destructive to the parasitic nematodes, as the nema-

capturing fungi (170, 184), the non-trapping fungal parasites, the predacious nematodes, the predacious mites, and different bacteria capable of destroying nematodes.

BACTERICIDAL ACTION OF MAGGOTS

Surgical maggots are said to have a bactericidal effect in wounds, in addition to removing necrotic debris. Simmons (825) demonstrated in the maggot *Lucilia sericata* the presence of an active bactericidal substance which is thermostable and active against *S. aureus*, hemolytic streptococci, and *Cl. welchii*.

CHAPTER 9

ANTAGONISTIC RELATIONSHIPS BETWEEN MICROORGANISMS, VIRUSES, AND OTHER NONSPECIFIC PATHOGENIC FORMS

ANTAGONISTIC phenomena in relation to viruses have been but little investigated. It has been established, however, that certain microorganisms are capable of destroying viruses, and particularly that some viruses possess the capacity of antagonizing other viruses. The rapid inactivation of poliomyelitis virus in the process of aeration of sewage sludge has also been indicated (106).

BACTERIA AND VIRUSES

B. subtilis was found (718) capable of inactivating the virus of vesicular stomatitis as well as staphylococcus phage, when in contact with them for 15 to 18 hours at 35° C. This phenomenon has been explained as due to the process of adsorption. The facts that it is selective in nature, that the phage cannot be reactivated, and that the virus is rendered impotent by the action of the bacterium, all point to an antagonistic effect rather than mere physical adsorption. The virus of rabies is said to be influenced in certain ways by *B. subtilis*, the culture filtrate of the organism suppressing the activity of the virus when a mixture of the two is injected into rabbits (173).

However, different antibiotic substances, including penicillin, tyrothricin, and subtilin, when used either alone or in combination with sulfonamides or acridine, have failed to prevent infection of mice with influenza virus (508).

A "nontoxic" inactivator has been defined (306) as a substance that inactivates plant viruses and is not detrimental to most forms of life. Various microorganisms are capable of producing such inactivators. Plant viruses differ in their sensitivity to "nontoxic" inactivators. According to Johnson (457, 458) various microorganisms are capable of forming such inactivators against tobacco-mosaic virus; *A. aerogenes*

was found to produce inactivators against a number of viruses. Takahashi (868) isolated from yeast a substance which was capable of rapidly inactivating the tobacco-mosaic virus. A chemical reaction between the inactivating principle and the virus was therefore suggested. The inactivator in this instance was destroyed by heating with 1 *N* NaOH solution, but not by 2 *N* HCl. It was not a protein and gave on analysis 39.7 per cent C and 5.85 per cent H. The substance was said to be a polysaccharide. Fulton (306) demonstrated that *A. niger* forms in the medium a substance capable of inactivating a number of different plant viruses; the effect of the inactivator was found to be exerted upon the virus itself and not upon the plant.

INTERRELATIONSHIPS AMONG VIRUSES

Andrews (20) reported that the cultivation of influenza virus in a simple tissue-culture rendered the culture unable to support the growth of a biologically distinct strain of the virus added 24 hours later. The tissue-culture, however, was still capable of supporting multiplication of a related virus such as that of lymphogranuloma venereum. When two strains of the influenza virus were added to the tissue-culture simultaneously, the one added in larger concentration suppressed the growth of the other.

Numerous reports have been made concerning the interference of one virus by another, and even of inactivated bacteriophage with the active agent of the same strain (1012, 1013). Henle and Henle (394) have shown that even an inactivated virus, whether a homologous or a heterologous strain, is capable of suppressing the development of the influenza virus.

Jungeblut and Sanders (467) suggested that poliomyelitis in animals may be aborted by the injection of another virus. A strong antagonism was observed between a murine virus mutant (virus passed through mice for many generations) and the parent strain of the virus. The murine virus was capable of counteracting large paralytic doses of poliomyelitis; the two viruses virtually counterbalanced each other.

Various other types of antagonism between viruses have been demonstrated, as in the case of canine distemper or of lymphocytic chorio-

meningitis virus against experimental poliomyelitis (162). An intramuscular injection of a neurotropic strain of yellow fever virus was found to protect animals against simultaneous infection with a highly pathogenic viscerotropic strain (433). The antagonistic agent was believed to be a chemical substance produced by the murine virus, for which the term "poliomyelitis inhibition" was proposed by Jungeblut. The "interference phenomenon" of two viruses can be used to advantage in bringing about immunity reactions.

A similar type of antagonism is frequently observed also among plant viruses. Yellow mosaic virus will not grow in the tobacco tissue cells already infected with the agent causing common mosaic disease (569). Other antagonistic phenomena between plant viruses have been observed by McWhorter (573). Kunkel (510) demonstrated that the peach-yellow virus prevented the invasion of the virus of little-peach and that the latter prevented invasion of the former. McKinney (569) concluded that virus domination in a plant may be looked upon as a type of antagonism, quantitative in nature, the degree of domination by a given virus being influenced by the host.

The ability of bacterial phages to interfere with the development of other phages has been studied in detail by Delbrück and Luria (167, 546). They have shown that a certain phage, after inactivation by ultraviolet radiation, retained its ability to interfere with the growth of another phage acting upon the same host. The partly inactivated first phage is adsorbed by the sensitive bacteria and inhibits their growth without producing lysis. The partly inactivated phage interferes also with the growth of the active phage. This interference between bacterial phages was explained as due to competition for a "key-enzyme" present in limited amount in each bacterial cell. This enzyme was also believed to be essential for bacterial growth.

BACTERIA AND TUMORS

The ability of certain bacteria to bring about hemorrhage in tumors (446, 1010) may also be classed among the antagonistic phenomena. Laszlo and Leuchtenberger (515) described a rapid test for the detection of tumor-growth inhibitors. Inhibition was judged by comparing

tumor sizes and weights in treated and untreated groups of mice bearing sarcoma, after a period of 48 hours of growth. The groups were matched as to initial size of the tumors. The selective damage of sarcoma cells, as compared with normal cells, said to be caused by penicillin (150a) was later shown (525a) to be due not to the pure penicillin itself but to some impurity present in crude penicillin preparations.

CHAPTER 10

CHEMICAL NATURE OF ANTIBIOTIC SUBSTANCES

CLASSIFICATION OF ANTIBIOTIC SUBSTANCES

ANTIMICROBIAL agents are of either chemical or biological origin. The first comprise inorganic (heavy metals, halogens) and organic (phenols, arsenicals, dyes, aromatic oils) compounds. The second include a variety of products of higher plants (quinine, chaulmoogra oil, wheat flour protein), higher animals (lactenin, lysozyme), and microorganisms, to which the term "antibiotic" is specifically applied.

The property possessed by culture filtrates of many bacteria of inhibiting the growth of bacterial cells has long been recognized (506). The suggestion has even been made that all bacteria, when tested at the right age and under proper conditions of culture, are able to produce antibacterial substances (70). It is now definitely established, however, that this property is characteristic of only certain strains of specific bacteria, fungi, and actinomycetes.

Antibiotic substances of microbial origin are primarily bacteriostatic in nature. They are selective in their action. Some substances affect largely gram-positive bacteria; their action upon gram-negative bacteria is more limited as regards both the kinds affected and the concentration required to bring about growth inhibition. Other substances may inhibit alike the growth of certain members of both groups of bacteria. One is fully justified, therefore, in speaking of a characteristic bacteriostatic spectrum for each antibiotic substance. The production of antibiotic substances by specific microorganisms is influenced by the strain of the organism, the composition of the medium, the temperature of incubation, the age of the culture, aeration, and certain other factors. Antibiotic substances also vary greatly in their mode of action upon the bacterial cells, in their toxicity to animals, and in their practical utilization for the treatment of human and animal diseases.

The more important antibiotic substances are described briefly in Table 32. They may be classified on the basis of their origin from specific microorganisms, their chemical properties, or their biological ac-

TABLE 32. SUMMARY OF CHEMICAL AND BIOLOGICAL PROPERTIES OF SOME OF THE MORE IMPORTANT ANTIBIOTIC SUBSTANCES

SUBSTANCE	ORGANISM	CHEMICAL PROPERTIES	BIOLOGICAL ACTIVITY
Actinomycetin	*S. albus*	Soluble in water, precipitated by alcohol; thermolabile; protein-like in nature	Lytic to dead gram-negative bacteria and some living gram-positive bacteria
Actinomycin	*S. antibioticus*	Soluble in ether and in alcohol, insoluble in petrol ether; orange colored; thermostable; nitrogen (13 per cent) bearing ring compound; m.p. 250° C.; m.w. 800	Gram-positive bacteria; higher concentrations needed for gram-negative bacteria; highly toxic to animals
Aspergillic acid	*A. flavus*	Soluble in water, alcohol, ether, acetone, insoluble in petroleum ether; acid nature; m.p. about 96° C.; m.w. 224; about 13 per cent nitrogen	Both gram-positive and gram-negative bacteria
B. simplex factor	*B. simplex*	Diffusible, heat stable, absorbed on active charcoal, removed by hot alcohol	Certain plant pathogenic fungi
Chaetomin	*Ch. cochliodes*	Soluble, in an acid state, in ether, alcohol, and other solvents	Gram-positive bacteria; low toxicity to animals; inactive *in vivo*
Chlorellin	*Chlorella* sp.	Soluble in chloroform and benzene	Gram-positive and gram-negative bacteria
Citrinin	*P. citrinum*, *A. candidus*	Soluble in water and in alcohol; lemon yellow; quinone	Limited bacteriostatic action against gram-positive bacteria
Clavacin (patulin, claviformin, clavatin)	*A. clavatus*, *P. patulum*, *P. expansum*, *P. claviforme*, et al.	Soluble in ether, chloroform, alcohol, water; m.p. 110° C.; m.w. 154; ketone	Gram-negative and gram-positive bacteria, as well as fungi; highly bactericidal; toxic to animals
Diplococcin	Streptococci	Soluble in water, insoluble in absolute alcohol; protein-like substance; sulfur and phosphorus-free; stable at *p*H 4 at 100° C. but not at *p*H 6 to 7	Inhibits the growth of *S. cremoris* and other gram-positive cocci
Flavicin (flavatin, aspergillin, flavacidin, gigantic acid, parasiticin)	*A. flavus*, *A. giganteus*, *A. parasiticus*	Similar in all respects to penicillin	Similar to penicillin

TABLE 32 (*continued*)

SUBSTANCE	ORGANISM	CHEMICAL PROPERTIES	BIOLOGICAL ACTIVITY
Fumigacin (helvolic acid)	*A. fumigatus*	White, needle-shaped crystals; m.p. 216–220° C.; soluble in alcohol, ether, acetone, chloroform; inactivated by strong alkali; nitrogen-free	Gram-positive bacteria; limited toxicity
Fumigatin	*A. fumigatus*	Quinone, maroon-colored needles; m.p. 116° C.	Limited antibiotic activity
Gliotoxin	*Trichoderma*, *Gliocladium*, *A. fumigatus*	Soluble in chloroform and in alcohol; contains nitrogen and sulfur; m.p. 195° C.	Active against various bacteria and fungi; toxic to animals
Gramicidin	*B. brevis*	Soluble in ether and in alcohol; polypeptide; thermolabile	Lytic to gram-positive bacteria; active *in vivo*; hemolytic
Iodinin	*Ch. iodinium*	A purple-bronze pigment, identified as di-N-oxide of dihydroxyphanazine	Streptococci; activity neutralized by quinones
Micromonosporin	*Micromonospora* sp.	Produced abundantly under submerged and agitated conditions of growth	Gram-positive bacteria
Notatin (penatin, penicillin B, *E. coli* factor, corylophilline, mycoin)	*P. notatum*, *P. chrysogenum*, et al.	Insoluble in organic solvents, soluble in water; glucose-oxidase	Acts, in presence of glucose, on gram-positive and gram-negative bacteria
Penicidin	*Penicillium* sp.	Soluble in ether, alcohol, chloroform, dilute acid, insoluble in petrol-ether; destroyed by bases; absorbed by activated charcoal	*E. typhosa*
Penicillic acid	*P. puberulum*, *P. cyclopium*	Colorless; soluble in water; crystallizes from light petroleum; hydroxy-lactone	Gram-positive and gram-negative bacteria

Penicillin	*P. notatum*, *P. chrysogenum*	Soluble in alcohol and in water; soluble in ether at *p*H 2.0 and in other solvents; thermolabile; contains nitrogen	Gram-positive aerobic and anaerobic bacteria; active *in vivo*; low toxicity
Proactinomycin	*N. gardneri*	Soluble in ether, benzene, water; base	Primarily gram-positive bacteria; toxic
Puberulic acid	*P. puberulum*	Dibasic, yellow acid, quinol structure; m.p. 316–318° C.	Weak antibiotic agent, active largely against gram-positive bacteria
Puberulonic acid	*P. puberulum*	Bright yellow acid; m.p. 298° C.	Weak antibiotic agent
Pyocyanase	*Ps. aeruginosa*	Soluble in ether and in alcohol; thermostable; activity largely due to unsaturated fatty acids	Lytic action on many gram-positive and gram-negative bacteria; active *in vivo*; low toxicity
Pyocyanin	*Ps. aeruginosa*	Soluble in chloroform; blue pigment; thermostable	Largely gram-positive bacteria; limited toxicity
Spinulosin	*P. spinulosum*, *A. fumigatus*	6-hydroxyfumigatin; forms purple-bronze plates; m.p. 201° C.	Very weak antibiotic agent
Streptomycin	*S. griseus*	Similar to streptothricin	Spectrum similar to that of streptothricin; active against *B. mycoides* and more active against *Ps. aeruginosa* and certain other gram-negative bacteria; low toxicity
Streptothricin	*S. lavendulae*	Soluble in water and in acid alcohol, insoluble in ether; organic base; thermostable	Various gram-negative and some gram-positive bacteria (not *B. mycoides*); limited toxicity; active *in vivo*
Subtilin	*B. subtilis*	Soluble in alcohol; heat stable; stable in acid and unstable in strong alkali	Gram-positive bacteria
Tyrocidine	*B. brevis*	Soluble in alcohol, insoluble in ether; thermostable; polypeptide	Lytic to gram-positive and gram-negative bacteria
Tyrothricin	*B. brevis*	Mixture of gramicidin and tyrocidine; soluble in alcohol, insoluble in water	Largely active against gram-positive bacteria; used for topical applications
Violacein	*Ch. violaceum*	Violet-black pigment; soluble in acetone and in pyridine	Largely active against gram-positive bacteria

tion. Differences between various compounds may often be in degree rather than in kind. Different organisms may produce the same kind of compound. Many organisms are able to produce more than one antibiotic substance: *B. brevis* produces tyrocidine and gramicidin; *P. notatum* forms penicillin and notatin; *S. antibioticus* produces actinomycin A and B; *A. fumigatus* forms fumigatin, fumigacin, spinulosin, and gliotoxin; *A. flavus* produces aspergillic acid and flavicin.

On the basis of their solubility, the antibiotic substances may be divided into three groups:

Group A. Soluble in water at different reactions, and insoluble in ether. These substances usually represent polypeptides, proteins, organic bases, or adsorption compounds on protein molecules. Most of them have not been isolated in a pure state. They comprise the bacterial enzymes acting upon microbial polysaccharides, actinomycetin, microbial lysozyme, streptothricin, streptomycin, notatin, and pyocyanin.

Group B. Soluble in ether and in water at proper reactions. Here belong some of the most important antibiotic substances so far isolated and described, namely, penicillin, flavicin, citrinin, clavacin, proactinomycin, penicillic acid, and aspergillic acid.

Group C. Insoluble in ether and in water. These include gramicidin, tyrocidine, subtilin, and the *B. simplex* factor.

Group D. Soluble in ether and insoluble in water. Here belong fumigacin, fumigatin, gliotoxin, actinomycin, pyocyanase, and others.

Some of the antibiotic substances have been crystallized, and information has been gained concerning the approximate chemical nature of others; many others are still imperfectly known. On the basis of their chemical nature, the antibiotic substances may be divided as follows:

Lipoids and lipoid-like bodies, including pyocyanase and certain little known microbial extracts

Pigments, namely, pyocyanin, hemipyocyanin, prodigiosin, fumigatin, chlororaphin, toxoflavin, and actinomycin

Polypeptides, comprising gramicidin, tyrocidine, subtilin, and actinomycetin

Sulfur-bearing compounds, such as gliotoxin and chaetomin

Quinones and ketones, namely, fumigatin, citrinin, spinulosin, clavacin, and penicillic acid

Organic bases, including streptothricin, streptomycin, and proactinomycin

On the basis of their biological activity, the antibiotic substances also vary considerably. They may be divided into three groups:

Primarily bacteriostatic agents, such as penicillin, actinomycin, and proactinomycin

Substances which are bactericidal but not bacteriolytic, including pyocyanase, gliotoxin, fumigacin, clavacin, and pyocyanin

Bacteriolytic substances, namely, gramicidin, actinomycetin, and lysozyme

On the basis of their toxicity to animals, antibiotic substances may also be divided into three groups:

Compounds that are nontoxic or but slightly toxic; here belong penicillin, streptomycin, flavicin, pyocyanase, and actinomycetin

Compounds of limited toxicity, including gramicidin, tyrocidine, citrinin, streptothricin, and fumigacin

Highly toxic compounds, such as actinomycin, gliotoxin, aspergillic acid, and clavacin

Many of the antibiotic substances are thermostable, others are thermolabile; some pass readily through Seitz and other filters, others are adsorbed. The various methods of isolation of these substances are based upon their chemical nature, solubility, and properties of adsorption.

SUBSTANCES PRODUCED BY BACTERIA

Lipoids and Pigments. *Ps. aeruginosa,* discovered by Gessard in 1882 (320) and formerly known under the names of *Bacterium pyocyaneum* and *Bacillus pyocyaneus,* was the first organism found to produce two antibiotic agents, the colorless pyocyanase and the pigment pyocyanin. Pyocyanase, believed to be of the nature of an enzyme, is now recognized as a lipoid containing unsaturated fatty acids (55, 409, 410). Recently this organism was shown to form (809) three compounds that possess antibacterial properties, namely, pyocyanin,

α-hydroxyphenazine, and an oil that forms insoluble salts with calcium, barium, and heavy metals. The last appears to be similar to what has previously been described as pyocyanic acid, a substance strongly lytic to *V. comma*. All three compounds were isolated by extraction with chloroform.

Different strains of *Ps. aeruginosa* may produce either pyocyanase or pyocyanin or both, the production of the two not proceeding in a parallel manner. Among the amino acids, alanine and tyrosine were found to be favorable to pyocyanin production (27, 320), although the effect of tyrosine is not very significant (340, 341, 452, 529). It was suggested (407) that the antagonistic action of young cultures of *Ps. aeruginosa* is due primarily to the presence of pyocyanin, whereas in older cultures pyocyanase is largely concerned.

The determination of the nature of the antibacterial substances of *Ps. aeruginosa* can be carried out in the following manner (407): the organism is grown in bouillon for 14 days; the cultures are heated for a half hour at 75° C. to kill the living cells; they are then centrifuged, the liquid is treated with chloroform which extracts the pigment, and the chloroform solution is concentrated *in vacuo* at 50° C.; the aqueous solution remaining after chloroform extraction is acidified with hydrochloric acid and again shaken five times with chloroform, thus extracting the fatty acids. It was found that, on removing the pigment, the antibacterial properties are very little diminished; however, when both the pigment and the fatty acids are removed, no antibacterial action is left in the culture. *S. aureus* is commonly used as the test bacterium.

In most cases the broth culture of the organism is first extracted with ether, giving pyocyanase, and the residue treated with chloroform, yielding pyocyanin. The solution left after the removal of the blue chloroform extract may be again treated with ether, giving a yellow pigment, which also has some activity (501). This pigment is a derivative of pyocyanin and is often designated (1006) as hemipyocyanin. It may also be obtained by acidifying pyocyanin with acetic acid and heating. The fluorescin remaining in the culture after the ether and chloroform extraction was found to be inactive. In old cultures, pyocyanin is changed into a brown pigment, pyoxanthose. A fourth pigment, which is yellow in transmissible light and fluorescent-green in reflected light,

may be produced under certain conditions. It was excreted into the medium as a leuco base.

Pyocyanase is soluble in ether, benzol, benzene, and petrol ether. It can be separated (370) into several lipoids, the action of which shows slight variation. This preparation consists of a phosphatide, a neutral fat, and a free fatty acid. The antibacterial properties have been attributed to the last constituent (410). A definite relation has been observed between the number of double bonds and the activity of the substance (55, 409). According to Dressel (185), most fatty acids exert bactericidal and bacteriolytic effects upon gram-positive bacteria, whereas gram-negative organisms are not lysed. Pyocyanase acts upon various bacteria, including the colon-typhoid group, though the ability of the substance to inhibit the growth of this group of bacteria has been denied by some workers (370).

Many commercial pyocyanase preparations have been found to be of little practical value. This is believed to be due largely to a lack of recognition of the importance of strain specificity, conditions of cultivation of organism, and methods of extraction of the active substance (501, 763).

Since *Ps. aeruginosa* is an extremely variable organism, the nature and abundance of the pigment are also influenced by these conditions. Keeping the organism for five minutes at 57° C. or cultivating it in liquid egg-albumin has been found to result in destruction of some of its pigment-producing properties (129, 321, 522).

Pyocyanin was first studied by Fordos in 1860 (279). Since then many contributions have appeared dealing with formation and nature of this pigment. Several formulae have been suggested for pyocyanin (452, 603, 913, 1006), one of which is shown in Figure 16. The structure of pyocyanin has considerable similarity to chlororaphin and iodinin, obtained from *Chromobacterium* (555, 556) and two synthetic compounds, phenazine and acridine (919).

Besides *Ps. aeruginosa*, spore-forming bacteria, including *B. mesentericus*, were also found to produce antibiotic agents of a lipoid nature. The cell-free filtrate of this organism killed diphtheria bacteria in 4 minutes (1016), but when diluted to 1 per cent it required 24 hours to effect a kill. The substance was not affected by heating for 30 seconds

at 100° C. but was weakened at 115° C. for 10 minutes. It is considered similar in its bactericidal properties to pyocyanase.

Alcohol and acetone extracted from *B. mesentericus* a weakly active substance (408) that diffused through a cellophane membrane and could be partly absorbed on a Berkfeld filter. When shaken directly with ether, the culture lost its antibacterial properties. The ether extract was concentrated and ammonia added, and the solution was treated with 50 per cent alcohol. The alcohol was then removed, and the residue was

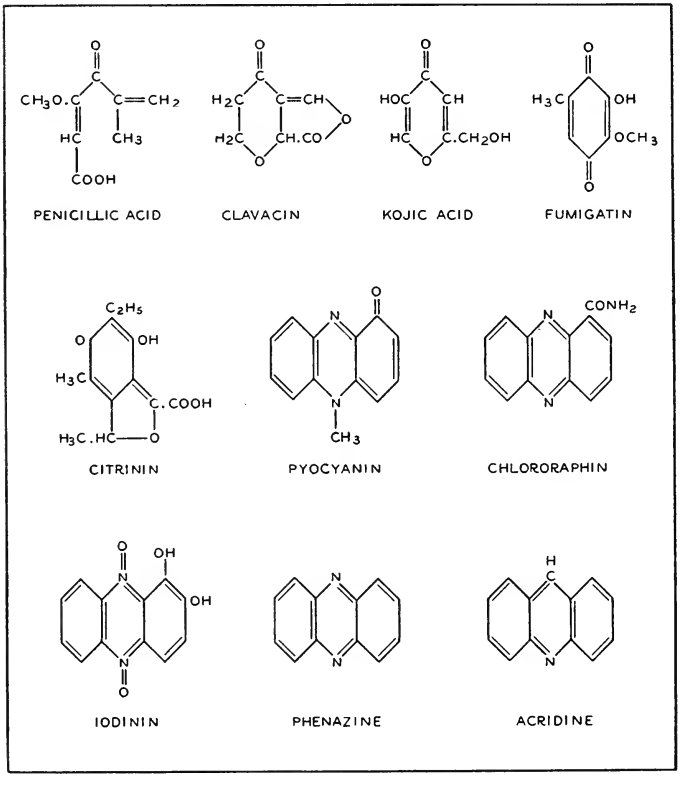


FIGURE 16. Structural formulae of some antibiotic substances.

acidified and treated with petrol ether, which brought the active substance into solution. The active substance was again dissolved in alcohol and taken up in ether. The ether solution was washed with water, evaporated, and dried. One liter of a 30-day-old culture of *B. mesentericus* gave 162 mg. of petrol-ether-soluble fatty acids and an oily substance of a brownish color. It was neutralized with NaOH solution and tested. The extract diluted to 1:7,500 killed diphtheria; a 1:1,000 dilution was required to kill staphylococci. Iso-valerianic acid and oleic acid, isolated from this material, had a similar bactericidal action. Weakening of the substance by heating was demonstrated and was believed to be due to a break in the double bond of the oleic acid.

E. coli exerts an antagonistic effect *in vivo* when injected subcutaneously or when used for feeding. It produces (365, 367) a thermolabile substance that was considered to be a lipoid in character. According to Hettche (408, 409), one is dealing, in the case of bactericidal constituents of the bacterial cell, with lipoids that contain unsaturated fatty acids.

Chromobacterium iodinum produces (555, 556) a purple-bronze pigment designated as iodinin and found to be a di-N-oxide of dihydroxyphenzine. This substance inhibits the growth of streptococci (*S. hemolyticus*) in concentrations of 1.2 to 2.0 x 10^{-6} M.

It may be added here that certain aromatic oils possess marked bactericidal properties. Ordinary peptones have also been found to contain a bacteriostatic substance that is active against various bacteria, especially when small amounts of inoculum are used (191). The active substance is thermostable and is associated with an acid-precipitated fraction that is pigmented and changes color upon oxidation and reduction. The bacteriostatic effect of this material can be corrected by the addition of reducing agents, such as thioglycollic acid. The bacteriostatic action of dyes is well known and need hardly be discussed here. It is sufficient to mention, for example, methylene blue and indophenols in oxidized forms (197).

Polysaccharidases. Among the antibiotic substances of microbial origin may also be included the enzyme systems that have the capacity of decomposing the capsular substance of certain bacteria, thereby rendering them more readily subject to destruction in the blood stream or

in other substrates. The first enzyme of this type was isolated by Dubos and Avery (195, 199, 202) from certain soil bacteria. These enzymes are highly specific, some being able to act only upon one type of pneumococci. As a result of their action, the pneumococcus cell is rendered susceptible to destruction by phagocytosis (819, 821, 822). This enzyme was produced by the soil bacteria under selective conditions of culture, that is, when the capsular polysaccharide of the pneumococcus was present in the medium; the only other substance that could be used for its production was aldobionic acid, derived from the above polysaccharide. Yields of the enzyme were increased by increasing the concentrations of the specific substrate in the medium from 0.01 to 0.1 per cent. Above 0.1 per cent, the yields decreased, 0.3 to 0.4 per cent inhibiting the growth of the bacterium. The addition of 0.1 per cent yeast extract favored the production of the enzyme; proper aeration was essential, the bacterium making the best growth in shallow layers of medium. The enzyme was concentrated by distillation *in vacuo* and by ultrafiltration. Toxic substances accompanying the active preparation could be largely removed by the use of an aluminum gel. The enzyme is associated with a protein which passes through a collodion membrane with an average pore size of 10.6 μ, but is held back by pores having a diameter of 8.2 μ. After filtration, the enzyme can be recovered in solution by immersing the membrane in distilled water or in physiological salt solution (30, 195, 293, 337).

Dubos (188) believed that it is possible to develop "adaptive" bacterial enzymes against many organic substances. These enzymes exhibit a great degree of specificity, as in the case of the enzyme that hydrolyzes the capsular polysaccharide of the pneumococcus. The cell of this organism contains an enzyme that changes the cell from the gram-positive to the gram-negative state, but is ineffective against streptococci or staphylococci.

Active preparations of the enzyme protected mice against infection with as many as 1,000,000 lethal doses of the specific pneumococcus. The enzyme retained its activity for 24 to 48 hours after its injection into normal mice; it also exerted a favorable influence on the outcome of an infection already established at the time of treatment. A definite rela-

tionship was found to exist between the activity of the enzyme *in vitro* and its protective power in the animal body.

Polypeptides. The credit for first isolating, in crystalline form from spore-forming aerobic soil bacteria, specific chemical compounds of the polypeptide type is due Dubos (190, 193, 203, 436, 530). The antagonistic organism (*B. brevis*) is grown in shallow layers of a medium containing 1 per cent casein digest or tryptone and 0.5 per cent NaCl in tap water, adjusted to *p*H 7.0. After inoculation, the medium is heated for 20 minutes at 70° C., in order to kill the vegetative cells of the bacteria, leaving only the spores to develop. The culture is allowed to grow for 72 hours. The reaction of the culture is then adjusted to *p*H 4.5 by the use of about 3 or 4 cc. concentrated HCl per liter of culture. A precipitate is formed which is removed by filtration through paper; it is then suspended in 95 per cent alcohol (20 cc. of alcohol per liter of culture) and allowed to stand 24 hours. The active substance is dissolved and is separated from the residue by filtration; when the alcoholic solution is diluted with 10 volumes of 1 per cent NaCl, the substance is precipitated out. It carries all the activity and can be desiccated *in vacuo*, over P_2O_5, giving a yield of about 100 mg. of final dry substance per liter of culture medium. The protein-free, alcohol-soluble active material was designated as tyrothricin. When an attempt was made to produce tyrothricin in aerated submerged cultures, none was obtained in complex nitrogenous media; however, simple amino compounds, like asparagine, gave good growth and yielded the antibiotic substance. The presence of cystine in the mixture of amino acids appeared to inhibit growth (856).

Tyrothricin can be separated into two crystalline preparations, gramicidin and tyrocidine. Gramicidin is obtained by treating tyrothricin with a mixture of equal volumes of acetone and ether, evaporating, and dissolving in boiling acetone. On cooling, it crystallizes out as spear-shaped colorless platelets, melting at 228° to 230° C., with a yield of about 10 to 15 grams from 100 grams of the crude material. Gramicidin is soluble in lower alcohols, acetic acid, and pyridine, and moderately soluble in dry acetone and dioxane; it is almost insoluble in water, ether, and hydrocarbons. When a solution containing 20 to 50 mg. per

milliliter alcohol is diluted to 1 mg. per milliliter, with distilled water or with glucose solution, an opalescent solution is produced without flocculation. On dilution with electrolyte solutions, an immediate flocculation occurs.

The specific rotation of gramicidin in 95 per cent alcohol solution is approximately $[\alpha]_D^{25} = +5°$. On analysis, it gives 62.7 per cent C, 7.5 per cent H, and 13.9 per cent N. The molecular weight, as determined in camphor, is about 1,400. The empirical formula of $C_{74}H_{106}N_{14}O_{14}$ has been suggested. On further study, the molecular weight of gramicidin was found (885) to present an anomaly in that it appeared to depend on the nature of the solvent and on the concentration of the solute, giving values from 600 to 1,200; isothermal distillation in methanol, however, indicated a molecular weight of 3,100. Sulfur and carbon analyses of gramicidin flavianate gave a molecular weight of 3,000. It gave neither free amino nor carboxyl groups (126, 434). Gramicidin is a polypeptide with 10 molecules of α-amino acids, of which two or three are tryptophane residues. These and a saturated aliphatic acid, with 14 to 16 carbons, account for about 85 to 90 per cent of the weight of substance. Amino acids that have definitely been identified are *l*-tryptophane, *d*-leucine, *l*-alanine, *dl*-valine, and glycine (339). A study of the configuration of the dipeptide valyvaline separated from gramicidin brought out the fact that only valines of like configuration have been joined together by the bacterium (125). About 45 per cent of the α-amino acids gave the *d* configuration (435, 436, 530). An unknown hydroxyamino compound has also been indicated (339).

Tyrocidine hydrochloride is moderately soluble in alcohol, acetic acid, and pyridine; it is sparingly soluble in water, acetone, and dioxane, and is insoluble in ether and hydrocarbon solvents. An alcohol solution can be diluted with water to give a clear solution containing 5 to 10 mg. per milliliter; electrolytes produce an immediate precipitate. A solution in distilled water containing 1 mg. or even less per milliliter has a low surface tension and behaves like a soap or detergent solution. Unlike gramicidin, it precipitates a number of soluble proteins in a manner similar to some of the cationic detergents.

Tyrocidine is dissolved in four times its weight of boiling absolute

alcohol, to which is added alcoholic HCl (0.1 mol. per liter). On cooling, a precipitate is formed. This is recrystallized from absolute methanol plus small amounts of HCl; clusters of microscopic needles are obtained, melting at 237–239° C., with decomposition; the specific rotation is $[\alpha]_D^{25} = -102°$ (1 per cent in 95 per cent alcohol). Tyrocidine

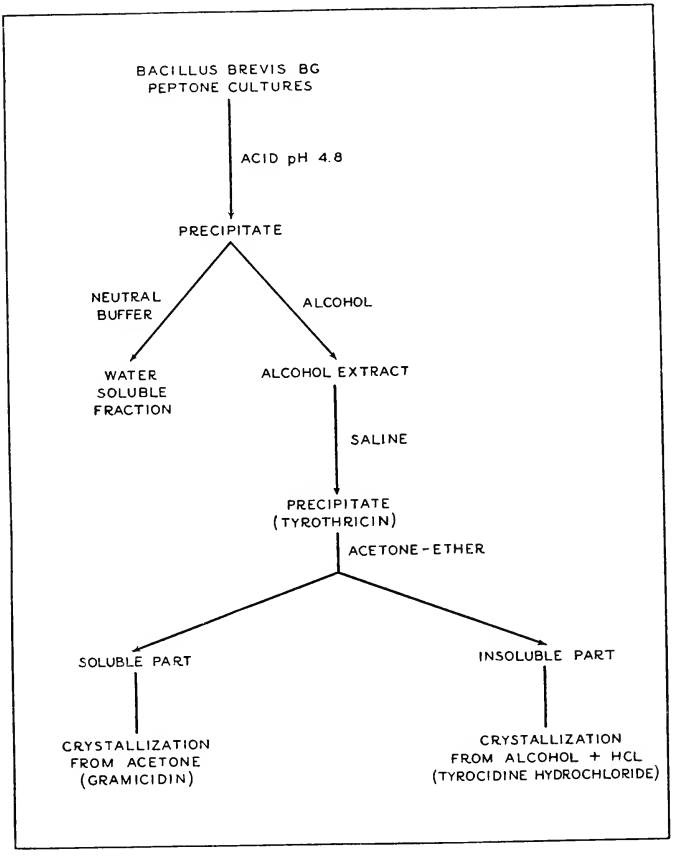


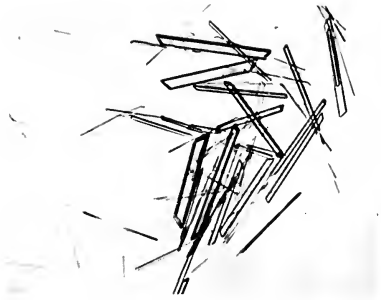
FIGURE 17. Preparation of tyrothricin, gramicidin, and tyrocidine. From Dubos (192).

analyzes: 59.4 per cent C, 6.8 per cent H, 13.5 per cent N, 2.7 per cent Cl. The molecular weight is about 1,260 or a multiple of this number. Tyrocidine is a salt of a polypeptide having free basic amino groups. The *d*-amino acids make up 20 per cent of its α-amino groups. The most probable molecule was shown to contain two amino groups, three amide groups, and one weakly acidic carboxyl or phenolic group, with a molecular weight of 2,534. Among the amino acids, tryptophane, tyrosine, and dicarboxylic-amino acids, including aspartic acid, have been detected (126, 434).

The tyrothricin type of antibiotic substance appears to be widely distributed among spore-forming aerobic soil bacteria (427, 428, 857). The following method for its extraction has also been employed: A seven-day-old bacterial culture was treated with 2 to 5 per cent of an electrolyte and HCl added to give a *p*H of 4.0. A precipitate was formed which was centrifuged and extracted with 95 per cent alcohol, until no more turbidity could be observed after dilution with an equal volume of water. The alcoholic extracts were evaporated to dryness and extracted with ether, petroleum ether, and benzol, in which the active substances are insoluble. The residue was then dissolved in absolute alcohol, and the concentrated solution dialyzed for 24 hours against running tap-water and for 24 hours against distilled water. The active substance was obtained partly in a precipitated form and partly in a colloidal solution in the dialysis bag. Upon evaporation of the water, a highly active, grayish-white powder was obtained. One hundred liters of medium gave 15 grams of purified active substance. The activity could be tested by inhibition of encapsulation of Friedländer's bacterium; this was brought about by the addition of 4 mg. to 1 ml. of culture medium. This preparation was later found to be identical with gramicidin (427a, 885).

A thermostable substance was obtained (149) from *B. simplex*, an organism capable of bringing about the destruction of various pathogenic fungi. It was produced by the bacterium grown both on synthetic and organic media. It can be adsorbed on activated charcoal and recovered from the latter by the use of hot alcohol.

To what extent substances of bacterial origin that are toxic to brain tissues, like toxoflavin ($C_6H_6N_4O_2$), are also effective against bacteria

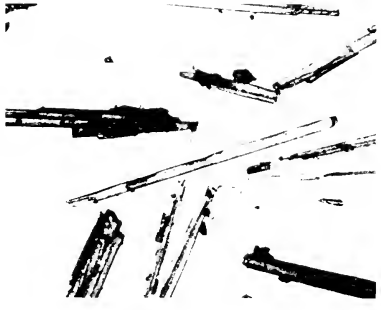


Tyrocidine hydrochloride. From Hotchkiss (435)

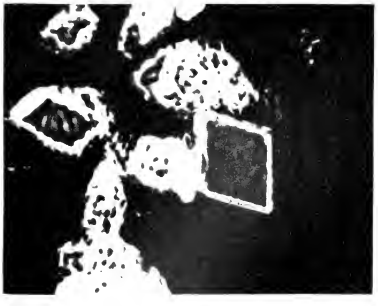
Gramicidin. From Hotchkiss (435)

Fumigacin. From Waksman and Geiger (933)

Gliotoxin. From Waksman and Geiger (933)



Citrinin. Prepared by Timonin



Actinomycin. Prepared by Tischler

FIGURE 18. Crystalline preparations of antibiotic substances.

and other microorganisms still remains to be determined. Toxoflavin, formed by *Bacterium cocovenenans*, is extracted from the culture saturated with salt by means of chloroform; from this it is recovered by an aqueous solution and purified (908, 909). Other bacterial toxins, like botulinus toxin, various amines and purine bases, and numerous toxins produced by bacteria in living plant and animal systems, are beyond the scope of this treatise.

SUBSTANCES PRODUCED BY ACTINOMYCETES

The antibacterial substances produced by actinomycetes can be divided into three groups:

Water-soluble and alcohol-insoluble compounds of the protein type, including actinomycetin (346, 347, 971–973), micromonosporin, and the compounds of the lysozyme type (507)

Ether-soluble and alcohol-soluble pigmented compounds, including actinomycin

Basic substances, soluble or insoluble in ether and soluble in aqueous or alcohol acid solution, including streptothricin, streptomycin, and proactinomycin

Actinomycin is an ether-soluble and alcohol-soluble pigmented substance produced by only a few organisms, notably *S. antibioticus*. The culture medium is treated with ether, giving an orange-colored extract. The residue is evaporated and separated (946) into two fractions: A, soluble in ether and in alcohol but not in petrol ether, giving a clear yellow-colored solution when diluted with water; B, soluble in ether and petrol ether, soluble with difficulty in alcohol, and giving a turbid suspension with water. Actinomycin A is bright red; it possesses extremely high bacteriostatic properties but is rather slowly bactericidal. Actinomycin B is colorless; it has comparatively little bacteriostatic action but possesses strong bactericidal properties. Despite the fact that the organism produces a dark-brown pigment on organic media, actinomycin does not possess the enzymatic properties of a tyrosinase.

The purification of actinomycin A was effected by chromatographic adsorption, followed by fractionation of eluate. The orange-brown residue left after treatment with petroleum ether was dissolved in benzene,

filtered, and allowed to pass through a tower packed with aluminum oxide. On washing the tower with large amounts of benzene, a chromatogram slowly developed. The column was then washed with a solution of 15 parts acetone to 85 parts benzene until the yellow-orange band approached the bottom of the column. The elution of the pigment from the column was accomplished finally by further washing with 30 per cent acetone in benzene until the eluate was faintly yellow in color. The later eluates were found by assays to contain all the active pigment, whereas all previous eluates, as well as the fractions remaining on the adsorbent, showed no bacteriostatic or bactericidal activity.

Pure actinomycin A was obtained by concentrating the 30 per cent acetone-benzene eluates to dryness, then recrystallizing the red solid residue from acetone-ether mixtures or from ethyl acetate. From these solvents, the pigment separated as vermilion-red platelets which melted at 250° C., with slow decomposition. The pigment is readily soluble in chloroform, benzene, and ethanol; moderately in acetone and hot ethyl acetate; and slightly in water and ether. The color of the solid pigment depends on its state of subdivision; when ground very fine, its color is orange-red.

Actinomycin A is optically active, a solution of 5 mg. in 2 cc. ethanol in a 1 dm. tube having a rotation —1.60°; $[\alpha]_D^{25} = -320° \pm 5$. Its molecular weight was found to be around 1,000. Cryoscopic measurements in cyclohexanol and in phenol gave molecular weights of 768 to 780 and 813, respectively. The approximate molecular formula was found to be $C_{41}H_{56}N_8O_{11}$. Actinomycin A exhibits characteristic absorption in the visible and ultraviolet regions. In ethyl alcohol, it shows strong absorption at 450 ($E_{1\text{ cm.}}^{1\%} = 200$) and between 230 and 250.

Actinomycin A is not soluble in dilute aqueous alkali or in dilute mineral acids. It is soluble in 10 per cent hydrochloric acid and appears to be regenerated by diluting such solutions with water. With strong alcoholic alkali a purple color is formed, which rapidly disappears. Actinomycin A is readily reduced by sodium hydrosulfite and by stannous chloride, but is unaffected by sodium bisulfite. With sodium hydrosulfite the reduction is characterized by a change in color from red to pale yellow. The color change is reversed by exposing the reduced pigment to air. The same reversibility of color occurs when the pigment

is subjected to catalytic hydrogenation in the presence of platinum oxide. The pigment has one or more functional groups capable of reversible reduction-oxidation (probably quinone in nature) and several others capable of acetylation (probably hydroxyls). The quinone-like structure of the pigment is borne out by its sensitivity to alcoholic alkali, and to hydrogen peroxide in the presence of sodium carbonate. In the latter instance, the color rapidly disappears and a cleavage seems to occur.

Actinomycin in alcohol-water solutions is resistant to the action of heat, being able to withstand boiling for 30 minutes. When such solutions are made acid, however, boiling has a destructive effect upon the activity of the substance, the extent of destruction being directly proportional to the concentration of acid. The effect of alkali, however, is much greater. Dilute alkali changes the color of the substance to light brown, accompanied by a reduction in activity, which can be largely restored when the solution is made neutral again. At a higher alkalinity (0.25 N), especially at boiling temperature, the activity and reversibility are destroyed. Exposure of solutions to light for 1 to 3 months reduces the activity of the pigment very little.

Streptothricin is produced by *Streptomyces lavendulae* grown in a medium containing glucose or starch (1 per cent) as a source of energy, and tryptone, glycocoll, glutamic acid, or other organic nitrogenous compound (0.3 to 0.5 per cent) as a source of nitrogen. Sodium nitrate is a somewhat less favorable source of nitrogen. The organism is grown in stationary, shallow cultures containing starch as a source of carbon or glucose and a small amount of agar, or in submerged cultures. The optimum temperature for the production of streptothricin is 23° to 25° C. (926). The relation between growth of the organism and production of the antibiotic substance is brought out in Table 33.

Streptothricin is soluble in water and in dilute mineral acids, but is destroyed by concentrated acids. It is insoluble in ether, petrol ether, and chloroform. In the crude culture-filtrate and in the alcohol-precipitated form, streptothricin is thermolabile, whereas in the purified state it is thermostable, withstanding 100° C. for 15 minutes. Treatment with proteolytic enzymes does not reduce its activity. On adjusting the reaction of the medium, when growth is completed, to *p*H 3.5 with

acid, a precipitate is produced, the filtrate containing virtually all the activity.

Streptothricin is completely adsorbed, at neutrality, on charcoal (norit A), from which it can be removed by treatment for 8 to 12 hours with dilute mineral acid or acid alcohol. The acid extract is neutralized and concentrated *in vacuo*, at 50° C., just to dryness; the residue is extracted with absolute alcohol, filtered, evaporated, and taken up in

TABLE 33. GROWTH OF STREPTOMYCES LAVENDULAE AND PRODUCTION OF STREPTOTHRICIN ON TRYPTONE-STARCH MEDIUM

AERATION	INCUBATION IN DAYS	STARCH LEFT	DRY WEIGHT OF MYCELIUM IN MILLIGRAMS	NITROGEN IN MYCELIUM IN MILLIGRAMS	ACTIVITY IN UNITS *E. coli*	ACTIVITY IN UNITS *B. subtilis*
Shaken	2	+++			10	5
Shaken	3	+	225	18.2	10	50
Shaken	4	0	293	26.2	75	250
Shaken	6	0	231	17.3	100	300
Shaken	8	0			75	200
Shaken	12	0	142	9.6	30	50
Stationary	7	+++			50	200
Stationary	10	Tr	235	18.8	50	300
Stationary	14	Tr			60	250

From Waksman (926).

water. It can also be precipitated from the neutralized solution with ether. These preparations usually contain 40 to 50 per cent inorganic matter and 2 to 3 per cent nitrogen, on an ash-free basis. Further concentration and reduction in ash content can be obtained by subsequent treatments. On electrodialysis, the active substance moves to the cathode at *p*H 7.0. A highly active fraction has been isolated by chromatographic adsorption on aluminum oxide.

Streptothricin acts as a base, with an optimum at *p*H 8.0. It is repressed by dextrose and by acid salts. Bacteria subject to the action of streptothricin show greatly enlarged cells, due to incomplete fission (287, 1002).

Another substance, designated as streptomycin (795), is similar in

many respects to streptothricin, although it differs somewhat in its antibacterial spectrum and its lower toxicity for animals.

PROACTINOMYCIN is produced by *N. gardneri* grown in soft agar media, from which it is extracted by organic solvents, such as ether, amyl acetate, benzene, and carbon tetrachloride. It can be re-extracted in water by adjusting the *p*H to 4.0 with HCl or H_2SO_4. The aqueous extract is concentrated *in vacuo* and evaporated to dryness from the frozen state. A white powder, very easily soluble in water, is obtained. The yield of the material is 60 mg. from 1 liter of culture. The substance is fairly stable, though boiling for 10 minutes at *p*H 2.0 or *p*H 7.0 results in a small loss of activity. Boiling at *p*H 10.0 destroys the greater part of the antibacterial activity. Proactinomycin has basic properties and is precipitated from aqueous solution by such base precipitants as picric acid, picrolonic acid, and flavianic acid.

SUBSTANCES PRODUCED BY FUNGI

The early studies of the phenomenon of staling accompanied by the production of antibacterial and antifungal substances (83), some of which could be removed from the acidified medium by ether or by colloidal clay (700), have recently been superseded by more exact and detailed chemical studies. Only a few of the many antibiotic substances produced by fungi have so far been identified, however. Some are produced in complex organic media, others in simple synthetic media. Only the more important substances will be discussed here. Among these, penicillin occupies a leading place because of its low toxicity and its activity *in vivo*.

PENICILLIN is produced by various strains of *P. notatum* and *P. chrysogenum*, and probably by a variety of other fungi (272a, 940a). The penicillin-like nature of an antibiotic substance is usually established by its biological and chemical properties: activity against *S. aureus* and not against *E. coli*; extraction in organic solvents at *p*H 2 and re-extraction in water at *p*H 7; inactivation by acid and alkali; partial inactivation by heating at 100° C. and *p*H 7 for 15 minutes; complete inactivation by penicillinase and by copper ions; inactivation by methyl alcohol (272a).

The strain of the organism used, the composition of the medium, and the conditions of growth greatly influence the yield of penicillin. Complex organic media containing glucose or brown sugar as a source of carbon are essential. Nitrate is used as a source of nitrogen; the medium also must contain a phosphate and certain other minerals. The supplementary addition of a stimulating substance in the form of yeast extract, corn steep, or certain vegetable juices is essential for the maximum production of penicillin. Since the organism produces an acid, probably gluconic, in the medium, some $CaCO_3$ must also be added. The metabolism of *P. notatum* in relation to penicillin production is illustrated in Figure 13 (page 129).

Four methods have been proposed for the growth of the fungus and production of penicillin. These are:

Surface growth in shallow liquid media; usually flasks, bottles, and other containers are employed, the depth of the medium being 1.5 to 2.0 cm.

Submerged growth in liquid media; the vessels must be provided with proper stirrers and aeration

Surface growth upon semi-solid media, including grain and bran (730)

Circulation of medium through a column, the supporting material being made up of wood shavings or pebbles; the rate of flow of the medium is very important

Since the various strains of penicillin-producing organisms vary greatly in their optimum conditions for the production of this antibiotic substance, different strains must be used for different conditions of cultivation.

Penicillin is produced in the medium when active growth begins but reaches a maximum soon after the growth maximum, which occurs in 7 to 14 days in stationary cultures and in 3 to 7 days in submerged cultures, at 20° to 25° C.

Penicillin is soluble in ether, acetone, esters, and dioxane; it is moderately soluble in chloroform, slightly soluble in benzene and in carbon tetrachloride. It is soluble in water to the extent of 5 mg./ml.

It is inactivated by oxidation and by evaporation at 40° to 45° C. in acid and in alkaline solutions, although it is fairly stable at *p*H 5 to 6.

If the solutions are adjusted to *p*H 6.8, it retains its potency for 3 months. The crude penicillin does not dialyze through a collodion membrane and resists heating at 60° to 90° C. for short periods; it remains active when heated at 100° C. for 5 minutes but not for 10 minutes (737).

Fleming first reported that penicillin is insoluble in ether. This was found (135) to be due to the alkaline reaction of the filtrate; for at *p*H 2.0 ether removes completely the antibacterial substance. The ether extract is evaporated with some water *in vacuo* at 40° to 45° C., the residual water containing the active substance, which is extremely labile.

For practical purposes, penicillin is extracted from the acidified culture by means of different organic solvents, such as ether or amyl acetate (7, 8). It is then removed from the solvent by shaking with phosphate buffer or with water at *p*H 6.7. Since penicillin is rapidly destroyed at a high acidity, the first extraction must be carried out very quickly and at a low temperature. In the presence of the solvents, penicillin is stable for several days. The aqueous extract may be partly decolorized by shaking with charcoal and filtering. The solution is cooled, acidified, and extracted several times with ether or amyl acetate; the extracts are passed through an adsorption alumina column, or through a 2.5 per cent precipitate of an alkaline earth carbonate on silica gel (109). Water may often contain a pyrogenic or heat-producing substance that must be removed from the penicillin.

The following four main zones were recognized in the chromatograms, beginning from the top:

1. A dark brownish-orange layer, the depth of which is inversely proportional to the amount of charcoal used for the decolorization; this zone contains some penicillin
2. A light yellow layer containing most of the penicillin but none of the pyrogen
3. An orange layer which contains some penicillin and some or all of the pyrogen
4. A brownish or reddish-violet layer which contains almost no penicillin; the pigment disappears on exposure to light

The fourth fraction is discarded, and the others are eluted with M/15 phosphate buffer (*p*H 7.2). The penicillin is again extracted

with ether, then with water, sodium hydroxide being used to adjust the *p*H. Since penicillin is destroyed readily in alkaline solution, care must be taken in adding the alkali. The "nonpyrogenic" or "therapeutic" fraction, which contains about 80 per cent of the penicillin, is extracted with pyrogen-free water. It is a deep reddish-orange liquid, yellow in dilute solution, with a characteristic smell and bitter taste.

By means of adsorption, distribution between solvents, and reduction, a barium salt or penicillin was finally obtained (3, 7, 8) which was homogeneous by chromatographic analysis and gave 450 to 500 Oxford units per milligram of dry material. The active substance was found to be a salt of a strong dibasic acid with *p*H values approximately 2.3 and 3.5, having a formula of $C_{24}H_{32}O_{10}N_2Ba$. The molecule contained one carboxyl, one latent carboxylic, two acetylatable, at least five C-Me groups, and no easily reducible double bond. The penicillin thus prepared was more sensitive to oxidizing agents than to reducing agents; it was unstable toward dilute acids and alkalies, and to heat (loss of CO_2), primary alcohols, and various heavy metal ions. Tentative suggestions were made concerning its chemical nature as follows: (a) a polysubstituted hydroaromatic ring structure; (b) the acidic groups (carboxyl) not conjugated with the chromophore responsible for the absorption; (c) the possible presence of a trisubstituted α-unsaturated ketone grouping.

Another method for obtaining penicillin has been suggested (598). In this method, the culture medium was adjusted to *p*H 3 to 4, saturated with ammonium sulfate and extracted with chloroform. The concentrated chloroform extract was treated with phosphate buffer at *p*H 7.2 to remove the active substance. This process was repeated, the less active substance being separated from the active fraction by extraction with chloroform at different ranges. By precipitating the concentrated extracts from petroleum ether, the free acid form of penicillin was obtained. By saturating the chloroform-benzol solution with dry ammonia gas, an ammonium salt was obtained which gave a dark yellow microcrystalline powder. The salt was more stable than the acid form. By acetylating or benzoylating the ammonium salt a further increase in stability was obtained. The analysis of the penicillin prepared by this method was found to fit best the formula $C_{14}H_{19}NO_6$ or $C_{14}H_{17}NO_5$ +

H_2O. This penicillin was strongly dextrorotatory and had an adsorption maximum of 2,750 A°. The preparation had an activity of 32,000,000 dilution units against hemolytic streptococci, which corresponds to about 240 Oxford units per milligram.

The most suitable form for general use is the barium salt. In this form, it retains its antibacterial activity for an indefinite period. The Ba salt of penicillin is soluble in absolute methyl alcohol, but is insoluble in absolute ethyl alcohol. Penicillin forms water-soluble salts with most heavy metals, except Fe^{+++}. It is inactivated by a number of heavy metal ions, especially Cu, Pb, Zn, and Cd. Penicillin is stable toward atmospheric oxygen, but is oxidized by H_2O_2 and $KMnO_4$, the antibacterial activity being lost (2, 5, 111).

In assaying penicillin, tests are made for potency by one of several procedures: sterility, moisture content, presence of pyrogenic substances, and toxicity (249).

Several derivatives of penicillin have been obtained:

1. Penicillamine, a degradation product (6), is produced by hydrolysis of the barium penicillin for one hour at 100° C. with N/10 sulfuric acid; the formula $C_6H_{11}NO_4.HCl$ has been suggested for it; the presence of a glyoxal nucleus has been indicated.

2. Penillic acid (210), a dextrorotatory substance having a pale bluish fluorescence, is insoluble in ether and soluble in butyl alcohol; it is produced by keeping penicillin in aqueous solution at *p*H 2.0; it has some of the properties of an amino acid.

3. Methyl, ethyl, *n*-butyl, and benzohydryl esters of penicillin have been prepared (599, 600); these are insoluble in neutral or slightly alkaline buffers, but soluble in benzene; these esters are much less active than true penicillin *in vitro* but are active *in vivo*.

The chemical nature and mode of action of the second antibacterial substance produced by *P. notatum* are given in Table 34. This substance is a protein and acts as a glucose oxidase, oxygen being required. It is characterized by its action not only upon gram-positive but also upon many gram-negative bacteria, and by the fact that the presence of glucose is required for its activity. Its action is inhibited by the presence of catalase (151, 416, 751).

Flavicin, a substance found (100, 461, 565, 567, 929) to be similar

TABLE 34. NATURE OF SECOND ANTIBIOTIC FACTOR PRODUCED BY PENICILLIUM NOTATUM

PHYSICAL AND CHEMICAL PROPERTIES	DESIGNATION		
	Penatin (493)	Notatin (57, 151)	Penicillin B (751, 902)
Solubility	Soluble in water, insoluble in organic solvents	Soluble in water, insoluble in organic solvents	Soluble in water
Adsorption or precipitation	Adsorbed on kaolin, at *p*H 4.0, eluted with sodium phosphate, precipitated by dioxane and phosphotungstic acid	Precipitated by acetone or tannic acid	Adsorbed on benzoic acid, precipitated by acetone
Chemical nature	Protein	Flavoprotein	Protein
Glucose required for activity	Required	Required	Glucose and certain other carbohydrates required
Oxygen required for activity	Apparently required	Required	Required
Activity against gram-negative bacteria in addition to gram-positive bacteria	Decidedly active	Highly active	Definitely active
Sensitivity to alkalies	Sensitive	Activity destroyed at *p*H above 8.0	
Biological nature	Hydrogen peroxide produced; not very toxic	Glucose-oxidase	Fairly toxic to animals

Note. In view of the fact that the three preparations have been obtained by somewhat different chemical procedures and in view of the variation of the strains of *P. notatum* producing this substance, there is a possibility that the different preparations may vary in chemical nature and possibly also in biological behavior.

in every respect to penicillin, is produced by *A. flavus* (929); the same is true of gigantic acid produced by *A. giganteus* (688). Preparations of flavicin have also been designated as flavatin (716, 717), aspergillin (100), flavacidin (461), and parasiticin.

ASPERGILLIC ACID is produced by *A. flavus* (461, 978) grown on tryptone-glucose media, as shown previously (page 131). The pure acid has an m.p. of 93° C. (84° to 96°) and has optical activity of $[\alpha]_D = +14°$. The formula $C_{12}H_{20}N_2O_2$ has been proposed for this substance. It possesses a hydroxyl group which gives it its acid nature (*p*K 5.5). It is stable under acid and alkaline conditions and can be distilled with steam or *in vacuo* without loss of activity. When grown in brown-sugar-containing media, a closely related substance is formed, having the formula $C_{12}H_{20}O_3N_2$ and an m.p. of 149°, with lower biological activity. Aspergillic acid is active against both gram-positive and gram-negative bacteria.

CITRININ was isolated from *Penicillium citrinum* (714). It is produced by growing the organism on a synthetic medium, with inorganic salts of nitrogen and with glucose as a source of carbon. The culture filtrate is acidified with HCl, and the substance crystallized from boiling alcohol. Citrinin forms a monosodium salt which, at *p*H 7.0 to 7.2, gives virtually colorless solutions in water. Its bacteriostatic activity is much lower than that of penicillin (33). It is a yellow crystalline solid, m.p. 170–171° (with decomposition). Its formula is $C_{13}H_{14}O_5$.

Citrinin is a strong acid, changing in color from lemon-yellow at *p*H 4.6 to orange-pink at *p*H 5.6 to 5.8 and to cherry-red at *p*H 9.9 (352). Addition of $FeCl_3$ to the culture solution gives a heavy buff-colored precipitate, which dissolves in excess of reagent to give an intense iodine-brown solution (871). It has little if any activity against gram-negative bacteria and about 50,000 dilution units against *B. subtilis* and *S. aureus*.

PENICILLIC ACID was first isolated in 1913 by Alsberg and Black (19) as a metabolic product of *Penicillium puberulum*. A limited air supply and an acid reaction of the medium favor the production of this acid, to which the chemical formula $C_8H_{10}O_4$ was given. This acid is a rather weak antibiotic substance active largely against gram-positive bacteria; however, it is more active against gram-negative bacteria than penicillin, giving complete inhibition of *E. coli* in concentrations of 1:50,000,

whereas penicillin does not inhibit this organism even in concentrations of 1:1,000. It was found to possess antibiotic properties also against yeasts, and to be toxic to animals when injected subcutaneously in concentrations of 0.2 to 0.3 gm. per kilogram weight.

More recently, penicillic acid was isolated (661, 664) by evaporation of the culture solution, the crude acid crystallizing on cooling. It was purified by recrystallization from hot water. Yields greater than 2 gm. per liter of culture were obtained. The acid is a stable, colorless compound which is appreciably soluble in cold water and gives a series of colorless and readily soluble salts (56, 470a).

Penicillic acid was shown to have the constitution γ-keto-β-methoxy-δ-methylene-Δ-α-hexenoic acid, which exists in both the keto and lactone forms:

$$CH_2{=}CH{\cdot}(CH_3){\cdot}CO{\cdot}C(OCH_3){=}CH{\cdot}COOH \rightleftharpoons$$

$$CH_2{=}CH{\cdot}(CH_3){\cdot}C(OH){\cdot}C(OCH_3){=}CH{-}CO$$

$$\underbrace{\qquad\qquad\qquad O \qquad\qquad\qquad}$$

Fumigatin. Among the other quinones isolated from fungi, fumigatin deserves consideration (21, 712). It is a 3-hydroxy, 4-methoxy, 2:5-toluquinone or $C_8H_8O_4$ (Figure 16, page 164).

All quinones have been divided into three groups on the basis of their action on *Staphylococcus:* (a) those that have a markedly weaker antibacterial action than fumigatin, including toluquinone and some of its derivatives; (b) those that are somewhat more effective than fumigatin, including 3:4 dimethoxytoluquinone; (c) those with activity greater than that of fumigatin (methoxytoluquinones). The introduction of $-OCH_3$ into the quinone nucleus results in an increase in antibacterial activity. The introduction of an OH or the replacement of $-OCH_3$ by OH results in a decrease in activity. None of these quinones, however, has any very striking action on gram-negative bacteria, such as *E. coli* (660). Electrode potentials of quinones have been found to be unrelated to their bacteriostatic action against *E. coli,* but for *S. aureus,* the reduction potentials fall within certain limits (417, 666).

Clavacin is produced by *A. clavatus* (935), *P. patulum* (patulin [713]), *P. claviforme* (114, 115), *P. expansum* (22), and probably a number of other fungi (470). It can be isolated from the culture filtrate

either by preliminary adsorption on norit followed by removal with ether or chloroform, or by the direct treatment of the culture with ether. The extract is evaporated, leaving a brown substance; this is treated with a small amount of water, and the aqueous solution again extracted with ether. Clavacin crystallizes when the ether solution is concentrated, or after preliminary purification over a silica gel column. Clavacin thus isolated (429, 473) from *A. clavatus* cultures showed the following chemical properties: melting point, 109–110° C.; empirical formula, $C_7H_6O_4$; molecular weight (cryoscopic in benzophenone) 154; semicarbazone, darkens at 200°, decomposes at 290° C.; 2,4-dinitrophenylhydrazone, darkens above 190°, decomposes at about 300° C.; lactone group indicated by slow reaction with alkali; saponification number 70 (evidently molecule cleaved); Zerewitinoff determination (in *n*-butyl ether) shows slightly less than one active hydrogen per mol; esterification by the acetic anhydride-pyridine method shows one hydroxyl per mol. Clavacin, a neutral optically inactive compound, darkens and loses activity in the presence of alkali, reduces Fehling's solution strongly on heating, and readily decolorizes alkaline permanganate; it does not react with aqueous ferric chloride or Schiff's reagent, and reduces ammoniacal silver nitrate.

Clavacin (patulin) is anhydro-3-hydroxymethylene-tetrahydro-γ-pyrone-2-carboxylic acid, for which a formula has been suggested (713), as shown in Figure 16.

Clavacin is soluble in water and in most of the more common organic solvents except light petroleum. It is about equally active against gram-positive and gram-negative bacteria, its growth inhibition being about 200,000 dilution units. Its lethal action upon mice is about 25 mg. per kilogram body weight, when given intravenously or subcutaneously.

Claviformin isolated (114, 115) from *P. claviforme* has recently been shown to be identical with clavacin and patulin (47, 927).

Fumigacin is produced by different strains of *A. fumigatus*. It can be extracted from the culture medium either by preliminary adsorption on active charcoal followed by treatment with ether and alcohol, or by direct extraction of culture in accordance with the following method (593): The culture filtrate is acidified to *p*H 2 with phosphoric acid and extracted three times with ether, the combined extracts equalling

the volume of the filtrate. The ether is evaporated to one-tenth of its volume and the concentrate is shaken repeatedly with saturated sodium bicarbonate solution, which removes a dark-red pigment. The solution is then exhaustively extracted with 6 per cent sodium carbonate solution. The ether phase, on evaporation, yields gliotoxin. The sodium carbonate solution is acidified and distributed several times with benzene; the partly crystalline residue from the benzene (7–12 mg. per 1 L of culture filtrate), on repeated recrystallization from methanol, yields pure fumigacin in the form of filamentous needles. Fumigacin melts with some decomposition at 215–220° C., depending on the rate of heating. $[\alpha]_D^{25} = -132 \pm 2°$ (0.41 per cent in chloroform). The ultraviolet absorption curve shows only strong end absorption below 260 mμ with $E_{1\text{ cm.}}^{1\%} = 298$ at 234 mμ. Fumigacin is practically insoluble in water, sparingly soluble in cold methanol and ethanol, and more readily soluble in acetone, ethyl acetate, benzene, and ether. It is easily dissolved by chloroform, acetic acid, and dioxane.

The following reactions are negative: ferric chloride, Legal, fuchsin sulfurous acid, Tollens, Molisch, Rosenheim, Hammersten (for cholic acid), Jaffe-Tortelli, digitonin. The Zimmerman reaction with *m*-dinitrobenzene for ketones is strongly positive. In the Chabrol-Charonnet test for bile acid (phosphoric acid and vanillin) a strong red color is obtained. Likewise, the Liebermann-Buchard test gives an intense blood-red color. Fehling's solution is slowly but perceptibly reduced at 100° C. The formula that has been suggested is $C_{29}H_{38\text{-}40}O_7$. Purified fumigacin has recently been shown to be identical with helvolic acid, isolated from a strain of *A. fumigatus*.

Gliotoxin was isolated from cultures of *Trichoderma*, *Gliocladium*, and *A. fumigatus* (459, 960, 961), as well as from other fungi (593, 933), the greatest activity being produced in 2 days. It was extracted from the culture medium by the use of chloroform. The latter was distilled off, and the residue taken up in a small amount of hot benzene or 95 per cent alcohol, from which, on cooling, silky white needles crystallized. It was recrystallized from benzene or alcohol. It was found (965) to have a molecular weight of 347, an optical rotation of $[\alpha]_D^{19} = -239°$, and an m.p. of 121° to 122° C. It analyzed $C_{14}H_{16}N_2O_4S_2$, later shown (459) to be $C_{13}H_{14}N_2O_4S_2$.

Gliotoxin is sparingly soluble in water and readily soluble in alcohol. It is unstable, particularly in alkaline solutions, and is sensitive to oxidation and to heating (961); it is inactivated by heating for 10 minutes at 100° C. (17). Its potency was found to be destroyed by bubbling oxygen for 5 minutes.

Gliotoxin is toxic to *Rhizoctonia* hyphae in a dilution of 1:300,000, which is about two-thirds of the toxicity of $HgCl_2$. The crystals, as well as the crude material, were found to be toxic also to *Trichoderma*, but the minimum lethal dose was about 40 times greater than that required for *Rhizoctonia*. Its antibacterial properties are brought out later.

Other Substances. Several other antibacterial substances have been isolated from fungi, but have not been adequately studied either chemically or biologically. It is sufficient to mention the following:

Puberulic acid, $C_6H_8O_6$, a colorless crystalline dibasic acid, with an m.p. of 316–318°, and puberulonic acid, $C_8H_4O_6$, a bright-yellow acid with an m.p. of 298° C., produced (38, 58, 665) by *P. puberulum*. The first is a quinol and the second is quinonoid. They have little activity against gram-negative bacteria and some activity against gram-positive types.

Penicidin was isolated (26) from a species of *Penicillium*. It is soluble in ether, alcohol, chloroform, and dilute acids, but not in petrol ether. It is destroyed by bases, and is adsorbed on active charcoal. It was found to be active against *E. typhosa*.

Chaetomin is produced by a species of *Chaetomium* (*Ch. cochliodes*) grown in complex organic media. It is active largely against gram-positive bacteria (930).

Kojic acid (Figure 16), produced by *A. oryzae*, apparently also possesses some antibacterial properties, more against gram-negative than gram-positive bacteria (282).

SUBSTANCES PRODUCED BY YEASTS

According to Fernbach (253), certain yeasts produce volatile substances which are toxic not only to other yeasts but also to bacteria. Rose yeasts (*Torula suganii*), either fresh or heated to 120 to 130° C., were found (654) to contain a substance which has an antagonistic action

against fungi, especially in the young mycelial stage, but not against yeasts; the growth of *A. niger* was reduced by 60 to 70 per cent and that of *A. oryzae* by 25 to 30 per cent. The substance was not found in the ash of the organism and was not secreted in the filtrate, but remained in the yeast cells. An alkaline reaction was unfavorable to its formation and action. The active substance was soluble in acetone, alcohol, ether, and chloroform, and was adsorbed by kaolin, Seitz filter, paper, and by the fungus mycelium. It could be removed from the kaolin by treatment with ether or acetone. Acetone-treated yeast no longer had an antagonistic effect, but only a stimulating one.

According to Schiller (798), yeasts produce a bacteriolytic substance only in a state of "forced antagonism," that is, in the presence of staphylococci and certain other bacteria. The substance is thermolabile, since it is destroyed at 60° C. It is active also outside the cell. More recently (144), the active substance of yeast was concentrated. In a crude state, the active material was found to be nonvolatile and readily soluble in water, in 95 per cent alcohol, and in acetone containing a trace of water. It was stable at 100° C. at *p*H 7.3. It contained nitrogen but no sulfur. Although a positive biuret reaction was obtained, it appeared that the protein was present as an impurity.

MICROBIAL LYSOZYME

The enzyme lysozyme or an antibiotic substance similar to it has often been reported to be produced by microorganisms. Fleming (264) found that the lysozyme of egg white was soluble in water and in dilute NaCl solution. It was precipitated by chloroform, acetone, ether, alcohol, and toluene. It was not acted upon by pepsin or trypsin. It was particularly active against micrococci, bringing about their lysis.

Lysozyme has been found in nearly all mammalian tissues and secretions, in certain vegetables, and in bacteria (880). It was found to be a polypeptide containing 16 per cent nitrogen and 2 to 3 per cent sulfur and having a molecular weight of 18,000 to 25,000. It is soluble and stable in acid solution, insoluble and inactivated in alkaline solutions, and inactivated by oxidizing agents (601). It diffuses in agar and through cellophane, and thus is markedly different from bacteriophage

(323). It is fixed on the bacterial cells (264). It acts primarily upon the cell membrane of bacteria, the highly viscous component of the bacterial cell (the mucoids), especially the sugar linkages of the complex amino-carbohydrates (601), being disintegrated by the enzyme. The degradation of the bacterial polysaccharide to water-soluble products (N-acetylated amino-hexose and a keto-hexose) by lysozyme is accompanied by complete lysis of some of the bacteria. In the case of other lysozyme-sensitive bacteria, such as *B. subtilis*, no lysis occurs; apparently the morphological structure of these bacteria does not depend exclusively on the unaltered state of the substrate for lysozyme (243).

The formation of a lysozyme-like material was demonstrated by Fleming (262) for a coccus isolated from dust. A sarcina susceptible to egg-white lysozyme also was found (601) to produce an autolytic enzyme similar to it. It has been suggested that the antibacterial action of saliva may be due to the presence in it of antagonistic bacteria (704). The lysozyme of saliva is known to act primarily upon gram-positive bacteria (880). Auerswald (28) tested a large number of bacteria for their ability to antagonize diphtheria and pseudo-diphtheria organisms. Only the spore-forming *B. mesentericus* and *B. subtilis* groups produced antagonistic substances, but these bacteria were not found in the saliva. Cultures of bacteria isolated from the saliva had no antagonistic effect, thus proving that the action of saliva need not be due to its bacterial content.

MISCELLANEOUS ANTIBIOTIC SUBSTANCES

In connection with the antibiotic substances of microbial origin, attention may be directed to certain substances, of apparently similar nature, of plant and animal origin. No attempt will be made to discuss here the antibacterial blood reactions, including antibody formation, precipitin reactions, agglutination, phagocytosis; lysin formation; action of various body fluids; and other animal and plant reactions against bacteria and in response to bacterial infection.

Unbleached wheat flour was shown (864) to contain a protein which had bacteriostatic and bactericidal activity *in vitro;* although this activity was greatest against gram-positive organisms, it also had some ac-

tivity against gram-negative types. The antimicrobial action of this protein can be neutralized by means of a phosphatide (1004), a reaction which may be due to the formation of a lipoprotein that has no longer any antibiotic activity. Sherman and Hodge (817) demonstrated that the fresh juice of several plants has a marked bactericidal effect. The active substance in the juice could be adsorbed on activated carbon and by passage through fine Berkfeld filters. The substance was thermolabile, being destroyed at 60° C. in 10 minutes. It has been recently demonstrated (658) that antibacterial substances are widely distributed among plants.

Milk was found (657) to contain several thermolabile bactericidal substances and two thermostable compounds which acted injuriously upon lactic acid bacteria. Orla-Jensen emphasized that the growth of bacteria in milk is influenced by a combination of activators or growth-promoting substances and of inhibitors, the predominance of one or the other being determined by various conditions. These substances influence the development of specific lactic acid bacteria during the spontaneous souring of milk.

CHAPTER 11

THE NATURE OF ANTIBIOTIC ACTION

Sulfanilamide, penicillin, and gramicidin can be clearly set apart from the classical antiseptics which are general protoplasmic poisons. All three substances are primarily bacteriostatic rather than bactericidal in their action. Since they do not destroy the respiration of bacteria, one may assume that the inhibition of growth which they cause depends not upon interruption of the cellular metabolism as a whole, but rather upon some subtle interference with certain individual reactions. To interrupt the pathogenic career of an infectious agent, therefore, it is not necessary to kill the invading cell, but only to block one step in its metabolic path by some specific inhibitor. — Dubos (192).

ANTIBIOTIC SUBSTANCES AND CHEMICAL DISINFECTANTS

Since antibiotic substances vary greatly in their origin and in their chemical nature, they would be expected also to vary in their mode of action upon the cells of bacteria and other microorganisms, and in the effect upon the animal tissues when these agents are used for chemotherapeutic purposes. Comparatively little is known concerning these mechanisms. It is known, however, that antibiotic substances act chiefly by interfering with the growth of the bacterial cell, although in many cases they are able to bring about the lysis of the cell as well. Because of the first effect, it has been assumed that antibacterial agents are structurally related to bacterial metabolites that usually function as coenzymes (560). In this connection, the following properties of antibiotic agents are of particular significance:

Most antibiotic substances are strongly bacteriostatic in nature and only weakly bactericidal, though a few are also strongly bactericidal, and some are even bacteriolytic.

Some substances act primarily *in vitro* and only to a limited extent *in vivo* because of interference of the body tissues with their action; others, however, act readily upon bacteria *in vivo*.

A few antibiotic agents are fairly nontoxic to the animal body; others are somewhat more toxic but can still be utilized; and some are so highly toxic that they offer little promise as chemotherapeutic agents.

Antibiotic agents differ greatly in their solubility: some are water soluble; others are alcohol soluble and only slightly soluble in water; and some are acids and react with alkali solution to form soluble salts.

Some antibiotic agents are stable under a variety of conditions, whereas others are unstable.

Some antibacterial substances are hemolytic; others have apparently no injurious effect upon the blood cells. The latter can, therefore, be used for general body treatment, whereas the former are suitable only for local applications.

Since antibiotic substances are selective in their action upon microorganisms, none can be expected to be utilized as general agents against all bacteria. This also points to the remarkable physiological differences in the morphology and physiology of the bacterial cells, and to the differences in the mode of action of the different antibiotic substances upon the various bacteria.

A comparison of the antibacterial action of the antibiotic substances produced by two bacteria will serve to illustrate some of the foregoing points. Pyocyanin, produced by *Ps. aeruginosa*, inhibits the growth of many gram-positive and gram-negative bacteria in dilutions as high as 1:100,000; pyocyanase and hemipyocyanin have less activity upon the bacteria, but yeasts are more sensitive to them than to pyocyanin. Tyrothricin, produced by *B. brevis*, is far more specific in its action, which is limited largely to gram-positive bacteria. The sensitivity of pathogenic fungi to these compounds also differs markedly (855). Other striking differences are found on comparing two types of antibiotic substances produced by fungi, namely, penicillin and clavacin, and two substances produced by actinomycetes, namely, streptothricin and actinomycin. The bacteriostatic spectra of these four substances are recorded in Table 35. The first of each pair has a limited toxicity to animals, and the second is highly toxic. Whereas penicillin acts largely upon gram-positive bacteria and only upon a few gram-negative organisms, streptothricin acts alike upon certain bacteria within each group. Clavacin and actinomycin, both of which are highly toxic, differ similarly in their action upon bacteria, the first being largely active against

gram-positive and the second active against members of both groups. These four compounds show various other differences in the nature of their antibacterial action. Differences in the bactericidal properties of other antibiotic substances are brought out in Tables 36 and 37.

Various attempts have been made to compare the antibacterial action of antibiotic substances with that of organic antiseptics. According to Suter (866), the bactericidal action of a compound depends upon certain physical and chemical characters; a property that determines the bactericidal action of the compound upon *E. typhosa* may be relatively unimportant in the case of another organism such as *S. aureus*. A substance may have the same activity, as expressed by the phenol coefficient, against two organisms and still differ markedly in its relative

TABLE 35. BACTERIOSTATIC SPECTRA OF FOUR ANTIBIOTIC SUBSTANCES

TEST ORGANISM	GRAM STAIN	PENICILLIN	ACTINOMYCIN	STREPTOTHRICIN	CLAVACIN
S. aureus	+	9,500*	20,000	200	100
S. aureus	+	1,000†	–	–	–
S. lutea	+	38,000*	60,000	100	500
B. subtilis	+	19,000*	60,000	750	200
B. megatherium	+	1,900*	40,000	200	100
B. mycoides	+	5*	40,000	<3	200
Cl. welchii	+	1,500†	1,000	–	–
Actinomyces sp.	+	1,000†	10	10–50	–
Neisseria sp.	–	2,000†	20	–	–
Br. abortus	–	2†	10	100	–
Sh. gallinarum	–	2†	20	300	–
Pasteurella sp.	–	1†	<10	100	–
Hemophilus sp.	–	–	50	30	–
S. schottmülleri	–	<1†	<10	200	60
S. aertrycke	–	10*	–	–	–
Ps. fluorescens	–	<5*	10	<3	6
S. marcescens	–	<1*	<5	5	60
A. aerogenes	–	<5*	<5	30	50
E. coli	–	<1†	–	–	–
E. coli	–	<5*	5	100	100

Note. Activity is indicated in thousands of dilution units per gram.
* Our own data, based on a sample having 470 Oxford units.
† Data reported by Abraham et al. (1), based on a less active preparation.

TABLE 36. BACTERICIDAL EFFECTS OF PENICILLIN, GRAMICIDIN, AND TYROCIDINE UPON S. HEMOLYTICUS

INHIBITING AGENT*	NUMBER OF VIABLE ORGANISMS† At start	At 1 hour	At 3 hours	At 7 hours	At 24 hours
Penicillin	1,500	4,300	2,650	420	0
Gramicidin	1,500	2,430	1,140	7	2.4
Tyrocidine	1,500	0.1	0	0	0

From Dawson, Hobby, Meyer, and Chaffee (164).
* 10 γ of each preparation was added to 1 milliliter of culture.
† In thousands per milliliter.

TABLE 37. BACTERIOSTATIC AND BACTERICIDAL ACTION OF FUMIGACIN AND CLAVACIN

TEST ORGANISM*	FUMIGACIN Bacteriostatic action† *Crystals*§	*Crude material*¶	Bacteri-cidal action‡	CLAVACIN, CRUDE MATERIAL Bacterio-static action†	Bacteri-cidal action‡
A. aerogenes	<40,000	<40,000	>200	50,000	20
E. coli	<40,000	40,000	>200	100,000	20
S. schottmülleri	<40,000	<40,000	>200	60,000	20
Salmonella sp. (Breslau type)	<40,000	<40,000	>200	75,000	20
S. choleraesuis	<40,000	<40,000	>200	150,000	5
B. megatherium	1,250,000	1,000,000	20	100,000	5
B. cereus	500,000	500,000	200	125,000	5
B. mycoides	1,250,000	500,000	200	200,000	2
B. subtilis	750,000	500,000	200	200,000	2
S. aureus 3	750,000	750,000	200	100,000	2
S. aureus 2	500,000	250,000	>200	60,000	20
S. aureus H	750,000	500,000	>200	75,000	20
S. aureus W1	750,000	500,000	200‖	200,000	5
S. aureus W2	500,000	500,000	200‖	200,000	2
S. lutea	4,000,000	3,750,000	20	500,000	2

From Waksman, Horning, and Spencer (935).
* *Staphylococcus* cultures and gram-negative pathogens incubated at 37° C., others at 28° C.
† Units of activity = dilution in plate or tube inhibiting growth completely.
‡ Micrograms required to kill bacteria in 1 milliliter portions of 6-hour-old cultures.
§ Water saturated solution used.
¶ Crude mother liquor, from which 2 lots of crystals were removed on basis of dried material in solution.
‖ Incomplete sterilization of culture even in 24 hours

lethal effects. The conclusion was reached that the mechanism of bactericidal action must be considered as a separate problem for each type of organism, and, one may add, for each type of compound.

Although the major difference in the action of antibiotic substances and chemical antiseptics is based upon the selective antibacterial nature of the former, still an attempt may be made to correlate the two types of compounds. Marshall and Hrenoff (584) constructed a disinfectant spectrum for antibacterial substances with a flexible blending of differentiated degrees of activity. The first, or ineffective, band covers a range of dilutions of an agent between zero concentration and the highest dilution which still exerts no action on bacteria. The second, or stimulative, band comprises a range of relatively high dilutions in which there is a slight stimulation of bacterial multiplication; this range is ordinarily narrow, but it may become broad. The third, or inhibiting, and the fourth, or germicidal, bands merge indistinguishably. The fifth, or impractical, band covers a range of concentrations of the disinfectant that are too great for practical purposes (Figure 19).

By establishing the normal rate of multiplication of bacterial cells in a given culture without the disinfectant, one can determine the retardation of that rate by the disinfectant. This rate approaches zero at com-

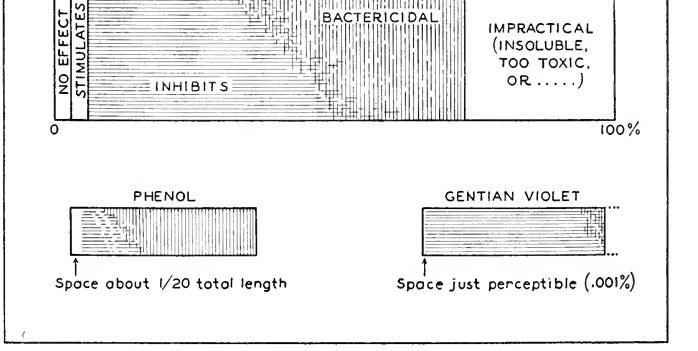


FIGURE 19. Disinfectant spectrum. From Marshall and Hrenoff (584).

plete inhibition with no multiplication and no deaths. A further increase in the concentration of disinfectant results in the death of some organisms per unit of time, and eventually a concentration is reached at which all organisms die rapidly (Figure 20). Any rate of multiplication

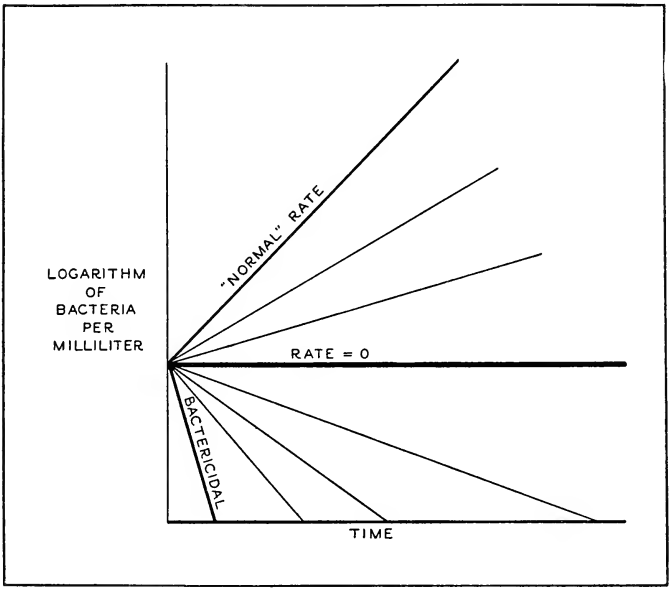


FIGURE 20. Disinfectant spectrum and rates of bacterial growth. From Marshall and Hrenoff (584).

greater than zero but less than normal can be considered as the bacteriostatic zone, and the rate less than zero as the bactericidal zone. According to this concept of bacteriostasis, bacterial growth may be delayed under the influence of a disinfectant for many days or for many hours; or the bacteria may progressively die over a period of many days.

The following factors influence the selective action of an antibiotic agent upon bacteria (189): the acidic and basic properties of the bacterial cell, the nature and property of its membrane, its permeability,

the relative importance for metabolism and viability of the specific biochemical systems affected by the agent, the activity of autolytic enzymes in the bacterial cell, as well as others.

Marked differences exist in the degree of sensitivity of various bacteria to different antibiotic substances and chemical agents. Gramicidin is most specific in its action, being limited to the cocci and acting upon actinomycetes to only a limited extent. Penicillin is next in its selective action. Actinomycin, tyrocidine, and gliotoxin act primarily upon the gram-positive organisms and actinomycetes, and much less upon gram-negative bacteria. The selective action is in contrast to the generalized, even if more limited, action of phenol and quinone, which act alike on both gram-positive and gram-negative organisms. Pyocyanase, pyocyanin, and the culture filtrate of *P. notatum* (due to the presence of notatin) are similar in some respects but not in others to the chemical compound in their action; they are found to be generally bacteriostatic over a wide range of test organisms, no sharp division being obtained upon the basis of the gram stain. Streptothricin is unique in its action; the gram-positive spore-former *B. subtilis* is most sensitive, but the other spore-former *B. mycoides* is not affected at all. The gram-negative *E. coli* is more sensitive to streptothricin than either *M. lysodeikticus* or *S. lutea*. Sulfanilamide has a definite, even if limited, retarding effect upon the growth of various organisms. The antibiotic substances of microbial origin are generally found to be stronger bacteriostatic agents than the chemicals tested. A high bacteriostatic effect is not necessarily accompanied by a correspondingly high bactericidal action. Gliotoxin, one of the most active bacteriostatic substances among those tested, possesses lower bactericidal properties than other preparations. Streptothricin, on the other hand, is highly bacteriostatic and bactericidal against certain gram-negative bacteria.

The specific morphological differences among the bacteria, based upon the gram stain (205), as shown by their sensitivity toward antibiotic substances, are thus found to be relative rather than absolute. Most of the gram-positive bacteria are more sensitive to the majority of antibiotic substances than are the gram-negative bacteria. But other antibiotic agents, such as streptothricin, streptomycin, and clavacin, act quite differently and show marked variations within each group.

MECHANISM OF ANTIBIOTIC ACTION

In an attempt to interpret the antibacterial activities of antibiotic substances, one may benefit from a comparison of the action of these substances and that of other antibacterial agents. Recent studies of the mechanism of antibacterial action of chemotherapeutic agents led to rather definite concepts concerning the nature of this action. The action was believed to consist in depriving the bacteria of the use of enzymes or metabolites by various types of interference (254). The nutritional requirements of the organisms thus inhibited are more exacting than in their normal state. *E. coli* and *S. hemolyticus*, when inhibited by acriflavine components, were found to require for further growth two types of material not normally added, one of which could best be replaced by nucleotides, and the other by a concentrate of amino acids, especially phenylalanine (557, 559).

On the basis of the information now available, the following mechanisms may be tentatively presented here:

The antibiotic substance interferes with bacterial cell division, thus preventing further growth of the organism. The cell, unable to divide, gradually dies. It has been shown (359), by the use of the manometric method, that certain bactericidal agents in bacteriostatic concentrations have no effect on the metabolic rates of bacteria, though they do inhibit cell multiplication.

The antibiotic substance interferes with the metabolic processes of the microbial cells, by substituting for one of the essential nutrients. It has been suggested (290a) that the antibiotic effect of certain polypeptides, such as gramicidin, may be due to the presence of a *d*-amino acid isomer of a natural amino acid, *l*-leucine, required for bacterial growth.

The antibiotic substance may interfere with the vitamin utilization of the organism. The staling effect of a medium, frequently spoken of in connection with protozoa as "biological conditioning" of the organism, may serve as an illustration. Such effects have been overcome by the addition of a mixture of thiamine, riboflavin, and nicotinamide (373).

The antibiotic agent brings about the oxidation of a metabolic substance

which must be reduced in the process of bacterial nutrition, or otherwise modifies the intermediary metabolism of the bacterial cell.

The agent combines with the substrate or with one of its constituents, which is thereby rendered inactive for bacterial utilization.

The agent competes for an enzyme needed by the bacteria to carry out an essential metabolic process.

The agent interferes with various enzymatic systems, such as the respiratory mechanism of the bacterial cell, especially the hydrogenase system (435) and the phosphate uptake by the bacteria accompanying glucose oxidation, as in the action of gramicidin. Penicillin, for example, was shown (892) to be capable of inhibiting the activity of urease. It was later proved (810a), however, that this was due not to the penicillin itself but to certain impurities in crude penicillin preparations.

The antibiotic substance may inhibit directly cellular oxidations, particularly those involving nitrogenous compounds, an action similar to that of propamidine (494).

The antibiotic substance acts as an enzyme system and produces, in the medium, oxidation products, such as peroxides, injurious to the bacterial cell. The glucose oxidase produced by *P. notatum* (153, 492, 751, 902) catalyzes the following reaction:

$$\text{Glucose} + O_2 \rightarrow \text{Gluconic acid} + H_2O_2.$$

Xanthine oxidase acts in a similar manner (531, 791).

The antibiotic substance favors certain lytic mechanisms in the cell, whereby the latter is destroyed; this mechanism may be either secondary or primary in nature.

The antibiotic substance affects the surface tension of the bacteria, acting as a detergent; tyrocidine lowers the surface tension of the bacterial cell, thereby causing its death, possibly by forming a stable complex with it (189).

The antibiotic substance may interfere with the sulfhydryl group which is essential for cell multiplication. This was shown (254a) to hold true for mercurials and other chemical antiseptics. The possible interrelationship between the sulfhydryl group and true antibiotics has recently been indicated (109a).

On the other hand, bacteria subjected to the action of an antibiotic substance may develop mechanisms that render them resistant to the

action of the substance, and some bacteria and fungi even may produce an enzyme, such as penicillinase, that brings about the destruction of the antibiotic substance.

The antibacterial action of gramicidin was found (391, 401) to be inhibited by a cationic detergent, phemerol, whereas penicillin was not affected by either gramicidin or two cationic detergents, phemerol and zephiran. When gramicidin and penicillin were used together, their effect was only slightly additive (388); however, penicillin and streptothricin exerted a marked additive effect upon bacteria sensitive to both of these substances (287).

The inhibition of the antibacterial action of sulfanilamide by *p*-amino-benzoic acid has been explained by the fact that the latter is a growth factor in bacterial nutrition (538, 813, 1003). Competition for this growth factor between the bacterial cell and the bacteriostatic agent is responsible for the inhibition of the agent. In a similar manner pantoyltaurine, which is related to pantothenic acid as sulfanilamide is to *p*-amino-benzoic acid, will inhibit the growth of hemolytic streptococci, pneumococci, and *C. diphtheriae*, by preventing the utilization of pantothenic acid by these bacteria, for which it is an essential metabolite (558). Fildes (254) emphasized that "chemotherapeutic research might reasonably be directed to modification of the structure of known essential metabolites to form products which can block the enzyme without exhibiting the specific action of the metabolite." The antibacterial activity of iodinin is neutralized by quinones; this is probably due to the destruction of the iodinin, since the N-oxide is reduced by the organism (555, 556). Different anti-inhibitors are known for other antibiotic substances, as shown later.

The concentration of the active substance and the composition of the medium are highly important in modifying the activity of the substance. Some antibiotic substances, like penicillic acid, lose considerable bacteriostatic activity when incubated with sterile broth or with sterile peptone water at *p*H 7 and 37° C. for 1 to 3 days (662); a similar effect was observed with certain simple amines and amino acids. The concentration of the substances reacting with penicillic acid is diminished on autoclaving the peptone broth in the presence of 2 per cent glucose. The neutralizing or anti-inhibiting agent interacts with the antibiotic

substance and neutralizes its antibacterial effect either in the absence or in the presence of the organism.

Since only few antibiotic substances of microbial origin have been isolated in a crystalline state, confusion often resulted from the use of crude preparations. Welsch (971, 972) found that concentrated and partly purified actinomycetin had no appreciable lytic action upon living cells. However, the presence of a small amount of a highly bactericidal substance, which was especially active against gram-positive bacteria, resulted in the lysis of living bacteria by actinomycetin. This action was thus a result of the activity of at least two different agents present in one preparation.

ANTIBACTERIAL ACTION

Two antibiotic agents have recently received special consideration, tyrothricin and penicillin. They will be considered here in further detail.

Tyrothricin

The phenomenon of antibiotic action by a specific substance can best be illustrated by the action of tyrothricin upon bacterial cells. Five distinct stages have been described (190):

1. Inhibition of growth. Certain gram-positive bacteria are inhibited by as little as 1 microgram or less of the substance per 10 milliliters of nutrient broth or agar, thus giving an activity of 1:10,000,000 or more.
2. Bactericidal action consists in the killing of the bacterial cells, either in a washed state and suspended in saline, or in a growing state in broth culture.
3. Lytic activity comprises the rate of lysis of a suspension of bacterial cells. Streptococci, for example, are readily lysed by gramicidin, whereas staphylococci are acted upon more slowly and less completely.
4. Inhibition of enzyme activity includes dehydrogenases or enzymes or respiration. Gram-positive cocci, incubated at 37° C., lose their ability to reduce methylene blue in the presence of glucose, upon addition of gramicidin. Since inactivation of the dehydrogenase takes

place before any morphological changes are observed in the cells, lysis was believed to be a secondary process, following cell injury; hydrolytic enzymes, however, remained unaffected.

5. Protection of animals by the antibiotic substance against infection.

Tyrothricin is made up of two compounds, gramicidin and tyrocidine, that differ in their chemical properties and in their biological activity (205). Gramicidin acts only against gram-positive bacteria, including pneumococci, streptococci, staphylococci, diphtheria bacteria, and aerobic spore-forming bacilli; meningococci and gonococci are not readily acted upon. Tyrocidine affects both gram-positive and gram-negative organisms. Gramicidin causes hemolysis of washed red cells, this hemolytic action being destroyed on heating. Tyrocidine causes lysis of many bacterial species. This action, however, is secondary, autolysis following the death of the cells. Peptones and serum inhibit the action of tyrocidine, but gramicidin is affected only to a limited extent by these agents (391, 579).

Tyrocidine behaves as a general protoplasmic poison. The effect of gramicidin, on the other hand, is reversible. Staphylococci "killed" with gramicidin and no longer able to grow on organic media can be made to grow in the presence of certain tissue components. Gramicidin is, therefore, not considered as a gross protoplasmic poison, but retains a good deal of its activity in animal tissues. When applied locally at the site of the infected area, gramicidin exhibits definite action against infection with pneumococci and streptococci. When injected intravenously, however, it is almost completely inactive against systemic infection.

It was demonstrated by tissue culture technique (401) that the hemolytic effect of tyrothricin was due to the presence of gramicidin. When tyrothricin or gramicidin was heated in an aqueous suspension there was a loss of hemolytic and bactericidal activity. Tyrocidine, which is not very hemolytic, showed no marked toxic effect upon the leucocytic elements of the human blood in amounts up to 100 mg. per milliliter for 8 hours.

Other investigators (728) have reported that the hemolytic activity of tyrothricin is inherent rather in the tyrocidine fraction, although gramicidin also exhibits a definite hemolytic action. The addition of

glucose caused only slight inhibition of the hemolytic effect. Gramicidin was found to be effective, in amounts as low as 1 mg., upon a billion gram-positive organisms, whereas tyrocidine acted in 25 to 50 times that concentration in the absence of inhibitors (435, 436). Tyrocidine appeared to block all the oxidative systems of the bacteria studied, whereas gramicidin seemed to affect only certain individual reactions. Both substances were found to exert a protective antibacterial action in mice infected intraperitoneally with susceptible bacteria; gramicidin protected the animals at a level one-fiftieth as high as that required for tyrocidine. Both substances are toxic to animals when injected into the blood stream; both are leucocytolytic; they show little toxicity when applied locally by the subcutaneous, the intramuscular, or the intrapleural route; oral administration is not accompanied by toxic effects, but such treatment is ineffective (729).

Gramicidin remains active in the blood stream, but it has only weak bacteriostatic properties and no bactericidal action. Tyrocidine is strongly bactericidal but it is inactivated by blood serum, hence it is limited to local applications. No specific effect was exerted by these substances on respiratory or circulatory systems (756).

According to Dubos (189, 201), the retention of the stain by gram-positive bacteria indicates a peculiar property of the cell wall of these organisms. The addition of 0.001 mg. of gramicidin to a billion pneumococci, streptococci, staphylococci, and others is considered sufficient to inhibit the growth of these organisms on subsequent transfers. This effect was said to be due not to an alteration of the protoplasm but to some specific interference with an essential metabolic function. Bacterial cells which have become inhibited under the action of gramicidin become viable again when cephalin is added to the medium. It was suggested that the ineffectiveness of gramicidin on gram-negative bacteria may be due to the presence of a phospholipid in these organisms.

Tyrothricin did not exert any effect upon staphylococcus bacteriophage (633). It did, however, inhibit the fibrinolytic activity of hetahemolytic streptococci as well as of the supernatant liquids of these bacteria but not of partially purified fibrinolysin. Although it prevented the neutralization of hemotoxin by antitoxin, it did not inhibit the protective action of antitoxin against the toxin in mice (64). This substance,

as well as actinomycin and clavacin, inhibited the coagulation of rabbit plasma by staphylococci but did not prevent coagulation by sterile culture filtrates of these organisms; none of these three substances destroyed the toxin, nor did they enhance its hemolytic or lethal action (64).

Different strains of *S. aureus* differ in their susceptibility to the action of tyrothricin. There is apparent adaptation of the organism to increasing concentrations of the substance. A marked increase in resistance of the infecting organism, after several weeks of therapy, was observed in one patient (720). Various other observations have been made (686) that staphylococci grown in the presence of increasing concentrations of gramicidin become resistant to inhibition by this substance (84, 689).

Both gramicidin and tyrocidine are said (196) to be surface-active compounds, their antibacterial action being inhibited by phospholipids. Tyrocidine behaves like a cationic detergent; it is bactericidal in buffer solutions for all bacterial species so far tested, with the exception of the tubercle bacillus; it destroys immediately and irreversibly their metabolic activity, such as oxygen uptake and acid production. For most tissue cells, with the exception of spermatozoa, gramicidin is much less toxic than tyrocidine. It behaves like a specific inhibitor of certain metabolic reactions. It retains much of its activity *in vivo*.

It remains to be determined to what extent the action of tyrocidine, as well as of other antibiotic substances, can be reversed by detoxication with high molecular anions in a manner similar to their action upon surface active cations. The bacteria were said (901) to function as cationic exchanges, both the surface and the adsorbability depending on the structure of the cation. The bacterial action of surface-active cations and of toxic metallic ions and dye cations was considered as a phenomenon of ionic exchange by bacteria. Harmless cations could thus exert a protective action on bacteria against the toxic cations.

Penicillin

Although penicillin is active primarily on gram-positive bacteria, it also has an effect on certain gram-negative bacteria, but not on the colon organism, *Hemophilus*, or *Brucella*. The gram-negative cocci can be

divided into two groups, on the basis of the action of penicillin: first, *Neisseria gonorrhoea, Neisseria intracellular,* and *Neisseria catarrhalis,* which are sensitive; and second, *N. flavus* and other nonpathogenic Neisseriae, which are not sensitive (261, 266, 267).

Different strains of *S. aureus* vary little in their susceptibility to the action of penicillin; however, by growing the organism in increasing concentrations of this substance, it is possible to obtain more resistant cultures. Strains of staphylococci possessing increasing resistance to penicillin were also isolated from infections treated with the substance (727).

The oxygen uptake of suspensions of staphylococci was not inhibited to any extent by the action of penicillin for 3 hours. In a concentration of 1:1,000, after incubation for 24 hours at 37° C., the bacteria gave larger numbers of colonies on plating (7). Although 0.01 to 0.1 mg. of penicillin per milliliter was found (418–424) to be sufficient to inhibit the growth of 2,500,000 hemolytic streptococci (Group A), no conclusion could be reached as to whether its action is truly bactericidal or bacteriostatic.

A comparison was made of the amounts of penicillin and gramicidin required to bring about total inhibition of growth of bacteria, on the basis of micrograms per milliliter of culture medium (rabbit's plasma and a serum extract of chick embryo). The results were as follows:

	PENICILLIN	GRAMICIDIN
D. pneumoniae	2.5–5.0	0.5–1.0
S. pyogenes	2.5	5.0
S. salivarius	20–40	2.5–60
S. faecalis	200*	40–60
S. aureus	2.5–10	300*

* Inhibition not complete at these figures.

The two substances appeared to be as effective against bacteria in cultures containing growth tissue as in cultures in which no tissue was present (388, 389).

Inhibition of growth of 2 to 4 million hemolytic streptococci was obtained by the use of 0.03 γ penicillin with an activity of 240 to 250 Oxford units per milligram (418–424). Peptone, *p*-amino-benzoic acid,

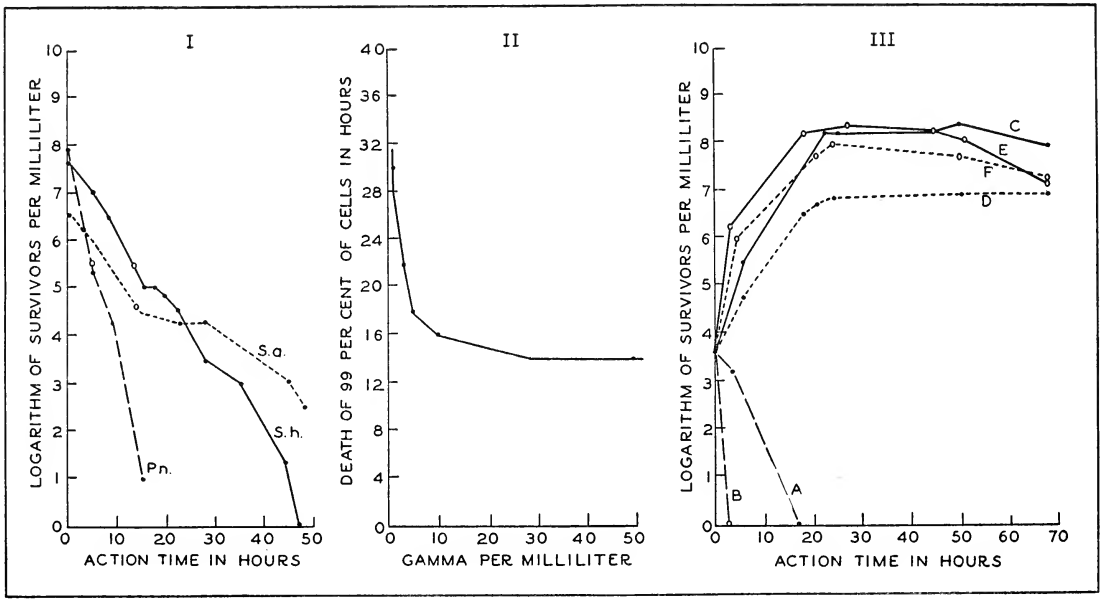


FIGURE 21. Bacteriostatic action of penicillin and of sulfathiazole. I. Action of penicillin, 50 γ per milliliter, on *S. albus* (S.a.), *S. hemolyticus* (S.h.), and *Pneumococcus* (Pn.). II. Effect of increasing amounts of penicillin on *S. albus*. III. Action of penicillin and sulfathiazole on *S. hemolyticus:* A. Penicillin, 100 γ per milliliter in broth; B. Penicillin, 100 γ per milliliter in blood broth; C. Broth control; D. Sulfathiazole, 100 γ per milliliter in broth; E. 50 per cent blood broth control; F. Sulfathiazole, 100 γ per milliliter in blood broth. From Hobby et al. (423).

blood, or serum exerted no inhibiting effect. A marked difference was found in the action of penicillin and sulfonamides, the latter merely decreasing the rate of multiplication and the former actually bringing about a decrease in the number of organisms present. This is brought out in Figure 21. The rates of activity of penicillin, gramicidin, and tyrocidine are compared in Table 36 (page 192). The bactericidal action of penicillin is not accompanied by lysis. No penicillin is absorbed or destroyed by the bacteria.

Penicillin is not very stable; it is sensitive to reaction and temperature changes. The effect of reaction upon the stability of penicillin is shown in Figure 22.

Para-amino-benzoic acid and sulfapyridine were found to have a synergistic effect on penicillin. A solution of sodium penicillin with 1,200 units per milliliter gave 100 *B. subtilis* units in a synthetic casein

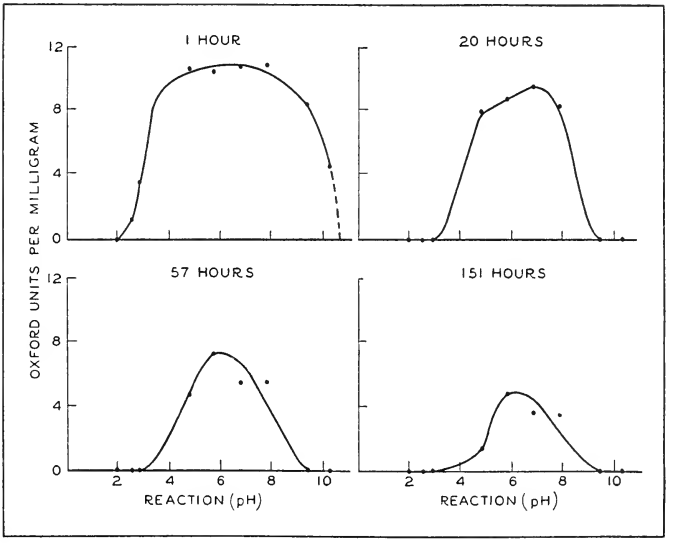


FIGURE 22. Inactivating effect of reaction upon penicillin. From Foster and Wilker (283).

hydrolyzate medium; the activity was increased to 6,000 by addition of *p*-amino-benzoic acid in dilution of 1:2,500 to 1:10,000; this was also true in presence of glucose in test medium. A similar, although somewhat lower, increase took place in case of *S. aureus;* no effect was obtained on *S. hemolyticus*. The addition of a dilute solution of sulfapyridine, which in itself had little inhibiting effect, exerted an even greater synergistic action upon penicillin. This effect was exerted not only *in vitro* but also *in vivo* (896).

Attention was directed previously to the production by *P. notatum* of an oxidative enzyme. It is interesting to find that *P. chrysogenum* also forms an oxytropic glucose-dehydrogenase that is not susceptible to CO and cyanide (523); the glucose is oxidized to gluconic acid. The active substances produced by both groups are thus similar (832).

Other Antibiotic Agents

Of the other agents, actinomycin, streptothricin, clavacin, gliotoxin, and several other mold products deserve particular attention.

Actinomycin is a bacteriostatic agent, active primarily against gram-positive bacteria. It is extremely toxic to animals, a factor which limits its practical utilization. One milligram of actinomycin given to mice, rats, or rabbits intravenously, intraperitoneally, subcutaneously, or orally proved (757) to be lethal for 1 kilogram weight of the animals. Doses as small as 50 γ per kilogram injected intraperitoneally daily for 6 days caused death accompanied by severe gross pathological changes, notably a marked shrinkage of the spleen. Actinomycin is rapidly removed from the blood and excreted. It has no effect upon bacteriophage or staphylococci, although 0.1 milligram per cent inhibits growth as well as blood coagulation by these organisms (633).

A comparison of the effect of actinomycin with that of tyrothricin and its constituents, tyrocidine and gramicidin, upon the growth of rhizobia (890) showed that, whereas gramicidin inhibited all strains alike, the other three substances inhibited the slow-growing rhizobia much more than the fast-growing ones. Effective and ineffective strains behaved alike. Of the four antibiotic substances, tyrocidine was usually bactericidal, actinomycin was bacteriostatic, and the other two pos-

sessed both properties. Some strains of rhizobia were stimulated by limited concentrations of actinomycin.

Streptothricin is far less toxic than these four substances. It acts largely upon gram-negative bacteria, and thus is quite distinct from the other four antibiotic agents. In this respect, it is similar to clavacin, which, however, is far more toxic.

Clavacin not only is bacteriostatic on gram-negative bacteria but possesses marked bactericidal properties, as is brought out in Table 37 (page 192) and in Figure 23. Fumigacin, on the other hand, is active only upon gram-positive bacteria and has far more limited bactericidal action.

Gliotoxin is active against both gram-positive and gram-negative bacteria (Table 38). The substance is rather toxic to animals, the minimum lethal dose being 45 to 65 mg. per kilogram body weight; hematusia is caused by even lower concentrations (459).

TABLE 38. BACTERIOSTATIC ACTION OF GLIOTOXIN

TEST ORGANISM	ACTIVITY
S. aureus	4,000,000
S. pyogenes	1,000,000
Pneumococci	4,000,000
S. enteritidis	250,000
A. aerogenes	200,000
K. pneumoniae	250,000
E. coli	80,000

From Johnson, Bruce, and Dutcher (459).
Note. Units of activity by dilution method.

On comparing the action of citrinin with penicillic acid, the first was found (661) to act largely upon gram-positive bacteria; the second, like quinones, had a more widespread action, especially against the colon-typhoid group.

BACTERIOSTATIC AND BACTERICIDAL AGENTS

Fleming (268) divided all selective bacteriostatic agents, exclusive of the action of oxygen on anaerobic bacteria, into three groups: (a)

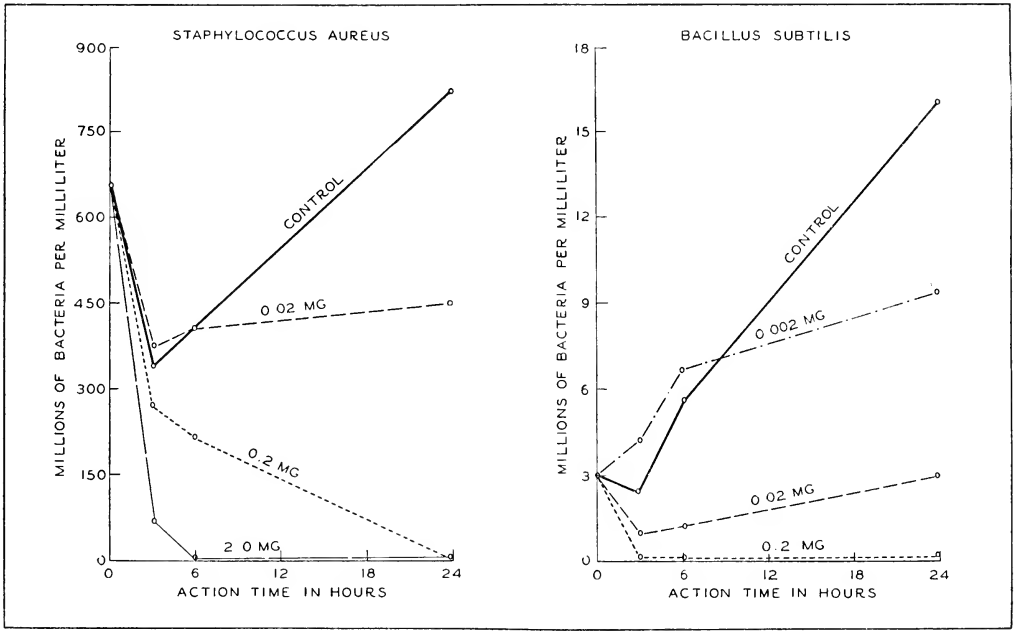


FIGURE 23. Bactericidal action of various concentrations of clavacin on *S. aureus* and *B. subtilis*. The cultures were 18 hours old. From Waksman, Horning, and Spencer (935).

physiological agents, including bile, serum, proteolytic enzymes, and lysozyme; (b) microbiological products, comprising the antibiotic substances; (c) chemicals of known composition, including dyes, salts (potassium, tellurite, mercuric salts), and other agents.

Concentrations of antibiotic substance smaller than those needed to cause inhibition often stimulate growth of the organisms or of their metabolic processes (705). In this respect these agents are similar in their action to synthetic detergents (35) and to other chemical disinfectants, as discussed previously. It may also be of interest to note here that the antibacterial action of straight-chain mono-amines and di-amines, amidines, guanidines, and quaternary bases increases with the length of the chain up to a maximum and then decreases, the gram-positive bacteria being more sensitive than the gram-negative organisms. Serum increases the activity of the shorter-chain compounds and decreases that of the longer-chain compounds, depending to a considerable extent upon the test bacteria (303).

The bactericidal action of antibiotic agents, as influenced by their concentration, can be illustrated by the action of pyocyanase (Table 39). In a study of the bactericidal action of actinomycin (946) it was found that the addition of 0.5 mg. of actinomycin to a 10 ml. suspension of *E. coli* reduced the number of viable cells from 6,400,000 to 493,000, the methylene blue reduction test remaining positive; 1 mg. actinomycin reduced the number of cells to 4,800, the reduction test becoming negative; 2 mg. of the agent brought about complete de-

TABLE 39. BACTERICIDAL ACTION OF PYOCYANASE UPON THREE BACTERIA

B. ANTHRACIS		E. TYPHOSA		M. TUBERCULOSIS	
Hours	Bacteria per milliliter	Hours	Bacteria per milliliter	Hours	Bacteria per milliliter
Start	11,060,000	Start	13,125,000	Start	2,105,000
24	6,890,000	3	1,242,000	3	980,000
72	1,360,000	9	105,000	8	71,500
96	654,000			24	0
120	329,000				
144	0				

From Emmerich, Löw, and Korschun (237).

struction of all the cells. The bactericidal action of actinomycin seems to be a result of a chemical interaction, similar to that of other antiseptics. On adding 0.1 mg. actinomycin to a suspension of *E. coli* cells in a 10 ml. buffer solution, the value of the constant K was found to vary from 0.021 to 0.026 for different periods of incubation. Figure 24 illustrates graphically the effect of different concentrations of actinomycin on the death rate of *E. coli* in buffer solution.

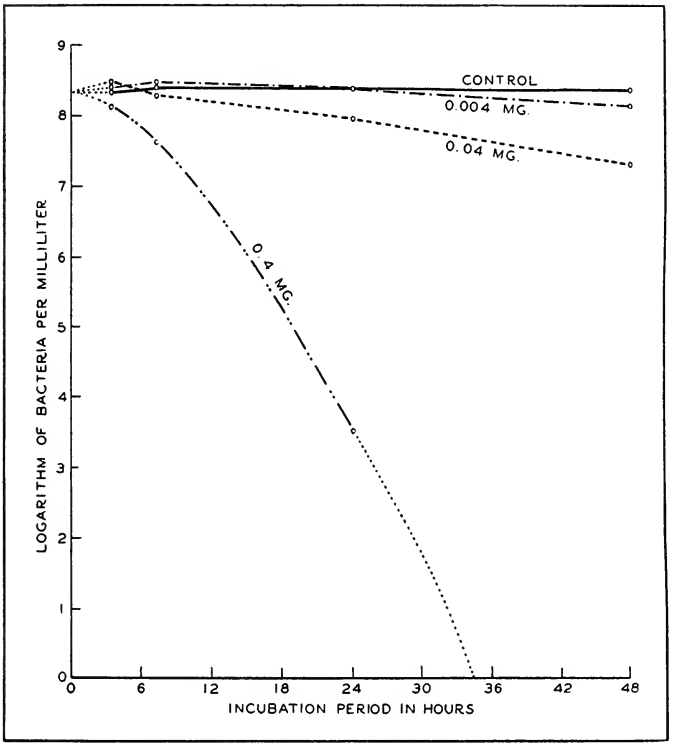


FIGURE 24. Action of actinomycin on *E. coli*; death rate in buffer solution. Amounts are given in milligrams of actinomycin per 10 milliliters of solution. From Waksman and Woodruff (948).

Quinones have a high bactericidal power (145, 147, 615, 948), due not to their chemical interaction with the cell proteins but to their reactivity with the simpler cell constituents such as some of the amino acids (146, 148). Only a slight difference was found in the apparent activity of quinones toward yeasts, bacteria, proteins, peptones, peptides, and certain amino acids. Alcohol increases the germicidal power of the quinones. Actinomycin contains a quinone group; however, it acts differently toward gram-positive and gram-negative bacteria; alcohol has no effect upon its action, thus pointing to marked differences in chemical and biological nature of this antibiotic agent and of quinones. On the other hand, many of the antibiotic substances produced by fungi are typical quinones and act as such.

By varying the concentrations of disinfectants, the types of curves of destruction of bacterial cells were found (694) to range from linear to an abrupt drop to zero at critical concentrations. This is brought out in a study of the spirocheticidal action of penicillin (Figure 25).

In general, chemical disinfectants act upon bacteria in four different ways: (a) some affect the lag phase of the growth period, (b) some influence the mechanism of cell division, (c) some influence the metabolic processes, and (d) some affect the death rate of the microbes. Similar, if not greater, variations are found in the nature of the action of antibiotic substances of microbial origin upon the bacterial cell.

Gramicidin inhibited dehydrogenase activity, since the antagonized bacteria rapidly lost their capacity to reduce methylene blue in the presence of glucose. *E. coli* suspension treated with actinomycin lost its capacity to reduce methylene blue before the cells were completely killed. The oxidation of succinic acid by tissue preparations, which require the cooperation of succinic dehydrogenase and a cytochrome system, was strongly inhibited by pyocyanin. This inhibition exhibited certain interesting peculiarities: in low concentrations, pyocyanin strongly inhibited the activity of the complete succinic cytochrome system but had no effect on the oxidation of succinic acid through methylene blue; in the presence of KCN, pyocyanin acted as an autoxidizable hydrogen acceptor similar to methylene blue; glutaminic acid did not affect the inhibitory action of pyocyanin. This inhibitory action was found to be due not to the formation of oxalacetic acid but to a direct effect on

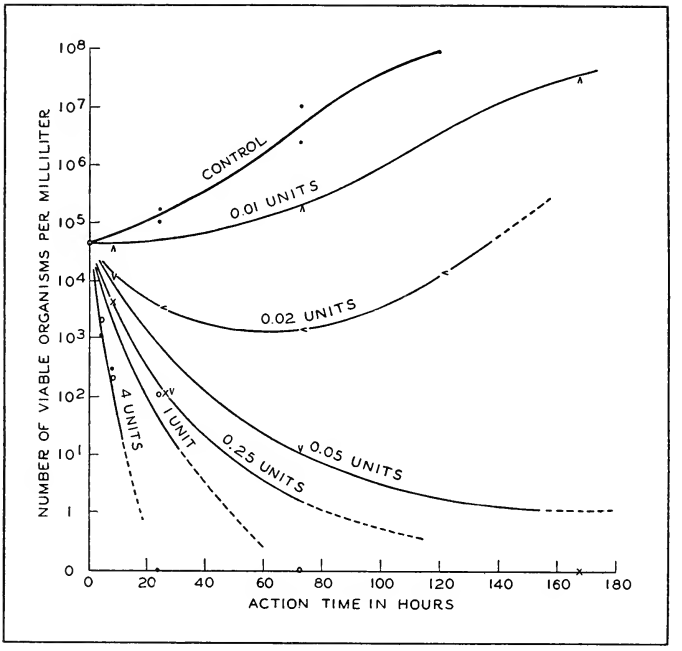


FIGURE 25. Spirocheticidal action of various amounts of penicillin *in vitro*. Inoculum, 4 x 10^4 organisms per milliliter. From Eagle and Musselman (unpublished).

succinic dehydrogenase. The influence of pyocyanin on bacterial respiration, as well as its ability to function as an accessory respiratory enzyme, has aroused much interest (227, 301, 854).

A strong lytic action of some of the antibiotic substances, similar in some cases to the action of enzymes, has also been indicated. This lytic mechanism may be a product of the antagonized cell itself. It is to be recalled that autolysis has usually been defined (865) as "the breaking down and solution of some of the essential chemical constituents of the cell by agencies (enzymes) originating within the cell." This does not hold true, however, for most of the antibiotic substances.

The relation between antibiotics and bacteriophage has attracted considerable attention. Gratia (345) observed a definite relation between the action of lysozyme and the liberation of bacteriophage. The action of antibiotic agents, however, usually exhibits a marked distinction from that of bacteriophage (218, 344, 634). Filtrates of cultures of homologous bacteria are able to inactivate the anti-coli phage; at 27° C., the inactivation is proportional to the phage and filtrate concentration; at 0° C., to the square root of the latter (232). Based upon the formation of iso-antagonistic substances, a method has been suggested (121) for the differentiation of bacteria belonging to the typhoid group.

EFFECT OF ANTIBIOTIC SUBSTANCES UPON THE MORPHOLOGY OF MICROORGANISMS

Emmerich and Saida (238) were the first to report that anthrax bacteria undergo morphological changes as a result of the action of pyocyanase. Since that early work, the effect of bacterial filtrates upon cell multiplication and cell growth has been made the subject of many investigations. It was reported (378), for example, that no complete cessation of the fission process of bacteria results from the action of the substance, but that growth itself is checked, the action being nonspecific as far as bacterial species are concerned. The conclusion was reached that this phenomenon is due to the production and accumulation of metabolic products injurious to growth. Nonspecific antibiotic substances were demonstrated (656) in filtrates of bacteria. They not only injured growth of other bacteria but prevented the production of the ectoplasmic antigen. These substances could be partly removed by the use of adsorbents.

The morphology of bacteria is greatly influenced by the presence of other organisms or their antibiotic substances. In the case of diphtheria bacteria this is accompanied by a reduction in virulence (406). The specific effect of the antagonistic *B. mesentericus* upon the morphology of antagonized bacteria has been established by Pringsheim (705). The antibiotic substances produced by actinomycetes were shown (80) to affect the growth of *B. mycoides* as follows: cell division is delayed; the cells become elongated, reaching enormous size and assuming most pe-

culiar forms; spore formation or, with lower concentrations of agent, the active substance is repressed; delayed nonspore-forming variants are produced with a modified type of growth on nutrient media (Table 40).

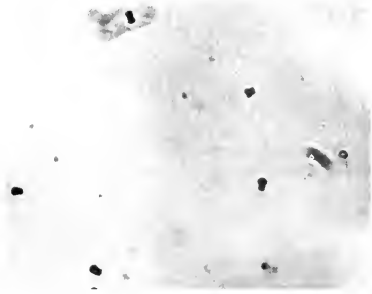
TABLE 40. INFLUENCE OF CULTURE FILTRATE OF STREPTOMYCES SP. ON MORPHOLOGY OF BACILLUS MYCOIDES

DAYS OF INCUBATION	MORPHOLOGY OF ANTAGONIZED BACTERIUM	MACROSCOPIC GROWTH IN BROTH	SPORE FORMATION	ROD FORMATION
MEDIUM PLUS 10 PER CENT CULTURE FILTRATE				
2	Long filaments	x	−	+
4	Filaments have divided into elongated cells	x	−	+
17	Cells altered	x	−	+
45	Cell fragments of various shape and length	x	−	−
MEDIUM PLUS 5 PER CENT CULTURE FILTRATE				
2	Elongated cells	x	−	+
4	Elongated cells	x	−	+
17	Greatly deformed cells	+	−	+
45	Greatly deformed cells	+	−	+
CONTROL MEDIUM				
2		++	−	+
4		++	+	+
17		++	+	+
45	Deformed cells rare	++	+	−

From Borodulina (80).
x indicates growth of *B. mycoides* in the shape of fluffy small balls inside liquid.

Gardner (308) reported that concentrations of penicillin lower than those required for full inhibition caused a change in the type of growth of *Cl. welchii* in liquid media. The majority of the cells became greatly elongated, giving rise to unsegmented filaments ten to twenty times longer than the average normal cells. The same was found to hold true for a number of other bacteria (Figure 26). Even gram-negative bacteria, which are relatively resistant to penicillin, showed the same effect. Many bacteria produced giant forms as a result of the autolytic

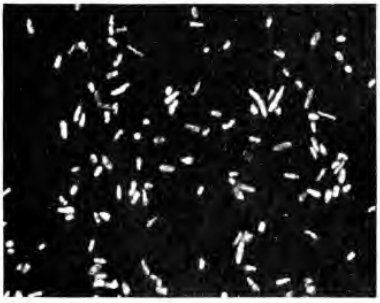
S. aureus, normal cells. Prepared by Foster and Woodruff



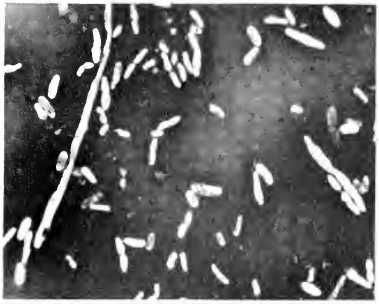
S. aureus, penicillin-inhibited cells. Prepared by Foster and Woodruff

B. subtilis, normal cells. Prepared by Foster and Woodruff

B. subtilis, penicillin-inhibited cells. Prepared by Foster and Woodruff



Az. vinlandii, normal cells. Prepared by Starkey



Az. vinlandii, actinomycin-inhibited cells. Prepared by Starkey

FIGURE 26. Influence of antibiotic substances upon the morphology of bacteria.

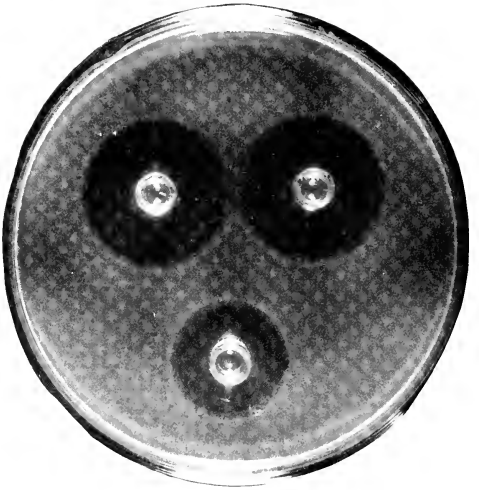


FIGURE 27. Mechanism of antibacterial action as illustrated by the gradual diffusion of an antibiotic substance in a bacterial agar plate. Effect of streptomycin on *B. subtilis*.

swelling and bursting of the elongated cells. It was recognized that these changes were due to a failure of fission. Cell growth not accompanied by cell division underwent autolysis. *Br. abortus* and *Br. melitensis*, which were not inhibited by penicillin even at 1:1,000 dilution, gave no enlargement of the cells but showed vacuolation even in lower dilutions. *Cl. welchii*, which was inhibited by 1:60,000 penicillin, showed filament formation in a dilution of 1:1,500,000. The phenomena of swelling and lysis were said (833) to be associated with the active growth of the bacterial cell. Suspensions of fully grown bacterial cells showed neither of these effects when added to concentrations of penicillin many times higher. It was suggested that penicillin either has some action on the cellular wall of *S. aureus* or that it interferes with the assimilation of one or more growth factors necessary for the fission of the growing cell.

A growth-depressing substance, which altered the type of growth of both fungi and bacteria, was also isolated (144) from yeast. Fungi treated with this substance produced thick gnarled mycelia and formed no conidia or pigment. Increasing the concentrations of the depressing agent changed the nature of the colony of *E. coli* from smooth to rough and finally to grainy; this was associated with an increase in the length of the cell and the formation of filaments. When the cultures thus modified were placed in media free of the agent, normal, highly motile cells were again produced.

The mechanism of disintegration of the hyphae of a plant pathogenic fungus *Rhizoctonia* by an antagonistic fungus *Trichoderma* as well as by the antibiotic product of the latter has been described by Weindling (962). The hyphae are usually killed in less than 10 hours, as shown by loss of the homogeneous appearance of the protoplasm and of the vacuolate structure of the hyphae, which either become empty or as if filled with granular material.

ANTIBIOTIC SUBSTANCES AND THE PHYSIOLOGY OF THE BACTERIAL CELL

Half a century ago Smith (838) emphasized that bacteria growing in mixed cultures undergo temporary and even permanent physiologi-

cal modifications. Aside from cell proliferation, the important metabolic processes commonly considered to be affected by antibiotic agents were oxygen uptake, acid production, and dehydrogenase activity. Some agents apparently can inhibit cell growth without destroying the viability of the cells and their capacity for taking up oxygen.

Gramicidin and tyrocidine were believed to affect bacteria (390, 391, 579) by depressing the surface tension of aqueous solutions. This effect was favored by the addition of organic solvents such as glycerin, which increases the solubility of gramicidin. The addition of serum resulted in a decrease in activity of tyrocidine, to a less extent, however, than of gramicidin. Heat destroyed the bacterial and hemolytic effects of gramicidin, but the property of altering surface tension was heat-stable. It has further been shown (395) that gramicidin, after an initial stimulation, inhibited oxygen consumption of bovine spermatozoa and rendered them immobile; aerobic as well as anaerobic glycolysis was depressed by about 40 per cent and motility of the spermatozoa impaired. Tyrocidine, however, caused a small reduction in the oxygen consumption and in glycolysis. The action of gramicidin upon the metabolic activities of *S. aureus* and *S. hemolyticus* was shown (206) to be influenced by the composition of the medium, the presence of potassium and phosphate ions giving a prolonged stimulation of metabolism, whereas ammonium ions favored a depression in oxygen uptake.

The specific effects of basic proteins, such as protamine and histone, upon the activity of selective inhibitors offered a possible explanation for the difference in the action of tyrothricin upon gram-positive and gram-negative bacteria (606). These basic proteins also possess antibacterial properties. They have the capacity of sensitizing gram-negative bacteria by means of substances which otherwise act only on gram-positive forms. This is brought out in Figure 28.

Pneumococci grown in media containing the specific enzymes which hydrolyze their capsular material are deprived of these capsules and fail to agglutinate in the specific antiserum. The enzymes do not interfere with the metabolic functions of the cells, but their action is directed essentially against the capsule (193). These enzymes were found not only to exhibit great selectivity but to be highly specific against the particular polysaccharides.

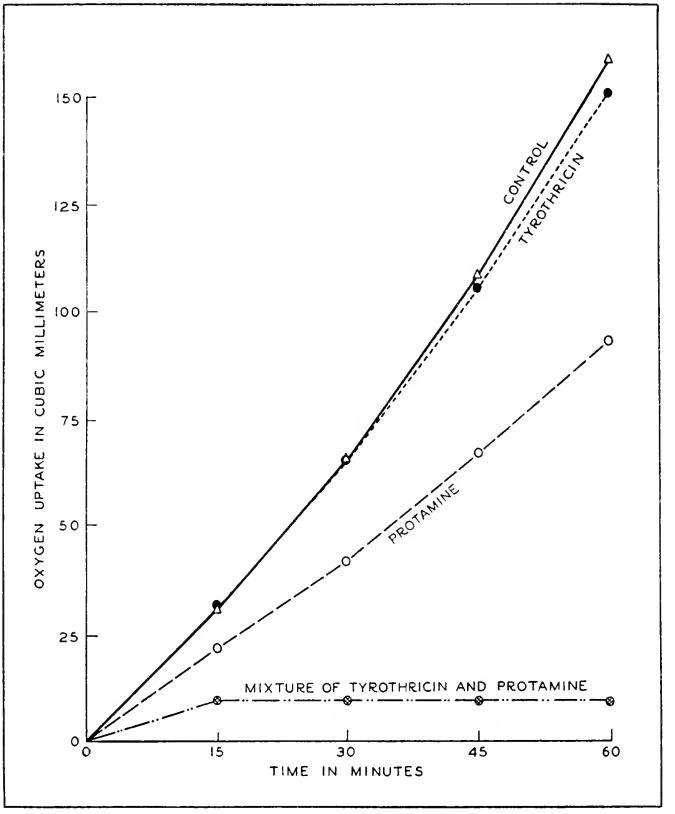


FIGURE 28. Effect of tyrothricin and protamine on the respiration of *E. coli* at 38° C. and *p*H 5.3. Number of cells per vessel: 5 x 10^9. Concentrations: tyrothricin 1:15,000; protamine 1:3,000; mixture, tyrothricin 1:15,000, protamine 1:3,000. From Miller, Abrams, Dorfman, and Klein (606).

INHIBITION OF BACTERIOSTATIC AND BACTERICIDAL ACTION

The formation of specific chemical compounds capable of inhibiting, inactivating, or even destroying bacteriostatic and bactericidal substances of microbial origin has been established for a number of antibiotic agents.

Yeasts were found to contain a substance which inhibits the action of sulfanilamide against *S. hemolyticus* as well as other streptococci and pneumococci. This substance has been identified (771) with the *p*-amino-benzoic acid referred to above. No relationship could be established, however, between the growth-promoting properties and anti-sulfanilamide activity of the yeast extract (544).

Br. abortus and certain other bacteria also contain (354, 355) a factor, designated as "p," which specifically inhibits the bacteriostatic action of sulfanilamide. This factor stimulates markedly the growth of many bacteria, and is not specific. The sensitivity of sulfanilamide depends on the rate of release of the factor from the bacterial cell and not on the total amount produced. This factor was believed to stimulate some enzyme reaction concerned with bacterial reproduction, whereas sulfanilamide inhibits this reaction. Similar factors have been isolated from yeast (870) and from hemolytic streptococci (538). It is also known that certain substances, like methionine, inhibit the growth-stimulating effect of biotin (259).

To what extent antibiotic substances can be inhibited in their action against bacteria still remains to be determined. Certain few facts have so far been established.

Bacteria not inhibited by penicillin were found (4, 1000) to be capable of producing a substance which destroys the growth-inhibiting property of the antibiotic agent. The penicillin-destroying substance is believed to be an enzyme, since it is destroyed by heating at 90° C. for 5 minutes. It was designated as "penicillinase." The optimum *p*H of its action was found to be 8 to 9. The presence or absence of this enzyme in bacteria is independent of the sensitivity of the organism to penicillin. Bacterial extracts, pus fluids, peptone, and *p*-amino-benzoic acid, which interfere with the action of sulfonamides, do not affect penicillin.

Cephalin and extracts of gram-negative bacteria, of milk, and of

blood serum were found to inhibit the action of gramicidin. Because of this, cephalin is capable of reviving bacterial cells killed with gramicidin. This phenomenon is similar to the inhibition by phospholipins of the action of synthetic detergents upon bacterial metabolism (35). Mucin inhibits the action of tyrothricin, especially on meningococci and pneumococci (182).

Tannic acid is able to neutralize the antibiotic action of actinomycin. Humus compounds of the soil have a similar capacity. This effect was believed to be responsible for rendering harmless, to living plant and animal forms, the actinomycin produced in the soil (947). Ascorbic acid also has an effect in reducing the activity of this substance. Jungeblut (466) demonstrated that vitamin C, interacting with atmospheric oxygen, leads to the production of destructive peroxides in a medium. Since vitamin C is a strong reducing agent and actinomycin is a reversible oxidation-reduction system, it is conceivable that actinomycin may be reduced through the action of the vitamin. Such an effect should be greatly increased under anaerobic conditions, where no reoxidation due to atmospheric oxygen could occur. Twenty-five to 50 times as great a neutralizing effect of ascorbic acid upon actinomycin was obtained under anaerobic conditions with *Cl. butyricum* as a test organism as under aerobic conditions with *B. mycoides*. Reduced actinomycin was inactive. It was concluded, therefore, that the neutralizing action of ascorbic acid upon actinomycin was due merely to its reducing properties. As far as the common growth factors are concerned, the action of actinomycin, like that of penicillin, differs from the mechanism proposed by Woods (1003) and others for sulfanilamide inhibition.

DIFFERENTIATION OF BACTERIA BY MEANS OF ANTIBIOTIC SUBSTANCES

Because of their selective action upon different bacteria, antibiotic substances can be utilized for separating bacteria from one another. Fleming (269) utilized penicillin to isolate Pfeiffer's bacillus and the pertussis organism of various cocci, diphtheria, and neisseria organisms; the substance was found to behave as the mirror image of tellurite in inhibiting specific bacteria. Penicillin was also utilized for the separation

of acne bacilli from accompanying staphylococci (153) and for the separation of streptococci from staphylococci (266).

Actinomycin was used to separate gram-negative from gram-positive bacteria (945).

Tyrothricin has been utilized (858) for the isolation of *N. gonorrhoeae* from contamination with gram-positive bacteria. Usually a 1:15,000 dilution of the substance in "chocolate" agar was found quite satisfactory for this purpose. By the use of tyrothricin, gram-negative microorganisms have been isolated from the nasopharynx even in the presence of overgrowth by gram-positive cocci. *Hemophilus influenzae* is resistant to the action of tyrothricin, and its isolation is facilitated by adding to the media on which it is cultured tyrothricin in a dilution which inhibits the cocci (808).

Streptothricin was found to help in distinguishing *B. mycoides* from *B. subtilis* (950).

SUMMARY

Comparatively little is yet known of the mode of action of antibiotic substances. This field offers great opportunities for research and utilization of bacterial activities. The solution to such important problems as the morphology of the bacterial cell, taxonomic relations of bacteria, various physiological reactions of microorganisms, the mechanism of causation of disease, and the very control of disease-producing microorganisms—all fundamental problems in microbiology—will be furthered by knowledge of the action of specific antibiotic substances upon bacteria and other microorganisms.

CHAPTER 12

UTILIZATION OF ANTAGONISTIC MICROORGANISMS AND ANTIBIOTIC SUBSTANCES FOR DISEASE CONTROL

Whether gramicidin or any other product of microbic origin will eventually be found to fulfill certain purposes better than either sulphonamides or any other class of antiseptic remains to be seen. That several classes of reagent should be competing for supremacy in different aspects of a task which not long ago was considered impossible of any real fulfilment is a truly remarkable position (343a).

Microorganisms and products of their metabolism have been utilized for the control of disease in man, animals, and plants with varying degrees of success. As early as 1877, Pasteur (675) noted that the production of anthrax in susceptible animals can be repressed by the simultaneous inoculation with *B. anthracis* and various other bacteria. This led him to make the following significant suggestion: ". . . on peut introduire à profusion dans un animal la bactéridie charbonneuse sans que celui-ci contracte le charbon: il suffit qu'au liquide qui tient en suspension la bactéridie on ait associé en même temps des bactéries communes."

Pasteur may thus be looked upon as the first to advance the subject of bacteriotherapy. Emmerich (233) reported that anthrax can be controlled by the use of streptococci such as the erysipelas organism; these bacteria were, therefore, looked upon as agents useful in bringing about immunity against all bacterial infections. Pawlowsky (676) obtained immunity against anthrax by inoculation with Friedländer's bacillus. Bouchard (81) was successful in the control of anthrax by means of *Ps. aeruginosa*. This organism, however, did not impart any immunity to the animals, but by the use of a sterilized ten-day-old culture of the antagonist, healing action was obtained against anthrax infection, or at least a delay in the course of its development. Rabbits infected with anthrax were also cured by means of a pyocyanase preparation (872); many other cases of successful treatment of anthrax with pyocyanase

have been reported (280). The pressed extract of *Ps. aeruginosa* had a similar effect when injected in the animal simultaneously with the pathogen (505).

Various methods of treating severe infections, like anthrax (97) or malignant tumors (138), with mild infective agents have been suggested. The reduction in pathogenicity of one organism by the presence of others has thus been well recognized (62, 120, 239). Nonpathogenic organisms apparently have specific effects upon the pathogens, the development of which was prevented or even suppressed. The very occurrence of specific types of pneumococci in healthy individuals and the causation of specific forms of pneumonia were found to be controlled by the antagonistic effects of other microorganisms (369).

It was thus definitely established that the growth of *B. anthracis* could be inhibited by antagonists (48). Guinea pigs survived large injections of washings from soil previously contaminated by *B. anthracis* through the slaughtering of a diseased cow. When cultures of this organism were isolated from the soil and injected, however, characteristic disease symptoms resulted. It was suggested that the anthrax spores are digested by the leukocytes which have been attracted to the site of injection by the accompanying bacteria (31).

Seitz (812), in discussing the problem of mixed infections, cited many cases not only of decreased but also of increased virulence of the pathogen as a result of accompanying bacteria. He warned, therefore, against too sweeping generalizations concerning the healing effect of antagonistic bacteria. He believed that in many cases of artificial infection, the favorable action of the antagonist may have been due entirely to increased body resistance. Nevertheless, he accepted the possibility of utilizing the antagonistic effects of microorganisms, provided it did not concern tissue or blood infection, but only skin surfaces, including those of the intestinal canal and the vagina.

Until very recent years, attempts to utilize the activities of antagonistic microorganisms for the control of disease did not always meet with success. This failure may have been due to an insufficient understanding of the nature of the chemical agent produced by the antagonizing organism, to a lack of knowledge concerning the mechanism of its action,

or to the variability of the antagonizing agent as regards strain specificity and the production of the active antibacterial substance.

MICROBIAL ANTAGONISTS AND DISEASE CONTROL

In 1885, Cantani treated a tubercular patient with a culture of a saprophytic organism, designated as *Bacterium termo;* the results were highly favorable (104). He expressed the hope that other infectious diseases readily accessible and of a local nature could be effectively treated with saprophytic bacteria which are antagonistic to the pathogens. Following this work of Cantani, Zageri (1009) inoculated *S. pyogenes* into animals suffering from anthrax; the rise in temperature caused by the streptococcus reduced the viability of the anthrax. The growth of an antagonistic organism was found to change the environmental conditions favorable to the pathogen, thus causing its attenuation. These results received the immediate attention of other investigators (226, 844).

Gaté and Papacostas (318) observed that mixed infections were usually mild, a phenomenon later confirmed. Mixed cultures of the Friedländer bacillus and of *C. diphtheriae* gradually gave a predominance of the former on repeated transfer; the morphology of the diphtheria organism changed toward a more homogeneous state on staining. The use of culture filtrates gave no evidence that the diphtheria toxin was neutralized by the antagonist, either *in vivo* or *in vitro;* however, when the two organisms were grown together no toxin was formed, nor was toxin produced when the filtrate of the culture of the antagonist was used to grow *C. diphtheriae*. The therapeutic use of filtrates was, therefore, suggested. Lactic acid bacteria were also employed successfully (636) in the treatment of diphtheria.

By allowing an antagonist to act upon a disease-producing organism that has previously been heated to 56° C., a hydrolyzate was obtained which could be employed as a vaccine (347). Bezançon (53) treated typhoid sufferers with a culture of *E. typhosa* lysed by means of *Ps. aeruginosa*. Gratia (347) said, however, that this type of hydrolyzate will bring about heat production, but the use of a preparation obtained

by means of an *Actinomyces*, designated as a mycolysate, will not. The use of living proteolytic bacteria (neocolysin) for treatment of chronic purulent conditions, such as osteomyelitis, gave favorable results; the bacteria were believed to continue growing as long as there was dead tissue available (99).

Besredka (51) used culture filtrates of bacteria for the treatment of various diseases in man. A filtrate of the anthrax organism was employed for dressings or for intracutaneous injections; the results were at least as good as those obtained with the bacterial vaccine. Staphylococci and streptococci were also utilized for similar purposes. Besredka believed that a substance, designated as antivirus, was secreted by the bacteria into the filtrate. This was said to check further growth of the bacteria. The mode of action of the antivirus was considered to be different from that of antibodies: the first affects the cells locally by stimulating their resistance; the second acts upon the organism as a whole and, through it, against the infecting agents. Antivirus was prepared by allowing bacteria to grow in ordinary bouillon for a long time, until the medium became unfavorable for further development of the bacteria. Staphylococcus antivirus prevented the growth of the staphylococcus organism in a medium in which it had grown previously. In the presence of the homologous antivirus, the organisms underwent active phagocytosis, this action being specific. The antivirus was nontoxic and could withstand a temperature of 100° C. It imparted to certain tissues a local immunity against the specific bacteria.

The favorable therapeutic results obtained from the use of antivirus have been confirmed, largely in France, Austria, and Germany. The antivirus apparently acts not upon the bacterium but upon the tissue of the host in such a way as to produce local immunization, thus preventing infection. Although unspecific filtrates may cause an occasional increase of resistance, the protection produced by specific filtrates is said to be more intense and more dependable (653, 741). Antivirus therapy was believed to offer some promise, although it was said not to give consistent results (381). Further studies of antivirus led to suggestions that its favorable effects were due entirely to the culture medium (6). The whole question thus appears to be still debatable, with proponents and opponents of the specific nature of the antivirus effect (110, 589).

The application of bacteriotherapy for the treatment of chronic infections of the middle ear (706) and actinomycosis in man has also been suggested. Filtrates of *E. typhosa* and of *E. coli* were found (810) to check the growth of the typhoid organism, whereas *E. coli* grew readily in such filtrates; the more sensitive typhoid bacterium was checked earlier in its growth than the colon organism. In general, *E. typhosa* was found to be readily inhibited by the growth of antagonistic bacteria. Because of this, it was believed that pasteurized milk contaminated with a pathogenic organism presents a particular danger, since no antagonists are present to inhibit the rapid multiplication of the pathogen. Metchnikov (596a) suggested utilization of the antagonistic relations between lactic acid bacteria and proteolytic bacteria for repressing the growth of the latter. Thus, pure cultures of the former are introduced into the food system of man, in order to repress in the intestinal canal the proteolytic organisms that are supposed to bring about intoxication in the system. In recent years, *L. acidophilus*, an inhabitant of the human intestine possessing antagonistic properties against pathogenic intestinal bacteria, has come into general use (744). The problem of combating pathogenic intestinal bacteria by means of nonpathogenic forms (703) has thus been given wide consideration. The utilization of yeasts for combating streptococci and staphylococci may also be classified among the phenomena of antagonism (893). On the basis of the rapid destruction of pathogenic bacteria added to natural water, the storage of drinking water in large reservoirs was recommended as an important safeguard against the water's becoming a carrier of bacterial diseases (295–297).

Clinical methods have been proposed for evaluating the results obtained by treating tooth gangrene by means of antagonists (325). Donaldson (181) found that *Cl. sporogenes* or a closely related form had a marked effect in suppressing the growth of pathogenic organisms in septic wounds. He believed the antagonistic anaerobe is present in the majority of gunshot wounds, but that its activities are held in abeyance by the method of wound-dressing. This antagonist acts by virtue of its proteolytic enzymes which hydrolyze the dead protein, from which the pathogenic organisms operate, as well as the toxic degradation products of other organisms.

Dack (159) reported that *Cl. sporogenes* formed in the soil was responsible for destroying the toxin of *Cl. botulinum*.

ANTIBIOTIC SUBSTANCES AND DISEASE CONTROL

In discussing the subject of antiseptics in war-time surgery, Fleming emphasized that the treatment of war wounds has become far more satisfactory during the second world war than it was during the first. It is now known, for instance, that carbolic acid lacks value inside the human body, as demonstrated by a diminution in efficiency with increasing concentrations, due to its destructive effect upon the blood leukocytes and body tissues. Dyes have been found also to be of little value, since they are absorbed by the cotton used in dressing the wounds. Fleming (260) warned against placing too much faith in antiseptics belonging to the sulfonamide group, since they are not general antiseptics but have specific effects upon certain bacteria, and their action is neutralized by chemicals, by pus, and by dead bacterial cells. They are, therefore, of little value in the treatment of seriously septic wounds, in which pus and bacteria are inevitably present. Their major importance is due chiefly to their great solubility, since they dissolve to form high concentrations in the wound.

In view of these limitations in the use of chemical antiseptics, bacteriostatic and bactericidal (antibiotic) agents produced by microorganisms may find particular application. Among the substances formerly utilized for this purpose pyocyanase has received special consideration (239). Unfortunately, the variation in the nature of the preparation of this material and the difficulty of keeping it in an active condition for very long periods of time have prevented its wider usefulness. Among the more recent preparations, penicillin occupies a leading place.

Penicillin

Nature of Action. The action of penicillin upon bacteria is chiefly bacteriostatic and not bactericidal (260). Penicillin shows *in vitro* a high degree of specificity; pyogenic cocci, anaerobic clostridia, and certain pathogenic gram-negative cocci (*Gonococcus*, *Meningococcus*, and *Micrococcus catarrhalis*) are sensitive, whereas the colon-typhoid, hemo-

philic chromogenic bacilli and certain micrococci (*Micrococcus flavus*) are resistant to its action (263); however, it has no effect upon *M. tuberculosis* (834), *Trypanosoma equiperdum*, and the influenza virus (753). The purest preparation of penicillin so far available completely inhibited (276) the growth of *S. aureus* in a dilution of between 1:24,000,000 and 1:30,000,000. Partial inhibition was obtained up to 1:160,000,000. *Salmonella* organisms were also sensitive. The antibacterial activity of penicillin is not interfered with by substances that inhibit sulfonamides, namely, bacterial extracts, pus fluids (7), tissue autolysates, peptones, and *p*-amino-benzoic acid. It is nontoxic in concentrations far greater than those required for therapeutic purposes (163, 164). However, it is rapidly excreted through the kidneys and frequent administration is essential in order to maintain a proper blood concentration.

In its biological properties, penicillin has been found, in general, to resemble sulfonamide drugs, with certain significant differences (7) which may be summarized as follows:

The bacteriostatic power of penicillin against streptococci and staphylococci is greater than that of sulfonamides, even when the tests are made under conditions optimum for the action of the latter. Saturated solutions of sulfapyridine and sulfathiazole showed no complete inhibition of bacteria on the assay plate, whereas penicillin, even in a dilution of 1:500,000, gave considerable inhibition.

The action of penicillin on streptococci and staphylococci, unlike that of the sulfonamides, is influenced very little by the number of bacteria to be inhibited. Bacterial multiplication could be completely prevented by as low a concentration of penicillin as 1:1,000,000, even if the inoculum contained several million bacterial cells. In the case of smaller inocula, inhibition occurred in even higher dilutions. This property of penicillin is believed to be of great importance in the treatment of heavily infected wounds, on which the sulfonamide drugs seem to have little beneficial action.

The bacteriostatic power of penicillin against streptococci and staphylococci is not inhibited to any extent by protein breakdown products or by pus, which neutralize the bacteriostatic action of sulfonamide drugs. The leukocytes remain active in any concentration of penicillin usually employed in intravenous injection.

Penicillin is active against strains of bacteria that are resistant to the action of sulfonamides (273, 566, 881). It is effective in the treatment of hemolytic streptococcus, pneumococcus, and gonococcus infections, which are resistant to sulfonamides. It has not been found effective, however, in the treatment of subacute bacterial endocarditis (748).

On repeated passage through broth containing penicillin, pneumococcus cultures as well as *Staphylococcus* sp. and *S. pyogenes* (564) increased in resistance to penicillin. This was accompanied by a proportional loss of virulence. Small colony variants (G forms) of *S. albus* showed a specially high resistance to penicillin (806). Two strains of pneumococcus developed resistance to penicillin as a result of serial passage through mice treated with it. The degrees of resistance developed and acquired varied significantly with the strains. In the case of one strain, resistance was not impaired by 30 serial passages through normal mice. The development of resistance *in vivo* was accompanied by an increase in resistance to penicillin *in vitro*. The response of the pneumococci to sulfonamides was not altered by the development of resistance to penicillin. The mechanisms whereby staphylococci become resistant to sulfonamides and to penicillin appear to be distinctly different (846, 847).

Toxicity. As to the toxicity of penicillin, it was found (7) that mice were little affected by the intravenous injection of 10 mg. of penicillin; they became ill from the use of 20 mg. but recovered shortly. One hundred milligrams of crude penicillin given intravenously to man caused a shivering attack with a rise of temperature in about an hour. The latter was due to the presence of a pyrogenic substance in the preparation. Certain isolated fractions of penicillin had no such pyrogenic effect. Penicillin was toxic to mice when given intravenously in single doses of 0.5, 1.0, 1.5, and 2.0 gm. per kilogram. More highly purified preparations were less toxic. Higher concentrations were required for lethal effect from subcutaneous administration. The toxic dose is 64 times greater than the effective dose (753).

The relative toxicity of various salts of penicillin was found (967) to be, in increasing order, Na, NH_4, Sr, Ca, Mg, and K. Based on milligrams of the cation at the LD_{50} dose of salts of penicillin, the relative

toxicity was Na, Sr, NH_4, Ca, K, and Mg. It was concluded that the toxicity of the salts of penicillin is primarily due to the cations used in their preparation.

Penicillin is not inactivated by saliva, bile, or succus entericus, but is destroyed rapidly by gastric juice, due not to the pepsin but to the HCl in the juice (724, 725).

Penicillin is slowly absorbed and excreted, usually within one hour, in the urine (725). The degree of its antibacterial action is proportional to its concentration in the serum, maximum effects against hemolytic streptococci being produced by concentrations of 0.019–0.156 Oxford units in 1 ml. of serum. The LD_{50} for an 18-gram mouse was 32 mg. of the sodium salt (422, 424). The cardinal symptoms of toxicity were choking, gasping, and rapid respiration. However, it is relatively nontoxic in doses used for therapeutic purposes (163, 164, 566, 753).

Penicillin was thus found to combine the two most desirable qualities of a chemotherapeutic agent, namely, a low toxicity to tissue cells and a highly bacteriostatic action against some of the most common and destructive bacteria with which man may become infected. It was possible to maintain a bacteriostatic concentration of penicillin in the blood without causing any toxic symptoms, and to recover a large proportion of the substance from the urine; this recovered penicillin could then be used again.

Animal Experiments. In animal experiments (699) it was established that penicillin is an effective chemotherapeutic agent against pneumococci, including sulfonamide-resistant types. In experiments with *S. aureus*, a survival ratio of 2:1 was obtained in favor of penicillin as compared with sulfathiazole, correction being made for the survival of control mice. Penicillin, when administered subcutaneously, intravenously, or intraperitoneally, was also found to be effective against hemolytic streptococci (418). Generalized staphylococcal infections were cured by penicillin and local lesions healed during parenteral administration. The best method for administering penicillin was by the intramuscular route at 3-hour intervals; the blood should contain enough penicillin (15,000 Oxford unit dose) to inhibit the growth of the infecting agent (276, 399, 404). Intraocular infection caused by *D. pneumoniae* was checked by local treatment with penicillin in solu-

tions of 0.25 and 0.1 per cent; the application was continued for 2 to 4 days (780).

Since penicillin readily loses its activity in an acid solution, it is used in the form of the sodium salt. Rabbits excreted in the urine as much as 50 per cent of the penicillin after intravenous injection, but less than 20 per cent after administration into the intestine; some excretion took place in the bile. The penicillin could not be detected in the blood within one-half hour after administration. Cats differed in this respect from rabbits, since they maintained an antibacterial concentration of penicillin in the blood for at least 1.5 hours after subcutaneous or intravenous injection, and for at least 3 hours after intestinal administration. They differed also in excreting about 50 per cent of the penicillin in the urine, even when the substance was injected into the intestine. In this respect man appeared to resemble cats more closely than rabbits. The excretion of penicillin could be blocked by simultaneous administration of diodrast (723–725).

A comparison of antibiotic agents against the anaerobes causing gas gangrene placed tyrothricin in first place, followed successively by penicillin, the sulfa drugs, and other antibiotic agents; however, *in vivo* treatment of mice infected intramuscularly with *Cl. perfringens* placed penicillin first, with tyrothricin and aspergillic acid at the bottom of the list (563). Penicillin also proved superior to sulfonamides and amino acridines in experimental infection with *Cl. welchii* and *Cl. aedematiens* (563).

The *in vivo* activity of penicillin against *Cl. septicum* and other anaerobes, as well as many other bacterial pathogens (113), is brought out in Table 41. A single subcutaneous treatment of mice with 50 Florey units of penicillin at the time of intramuscular inoculation with *Cl. welchii* protected 98 per cent of the infected animals, and repeated small doses gave as good protection as a single large dose. Delay in the institution of therapy lowered the survival rate, but not appreciably unless the delay was over 3 hours. Local lesions were completely healed within 3 weeks if penicillin was injected repeatedly into the site of infection (371).

An intravenous injection of 20 mg. of the sodium salt of penicillin was without apparent effect on a mouse, and human leukocytes survived

TABLE 41. THERAPEUTIC ACTION OF PENICILLIN ON MICE

	VOLUME OF INFECTING AGENT IN MILLILITERS	LENGTH OF TREATMENT PERIOD	EACH DOSE OF PENICILLIN IN MILLIGRAMS	TOTAL DOSE OF PENICILLIN IN MILLIGRAMS	TOTAL NUMBER OF MICE	NUMBER OF SURVIVORS AT END OF						
						6 hrs.	12 hrs.	24 hrs.	2 days	4 days	7 days	10 days
S. PYOGENES (LANCEFIELD, GROUP A)												
Control mice	0.5				25		15	9	8		4	4
Treated mice	0.5	12 hrs.	2.0	10.0	50				49		28	25
Control mice	0.5				25	24	3	0				0
Treated mice	0.5	45 hrs.	0.5	7.5	25	24						24
S. AUREUS												
Control mice	1.0				24	21	1	0				0
Treated mice	1.0	55 hrs.	0.5	9.0	25	25	12		11	10		8
Control mice	0.2				24	23	15	5	0			0
Treated mice	0.2	4 days	0.5	11.5	24		23	22		21		21
CL. SEPTICUM												
Control mice					25		21	0				0
Treated mice		10 days	0.5	19	25			24	21	18		18
Treated mice		10 days	1.0	38	25					24		24

From Chain, Florey, Gardner, Heatley, Jennings, Orr-Ewing, and Sanders (113).

for an hour in a 1 per cent solution (273). The efficacy of penicillin in protecting mice against streptococcal infections is brought out in Table 42.

TABLE 42. IN VIVO EFFICACY OF PENICILLIN COMPARED WITH SULFANILAMIDE IN STREPTOCOCCUS HEMOLYTICUS INFECTIONS IN MICE

DAILY DOSE IN MILLIGRAMS	DAILY DOSE IN OXFORD UNITS	NUMBER OF MICE OF ORIGINAL 20 SURVIVING AFTER 1 day	2 days	3 days	4 days	5 days	6 days	7 days	8 days	9 days	10 days
PENICILLIN											
0.0625	3.75	0									
0.125	7.5	16	0								
0.250	15.0	18	4	1	1	1	1	1	1	0	
0.500	30.0	20	4	0							
1.0	60.0	20	20	18	15	15	15	15	13	9	9
2.0	120.0	20	20	20	20	20	20	20	20	20	20
4.0	240.0	20	20	20	20	20	20	20	20	20	20
SULFANILAMIDE											
0.5		20	0								
1.0		20	20	20	10	8	8	5	4	1	1
2.0		20	20	20	20	20	20	20	20	14	3
4.0		20	20	20	20	20	20	20	19	19	16
8.0		20	20	20	20	20	20	20	20	20	18
16.0		20	20	20	20	20	20	20	20	20	20
CONTROLS											
Culture dilution 10^{-4}		0									
Culture dilution 10^{-8}		0									

From Robinson (752).
Notes. Infection: 0.5 cc. of 10^{-4} 6-hour-old culture dilution in broth. Treatment: Penicillin given subcutaneously and sulfanilamide given orally immediately after the inoculation of bacteria, then every 3 hours day and night for 5 days.

The effectiveness of penicillin has also been tested against various other infections in experimental animals, with varying degrees of success. It was found, for example, that the administration of penicillin in relatively large doses to mice after injection with murine typhus rickettsiae resulted in marked reduction in mortality, particularly when the initial dosage of the rickettsiae was relatively small (614).

CHEMOTHERAPEUTIC ACTION. Penicillin has also found an important

place in the treatment of local and generalized infections in man. Fleming was the first (265, 266) to recommend that it be employed for dressing septic wounds. It appeared to be superior to dressings containing purely chemical agents. Isolated penicillin in a dry state was many times more powerful than the most potent of the sulfonamide compounds (267, 271). Local applications include those to lesions of the eye produced by *S. aureus,* in which early treatment resulted in the elimination of the organism from the flora of the conjunctival sac (758). Weight for weight, penicillin was found to be four times as potent as sulfathiazole and 100 times as potent as sulfanilamide for the treatment of wound infections (7, 272).

Penicillin proved to be an especially effective agent for the treatment of staphylococcal and hemolytic streptococcal infections in man (725), including streptococcal meningitis (270). Many cases of infected war wounds treated with penicillin gave, in 24 hours, a uniform drop in the number of gram-positive organisms, including clostridia, staphylococci, streptococci, and corynebacteria; the gram-negative bacteria were not affected. Excellent therapeutic effects were obtained. Even crude culture filtrates of *P. notatum,* applied locally, gave good results (242, 707).

In order to lessen the frequency of effective invasion of the nose by bacteria and the subsequent infection of the nasopharynx, the use of penicillin as an antiseptic snuff was suggested (166). The material acts as a prophylactic against bacterial infections of the upper respiratory tract; the course of a cold could thus be checked by preventing secondary bacterial infection. The curing of nasal carriers of staphylococci or even the reduction of the number of vegetative organisms was considered important in order to reduce the danger of the carrier as a source of infection to others. Penicillin can also find application in certain chronic cases, as in the treatment of chronic dermatitis and in preparing infected surfaces of hands for skin-grafting and infected stumps for amputation (277).

Penicillin is an effective agent in the treatment of clinical infections due to sulfonamide-resistant bacteria (39, 136, 137, 143, 252, 400, 576). Several strains of *N. gonorrheae,* isolated from patients in whom the infection was resistant to treatment with sulfonamide preparations,

were found to be inhibited completely by penicillin. The number of organisms decreased greatly at the end of 1 or 2 hours' contact with the substance, and no viable organisms were found after 3 to 4 hours' contact. The complete absence of toxicity following the intravenous administration of penicillin, the lack of discomfort to the patient, and the rapid disappearance of clinical symptoms were observed in cases of sulfonamide-resistant gonorrheal infections. In all the cases reported, in addition to the clinical response noted, negative bacterial cultures were obtained some time between 17 and 48 hours after the institution of penicillin therapy. Sulfonamide-resistant gonorrhea cases responded to injections of 100,000 to 160,000 Oxford units (136, 479, 549, 863). Favorable responses have also been obtained in the treatment of sulfonamide-resistant strains causing staphylococcal pneumonia and empyema (44, 65) and other diseases. The susceptibility of various bacteria to penicillin can be determined by means of a very simple technique (879).

A favorable therapeutic response was obtained by administering penicillin intravenously to patients with staphylococcal infections and by mouth to a baby with a persistent staphylococcal urinary infection. In patients suffering from meningitis, penicillin was found to be absorbed more rapidly than in normal persons, and a larger part of the dose was excreted in the urine (723, 725).

Penicillin has not been found to be effective in trypanosome infections, but has been used successfully in the treatment of relapsing fever (29, 393), although excessive doses were required (220, 221).

Treatment of early syphilis cases with penicillin (575) indicated that the therapy was responsible for the rapid and complete disappearance of the infecting agent from the blood stream, as determined by various tests (112, 271, 275, 523a, 805). Penicillin was found to be actively spirocheticidal (222). A comparative study has been made of the action of penicillin and of other antibiotic agents upon *Treponema pallida* (217). The administration, at 3-hour intervals for a period of 15 hours, of 20,000 units of penicillin intramuscularly was found satisfactory in the control of gonorrhea in men (879a, 907). Penicillin was also found to have an effect upon experimental typhus rickettsiae (357, 614).

As a result of treatment of 300 patients with penicillin, it has been concluded (748) that this material is far superior to any of the sulfonam-

ides in the treatment of *S. aureus* infections with and without bacteriemia, including acute and chronic osteomyelitis, cellulitis, carbuncles of the lip and face, pneumonia and empyema, infected wounds and burns.

A study of 500 cases of infections treated with penicillin led to the following conclusions (479, 549): Penicillin can be administered intravenously, intramuscularly, or topically, but is ineffective when given by mouth. As it is excreted rapidly in the urine, it must be injected continuously or at intervals of 3 to 4 hours. Penicillin was found to be particularly effective in the treatment of staphylococcic, gonococcic, pneumococcic, and hemolytic streptococcus infections, especially sulfonamide-resistant gonococcic infections, but not bacterial endocarditis. The usual patient requires a total of 500,000 to 1,000,000 Oxford units, the best results being obtained when treatment is continued for 10 to 14 days, 10,000 units to be given every 2 to 3 hours at the beginning of treatment, either by continuous intravenous injection or by interrupted intravenous or intramuscular injections. Good results were obtained by injections of 100,000 to 160,000 units over a period of 2 to 3 days. In the treatment of empyema or meningitis it was found advisable to use penicillin topically by injecting it directly into the pleural cavity or the subarachnoid space. Toxic effects were extremely rare. Occasional chills with fever or headache and flushing of the face were noted.

A summary of the response of different bacteria in septic gunshot fractures is given in Table 43. Staphylococci and streptococci are rapidly responsive to penicillin therapy. Anaerobic cellulitis due to the proteolytic bacteria of putrid wound infection responds to penicillin, but the bacteria may persist in the presence of devitalized tissue or wound exudates. Pyocyaneus is not susceptible to penicillin and is considered to be relatively unimportant as a single pathogen in the surgical management of the wound (272, 548).

In view of the inefficacy of the sulfa drugs for the treatment of burns, a detailed study has been made (133) of the utilization of penicillin applied to the wound in the form of a cream. In 54 wounds thus treated, penicillin had a 100 per cent effect upon the hemolytic streptococcal flora, in 7 cases the strains being insensitive to sulfonamide. The staphylococci also disappeared, although somewhat more slowly. Healing was usually rapid and no toxic effects were observed. Gram-

negative bacteria (*E. coli*, *P. vulgaris*, *Ps. aeruginosa*), when present, were not affected, as further shown by Bodenham (67).

A summary of the results of extensive use of penicillin in the North African campaign of the present war led to the conclusion that in the treatment of recent soft-tissue wounds penicillin brought about the virtual elimination of infection and saved much hospitalization time. Treatment of fractures also gave good results, though some penicillin-resistant cocci appeared. Favorable results were also obtained in various other infections. It is suggested that an average of 750,000 units of sodium penicillin be allowed for systemic treatment and 50,000 units of the calcium salt for local treatment (276, 316).

Penicillin is thus found to form a valuable addition to the growing list of chemotherapeutic agents, to help man combat disease-producing bacteria. It is commonly used not as a pure acid but as either a calcium or a sodium salt, the former for local applications and the latter for intramuscular or intravenous treatments (276, 479). Since penicillin solutions are quite unstable, especially in the form of salts, the dry preparations are stored and are dissolved either in water or in saline just before required for use. Although penicillin has so far proved ineffective when administered orally, certain of its esters (e-butyl) that

TABLE 43. RESPONSE OF DIFFERENT BACTERIA FOUND IN WOUNDS TO PENICILLIN TREATMENT

TYPE OF INFECTION	PENICILLIN RESPONSE	
	Systemic	Local
Putrid:		
Proteolytic clostridia	+ (large dosage)	+
Proteus vulgaris	0	0
Nonhemolytic streptococci:		
Mesophilic	+	+
Thermophilic (*S. faecalis*)	0	0 (or slight)
Staphylococci	+ (3–5 days)	+ (often necessary)
Hemolytic streptococci	+ (1–3 days)	+ (not essential)
Pseudomonas aeruginosa	0	0

From Lyons (548).

are inactive *in vitro* can, when given by the oral route, become highly active against hemolytic streptococci (599, 600).

Production. Because of the limited amounts of penicillin available at the present time, many attempts have been made by physicians and hospitals to grow *P. notatum* on a suitable medium and use the crude culture filtrate for the treatment of wounds and infections. Since such cultures cannot be standardized and their activity cannot always be determined and since unforeseen toxic substances may be produced by certain contaminants in the culture, this practice should not be encouraged, unless carefully supervised by properly qualified bacteriologists.

The production of penicillin-destroying enzymes by bacteria and fungi (4, 379, 516) can be utilized for the purpose of testing the sterility of penicillin preparations. The penicillin, which would otherwise cause inhibition of growth of the contaminating organism in the test medium, is destroyed by the enzyme previous to the test. No apparent relation was said (1000) to exist between the resistance of an organism to penicillin and its ability to produce penicillinase, a fact not generally accepted (73–75). The mode of action of the enzyme is still not clearly understood, although there is apparently an increase in the number of carboxyl groups, as measured by *p*H change (1000).

Clavacin (*Patulin*)

The treatment of common colds that were prevalent in an English naval establishment by the use of clavacin in the form of nasal sprays or snuffed up by hand gave 57 per cent complete recovery in 48 hours, as compared with 9.4 per cent for the controls; no ill effects were observed (713). These results were not confirmed, however, the conclusion having been reached that, compared with the natural evolution of the disease, patulin has no demonstrable effect on the course of a cold (847a). This substance also proved to be unsatisfactory for the treatmnt of bovine mastitis by udder infusion (681a).

Tyrothricin

Dubos (193) reported that 0.002 mg. of gramicidin, one of the two chemical constituents of tyrothricin, when injected intraperitoneally into white mice, exerted a therapeutic action against experimental peri-

tonitis caused by pneumococci and streptococci (Table 44). This substance was found to be effective against five different types of pneumococci, eleven types of group A streptococci, and three strains of group C streptococci. It was, however, almost completely ineffective when administered into animal tissues by the intravenous, intramuscular, or subcutaneous route, because of its lack of activity under these conditions.

TABLE 44. BACTERICIDAL EFFECT OF TYROTHRICIN UPON DIFFERENT BACTERIA

	TYROTHRICIN IN MILLIGRAMS PER MILLILITER OF CULTURE						
	0.040	0.020	0.010	0.004	0.002	0.001	0.0
DIPLOCOCCUS PNEUMONIAE, TYPE I							
Viability*	–	–	–	–	–	+	++++
Reductase†	NR	NR	NR	NR	NR	NR	CR
Lysis§	C	C	C	C	C	P	N
STREPTOCOCCUS HEMOLYTICUS, GROUP A, TYPE 6							
Viability*	–	–	–	–	–	+++	++++
Reductase†	NR	NR	NR	NR	NR	PR	CR
Lysis§	N	N	N	N	N	N	N
STAPHYLOCOCCUS AUREUS							
Viability*	–	–	–	++++	++++	++++	++++
Reductase†	NR	NR	NR	CR	CR	CR	CR
Lysis§	C	C	N	N	N	N	N

From Dubos and Cattaneo (203).
* – no growth on blood agar, + reduced growth, ++++ abundant growth.
† NR no reduction of methylene blue, PR partial reduction, CR complete reduction.
§ N no lysis, P partial lysis, C complete lysis.

Tyrothricin exerted a lethal action *in vitro* on 18-hour broth cultures of *S. hemolyticus*, *S. aureus*, and *C. diphtheriae*, in a final dilution of 1:1,000,000; freshly isolated strains of meningococcus were affected in a dilution of 1:100,000 (807). Two monkeys which carried in the nasopharynx and throat gram-positive hemolytic streptococci and gram-negative hemolytic bacilli showed disappearance of these bacteria within 2 hours following the administration of tyrothricin. Five days after a single treatment no hemolytic organisms were found in one monkey, and, in the other, only throat cultures were positive. A second application of the material gave completely negative cultures within 3 hours.

No local or general reactions to these treatments were observed. This material was also administered to 5 human carriers of hemolytic streptococci, 2 of whom were persistent nasal carriers for two months following scarlet fever and the other three convalescent in the third week of this disease. In only one case was an immediate reduction in the number of streptococci obtained; a striking reduction or complete disappearance of the organisms occurred in the others on the fifth day, after 3 to 4 sprayings. These observations were said to be sufficiently encouraging to justify the use of the material against carriers harboring streptococci, diphtheria organisms, meningococci, and pneumococci. Injection of 3 to 40 mg. tyrothricin into the pleural cavity of rabbits with hemolytic streptococcal empyema brought about the sterilization of the pleural cavity and enabled the animal to survive. The injection of 10 mg. of tyrothricin into the pleural cavity of normal rabbits produced certain local tissue reactions. In excess of 10 mg., adhesions, thickening of the pleura, sterile abscesses, and other disturbances were produced (721).

The susceptibility of fecal streptococci to tyrothricin varies from strain to strain. Oral administration of the substance may produce inhibition of the growth of streptococci in the intestines of mice. This inhibition was most readily demonstrated when sulfasuxidine was administered together with the tyrothricin (761). Application of tyrothricin to ulcers brought about sterilization and healing of local infections. Application to the mastoid cavity following mastoidectomy also gave favorable results. In staphylococcic infections, resistant strains may develop during therapy (722). Certain sulfonamide-resistant strains of *S. pyogenes* were eradicated by application of gramicidin (292).

Tyrothricin and tyrocidine exert a bactericidal effect, and gramicidin is largely bacteriostatic (Figure 29); the first two are affected by blood and serum, but not the last. In order to be effective against bacteria, the organisms must be in contact with the material (754). Gramicidin is more toxic than tyrocidine, the toxic dose being larger, however, than the dose necessary to kill most gram-positive cocci. Penicillin is one-tenth as toxic as gramicidin (402, 403).

Both tyrothricin and tyrocidine cause hemolysis of erythrocytes, and both are leukocytolytic, gramicidin being less so. Both tyrothricin and gramicidin cause local and general toxic effects when injected into closed

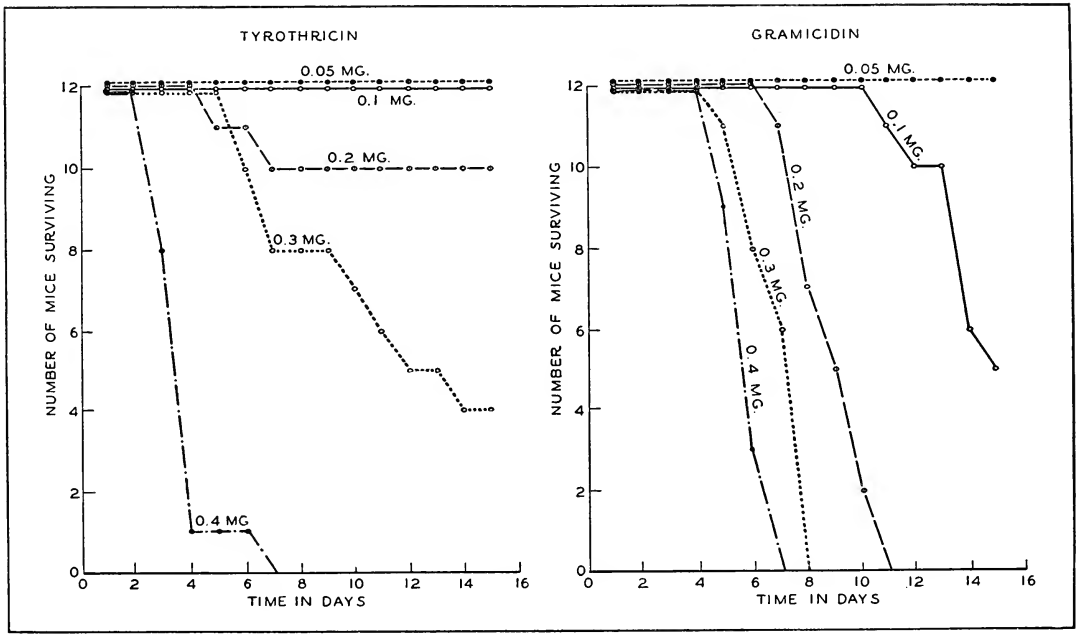


FIGURE 29. Survival of mice receiving daily intraperitoneal injections of varying amounts of tyrothricin and gramicidin. From Rammelkamp and Weinstein (729).

cavities of the body. Small amounts may bring about the sterilization of local infections without producing general toxic effects, giving only minimal local reactions. When injected into the skin, tyrothricin and gramicidin produce local reaction, the latter to a lesser degree. Oral administration is ineffective in reducing or destroying organisms which are susceptible *in vitro*. Local application of these substances has not been attended by toxic reactions even when large amounts were applied (729). Tyrothricin in high concentrations caused cytoplasmic and nuclear disintegration of the exudative rabbit polymorphonuclear leukocytes; in lower concentrations, it brought about altered staining reactions. When there was no apparent microscopic injury to the cells, phagocytosis of pneumococci took place. The presence of serum brought about some protection of the cells from the effects of these substances (132). Tyrothricin does not inhibit mitosis or migration of fibroblasts or activities of leukocytes, following direct applications to tissue culture (693).

The filtrate of *B. mesentericus* was found (956) to have a specific bactericidal action on *C. diphtheriae* in a dilution of 1:1,250. When injected parenterally into guinea pigs, it inhibited the toxic effect of the diphtheria organism. The filtrate of the antagonist was found useful in the treatment of diphtheria carriers.

Particularly favorable results were obtained from the use of gramicidin in the treatment of chronic mastitis (535). Several cows received treatment with increasing amounts of gramicidin diluted with distilled water. Following the morning milking, the residual milk in the cistern and in the teat was flushed out with 100 to 200 ml. of the gramicidin solution, containing 60 to 240 mg. in 1,000 ml. water; 800 to 900 ml. were then injected under pressure into the quarter and allowed to remain until the next milking. Within one hour after the injection, the treated quarter became distended and rectal temperature began to increase, reaching 41° C. at the fifth or sixth hour. The temperature returned to nearly normal in about 3 hours thereafter, the acute swelling having subsided at the next milking. The streptococci disappeared from most of the quarters treated, without an appreciable decrease in milk production. The fact that streptococci had been eliminated was established

by daily bacteriological examination of the milk over periods ranging from 15 to 81 days.

Sterile mineral oil was later found (536, 537) to be a suitable, non-irritating medium for the administration of the gramicidin, though some of the cows thus treated gave severe reactions. An alcoholic solution (2 to 3 ml.) of 80 to 120 mg. gramicidin was emulsified in 15 ml. sterile distilled water, and the emulsion mixed with 25 ml. of heavy mineral oil. The mixture was injected into the cistern shortly after the morning milking and allowed to remain until the evening milking. The treatment was repeated for several days in succession. Of 31 quarters naturally infected with *Streptococcus agalactiae* and treated by the gramicidin-oil mixture, 26 seemed to have responded by a complete disappearance of the streptococci. The infection in some of the cases was of severe chronic nature.

Less satisfactory results were obtained in the treatment of bovine mastitis caused by *Streptococcus uberis;* of 4 cases treated, only one responded satisfactorily. The final recommendations consisted in using 20 to 40 mg. gramicidin in oil, daily, for four consecutive days. These results were confirmed by various investigators (16, 888), who reported 60 to 90 per cent cure for two to three treatments (585).

A comparison of tyrothricin, trypaflavin, and novoxil for eradicating *S. agalactiae* from infected udders showed best results for the first. Infections with *S. uberis* and *Streptococcus dysgalactiae* also responded well, but not staphylococcic infections (793). The efficacy of the treatment is influenced by several factors, namely, (a) site of chronic infection, (b) selection of suitable cases, and (c) stage of lactation. Too extensive administration may damage the secretory tissues (622). In some experiments, as many as 90 per cent of the cases were cleared up with tyrothricin (95).

Gramicidin-like preparations were also used successfully in the treatment of local infections in man, such as osteomyelitis (553), and for various local administrations, such as conjunctivitis, as well as for infected diabetic and ulcerating lesions of cancer (1007). The active material must be used locally on infected cavities which do not communicate with the blood stream (318a, 401). Tyrothricin has also been used (78, 154) successfully in the treatment of acute otitis media, acute and

chronic mastoiditis, and acute and chronic sinusitis. The substance does not damage the tissue or interfere with wound healing. The growth of most staphylococci, streptococci, and pneumococci is inhibited or the organisms are killed. When applied locally, tyrothricin does not reach the blood stream. It has also been found that tyrothricin has an excellent therapeutic effect when used for urethral irrigations in the male (187).

Tyrothricin possesses several limitations, from the point of view of practical utilization: (a) development of bacterial, notably staphylococcus, variants, which become resistant to this agent; (b) inhibition of its action by phospholipins; (c) hemolytic action, which prevents its intravenous use.

Tyrothricin was found to produce no lesions in the gastro-intestinal tract (728, 968), but it is not very active when administered by mouth, since it is destroyed by the proteolytic enzymes of the digestive system.

Streptothricin

The fourth agent that was found to offer practical possibilities is streptothricin. This agent gave favorable results for the treatment of *Br. abortus* grown on chicken egg embryos. Its toxicity is low enough to make possible the administration of doses sufficient to destroy this pathogen in the living tissues (Table 45). Both the *in vitro* and the *in vivo* activities of streptothricin against gram-negative bacteria suggested the probability that it will prove useful in the local treatment of infected wounds and burns, bacillary dysentery, typhoid fever, and food poisoning produced by Salmonella organisms (755, 756a).

Other Agents

The protective action of the specific enzyme (polysaccharidase) of a soil bacterium against type III pneumococcus infection has also been established (30, 293). The specific protection induced in experimental animals is determined by the nature of the polysaccharide of the pneumococcus type. The polysaccharidase destroys the protective capsular substance of the pneumococcus, thus rendering it susceptible to phagocytosis.

The possibility of utilizing antagonistic bacteria for the control of fungi causing skin infections has also been suggested (119).

Virulent strains of *M. tuberculosis* were found to lose their virulence in the presence of certain other organisms or their products. According to Vaudremer (912), this phenomenon occurs when the tubercle organism is kept for 24 hours at 39° C. in contact with a filtered extract of *A. fumigatus*, and a similar effect can be exerted by certain bacteria (708). On the other hand, extracts of *A. fumigatus* were used for the treatment of 200 tubercular patients with rather inconclusive results (912).

TABLE 45. IN VIVO EFFICACY OF STREPTOTHRICIN COMPARED WITH SULFADIAZINE IN SALMONELLA AERTRYCKE INFECTION IN MICE

INFECTION*	DAILY TREATMENT IN MILLIGRAMS†	ORIGINAL NUMBER OF MICE	NUMBER OF MICE SURVIVING AFTER 1 day	2 days	3 days	4 days	5 days
STREPTOTHRICIN							
10^{-4}	1	10	10	5	5	4	2
10^{-4}	2	10	10	10	10	10	10
10^{-4}	4	10	10	10	10	10	10
10^{-4}	8	10	10	10	10	10	10
SULFADIAZINE							
10^{-4}	16	10	10	10	10	4	4
CONTROL							
10^{-4}		10	0				
10^{-5}		10	0				
10^{-6}		10	0				
10^{-7}		10	0				

From Robinson (752).
* 0.5 cc. of a 6-hour-old culture dilution in broth.
† Streptothricin given subcutaneously immediately after inoculation of bacteria.

Treatments of intestinal disturbances by the use of antagonistic microorganisms, although highly promising, have not been sufficiently investigated as yet. It may be of interest to note, in this connection, that the presence in human intestines of *E. coli* with a high antagonistic index is considered as important evidence of immunity of certain individuals to intestinal disturbances (383, 580, 619, 643).

TOXICITY OF ANTIBIOTIC SUBSTANCES

The various antibiotic substances obtained from microorganisms vary greatly in their toxicity to animals. The therapeutic use of many of these agents, like actinomycin or clavacin, which are highly bacteriostatic, may be considered as either entirely excluded for the present or limited only to local applications. Some substances, as pyocyanase, penicillin, and streptothricin, are relatively nontoxic (754); others, like tyrothricin (756) and fumigacin (752), are slightly toxic; and still others, like actinomycin and clavacin, are highly toxic (757, 941). Some, like tyrothricin, are hemolytic (390, 579); others, like penicillin, actinomycin, and streptothricin, are not (Table 46).

TABLE 46. ACUTE TOXICITY TO MICE OF VARIOUS ANTIBIOTIC AGENTS

ANTIBIOTIC AGENT	ORAL ADMINISTRATION	SUBCUTANEOUS ADMINISTRATION	INTRAPERITONEAL ADMINISTRATION	INTRAVENOUS ADMINISTRATION
Streptothricin	>2,000	>1,000	3,000	1,000
Penicillin	>2,000	1,600	2,000	500
Fumigacin (helvolic acid)			800	
Citrinin	100		100	
Pyocyanin		100	80	
Tyrocidine	>1,000	>1,000	20	1.25
Tyrothricin	>1,000	>1,000	10	1.2
Gramicidin	>1,000	>1,000	10	1.2
Gliotoxin			5	
Clavacin (crude)			3.5	
Actinomycin	5	0.15	0.15	0.15

From Robinson (752).
Note: Figures represent maximal tolerated dose in mgm./kgm.

CHAPTER 13

MICROBIOLOGICAL CONTROL OF SOIL-BORNE PLANT DISEASES

THE possibility of controlling microorganisms, especially fungi, in the soil by favoring the development of antagonistic microorganisms is significant for several reasons: fungi are causative agents of some of the most important diseases of plants and are added constantly and often quite extensively to the soil, in plant residues and in diseased plant products; fungi capable of causing certain diseases of animals and of man also find their way sooner or later into the soil; many soil-inhabiting fungi have a marked antagonistic effect against fungus and bacterial plant pathogens.

Some fungi that produce plant diseases are able to survive in the soil for only short periods of time; others become established in the soil saprophytically and remain capable of attacking living plants when proper conditions arise. Some of these fungi are specific, their ability to attack different plants being limited, whereas others can cause diseases of a great variety of plants and many survive in infected soil for long periods. Some plant diseases, as in the case of virus infections, are transmitted by specialized means, as by insect carriers. This complicates further the interrelationship among the different organisms, in relation to plant and animal diseases.

Microorganisms causing diseases of plants may either reduce the vigor and productivity of the plants or destroy them completely. Plants appear to develop at times a certain degree of resistance to microbial infection. Whether this is in the nature of a phenomenon of immunization, similar to that of animals, is still a matter of speculation. Whatever the nature of the reaction, the degree of resistance depends to a certain extent upon the imperviousness of the outer layers of the plant tissues to penetration by the parasites, as well as upon the chemical composition of the plants. It is believed that an acid plant reaction, combined with the presence of tannins and lignins, retards the growth of many disease-producing agents. The survival of the pathogens outside

the host plant is due to the formation of resistant spores which remain viable in the soil for long periods. Because of this, the growth of many plants requires a long rotation if this system is to be used as a means of controlling the specific diseases.

Many fungi and bacteria causing plant diseases were at first thought capable of surviving in the soil for an indefinite time, even in the absence of the hosts. It has since been established, however, that, although the majority of these pathogens are facultative saprophytes, some are obligate parasites. The first can be grown easily on sterile soil and on artificial culture media, whereas the second, such as *Plasmodiophora brassicae* and *Synchytrium endobioticum*, have not been cultivated so far upon any artificial media and are known to die out in the soil in the absence of host plants.

Certain soil-borne plant diseases may be caused by more than one organism. In the pink-root of onions (375), *Phoma terrestris* is followed by *Fusarium malli;* in the take-all of wheat (314), *O. graminis* is followed by *Fusarium culmorum*. This type of sequence occurs with other diseases, where the primary parasite first attacks the root and is followed by a succession of other fungi, both parasites and saprophytes. By means of the direct microscopic technique, the sequence of microorganisms can be demonstrated in the infected roots of the plants. Certain less specialized parasites are able to live saprophytically on the dead tissues, whereas the saprophytes are found only in the later stages of decomposition.

It has been suggested (313, 739) that the root-infecting fungi be classified ecologically as soil inhabitants and soil invaders. The first may be looked upon as primitive or unspecialized parasites with a wide host range, their parasitism being considered incidental to their saprophytic existence in the soil. The second group comprises a majority of root-infecting fungi, the more highly specialized parasites. The presence of these in the soil is closely associated with the occurrence of the host plants: in the absence of a host, these fungi die out in the soil, because of their inability to compete with the soil saprophytes. The close association between this group of organisms and their host plants is believed to be enforced by competition with the microbiological population of the soil (312a).

ANTAGONISM OF SOIL-INHABITING MICRO-ORGANISMS TO PLANT PATHOGENS

The antagonistic interrelationships among the members of the microbiological population of the soil have received particular attention from the point of view of modifying the virulence of those plant pathogens, especially the fungi, that find temporary or permanent habitat in the soil (23, 310, 554, 696).

In the infection of wheat seedlings by *O. graminis*, a number of fungi and bacteria are able to exert a marked antagonistic action against the pathogen (784). Not only the living cultures of the antagonists, but, in many cases, the culture filtrates are also effective (511, 992). The growth of *H. sativum* and *F. graminearum* upon sterilized soil was completely suppressed (397) by the addition of small amounts of unsterilized soil or by the simultaneous inoculation with harmless fungi and bacteria, with the result that no infection occurred when wheat seeds were inoculated with this soil. Although *H. sativum* is able to sporulate readily in sterilized soil, this does not take place in nonsterilized soil, sporulation being inhibited by the soil microorganisms. Virulence of *H. sativum* on wheat seedlings was reduced by 11 to 57 per cent by certain cultures of *Penicillium; Trichoderma* reduced virulence by 50 to 58 per cent, *Absidia glauca* by 39 per cent, and *A. nidulans* by 30 per cent. Many fungi, however, had no effect on the virulence of the pathogen, and some even increased it (785). The fact that root-rot diseases are less severe on wheat grown on summer-fallowed land than on land cropped to wheat for several years was believed to be due to the soil saprophytic microorganisms, which in bare fallow have an advantage over the pathogenic organisms in competition for food (Figures 30 and 31).

The infection of wheat seedlings by *O. graminis* in sterile soil was found to fall off rapidly with the reestablishment of the original soil microflora (86). It was emphasized, however, that the effect of various organisms upon the pathogen grown in artificial culture media is no proof that the same organisms will be able to suppress the virulence of the pathogen on wheat in soil. An inverse correlation was shown (617) to exist between the degree of infection and the protective effect of the

general soil microflora; this was determined by comparing infection in an unsterilized soil with that obtained in a sterilized soil. An increase in soil temperature was found (312, 398) to increase the antagonistic action of the soil microflora against the parasitic fungi causing cereal root rots.

Various actinomycetes were shown to be antagonistic (884) to species of *Pythium*, a root parasite of sugar cane. The phenomenon of antagonism was independent of the *p*H changes; it has been ascribed to the formation of a toxic, partly thermostable, principle. A marked influence of the soil microflora on grass diseases caused by *Pythium* (691) is illustrated in Figures 32 and 33 (906). Clavacin (patulin) was found capable of inhibiting the growth of various species of *Pythium* (cause of damping-off disease of seedlings) in dilutions of about 1:400,000 (22), and of exerting a strong fungicidal action upon *Ceratostomella ulmi*, the causative agent of the Dutch elm disease; the last effect could partly be overcome by certain nutrients in the medium, especially peptone (928).

Numerous soil microorganisms are moderately or strongly antagonistic to such pathogens as *Hypochnus centrifugus*, *Hypochnus sasakii*, and *Sclerotium oryzae sativae* (241); culture filtrates from some of the antagonistic fungi were also able to reduce the damage caused by the pathogens.

Phytophthora cactorum was found (974) to be inhibited in the rotted tissues by the antagonistic effects of secondary organisms. In many cases, the rotting of fruits was suppressed by mixtures of organisms as compared with the pathogens; the type of rot was also modified, depending on the temperature and the specific nature of the antagonists (790).

The stimulating effect of mycorrhizal fungi on the host plant has been explained (298) by the capacity of the fungi to inactivate, destroy, or absorb certain plant-retarding principles found among the organic constituents of peat and other humus materials, or produced by fungi.

Certain fungi are also known (332) to be antagonistic to ants and their fungal symbionts. These antagonists are distributed by the insects, thus spreading agents that are destructive to themselves and to their fungus gardens.

GENERAL METHODS OF CONTROL OF SOIL-BORNE DISEASES

Soil sterilization by heat and chemicals has long been practiced as a method of control of soil-borne fungus diseases. This phenomenon is usually designated as partial sterilization of soil, since not all microorganisms are killed by these treatments. However, once a soil thus treated becomes reinfected with a disease-producing organism, the infection may become much more severe. It has, therefore, been suggested (380, 682) that partially sterilized soil be reinoculated with a mixture of saprophytic microorganisms before it is used as a seedbed, so as to counterbalance the injurious effect of the parasites (Table 47).

TABLE 47. EFFECT OF A BACTERIAL ANTAGONIST ON DAMPING-OFF OF PLANTS IN THE SOIL

TREATMENT OF SOIL	PERCENTAGE OF SEEDS PLANTED PRODUCING NORMAL SEEDLINGS IN 2 WEEKS	
	Cucumbers	Peas
Control soil	35	52
Fresh medium added	65	55
Diluted medium added	61	77
Washed bacterial cells added	58	75
Culture of bacterial antagonist added	55	80
Diluted culture added	87	90
Culture added continuously	81	90

From Cordon and Haenseler (149).

The importance of the soil microflora in modifying plant diseases caused by soil-borne pathogens is being realized more and more clearly. One of the earliest attempts to control a plant disease by microbiological agents was made in 1908 by Potter (697). He found that *Pseudomonas destructans,* the cause of rot of turnip, produces a potent, heat-resistant toxin. The bacteria failed to grow in the presence of this toxin, and were completely killed by the substance. By spraying turnips with this material, the disease could be checked; the toxin was more or less specific for the particular organism. Certain bacteria commonly found in soils were shown (304) to have a deleterious effect on the growth, in artificial

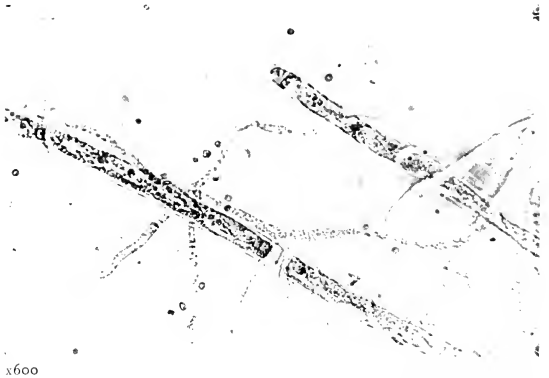


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FIGURE 30. An antagonistic fungus, *Trichoderma*, attacking a plant pathogenic fungus, *S. rolfsii*, showing one break of a septum. From Weindling (963).

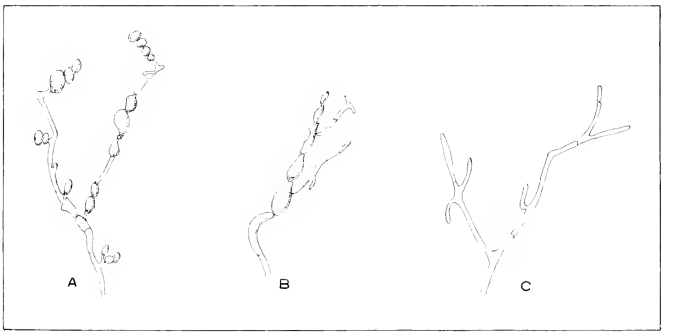

FIGURE 31. Influence of antagonists upon the growth of *Helminthosporium*. Distortion of mycelium by *Bacterium* sp. (A) and *B. ramosus* (B). C is a normal mycelium. From Porter (695).

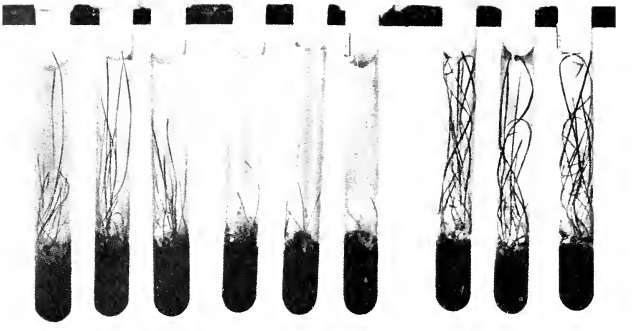

FIGURE 32. Antagonism of soil organisms against parasitism of *P. volutum* on *Agrostis*. From van Luijk (906).

FIGURE 33. Inhibiting effect of sterilized liquid medium of *P. expansum* versus *Pythium de Baryanum* on lucerne. From van Luijk (906).

media, of *Ps. citri,* which causes citrus canker. This effect was brought about by inhibiting the growth of and by killing the pathogen. By the use of an antagonistic bacterium, wheat seedlings were protected from infection by *Helminthosporium* sp. (695). In a similar manner, flax seedlings were protected from *Fusarium* sp.

A watermelon disease, caused by *Phymatotrichum omnivorum,* was considerably reduced when certain specific fungi and bacteria were present in the soil together with the pathogen (91, 101); *T. lignorum* was observed to attack and kill the hyphae of *Phymatotrichum* in culture. The severity of the seedling blight of flax, caused by *Fusarium lini,* was diminished when the pathogen was accompanied in the soil by various other fungi (875). The pathogenicity of *H. sativum* on wheat seedlings was suppressed by the antagonistic action of *Trichothecium roseum,* this effect being due to a toxic substance produced by the latter (353). *T. lignorum* prevented infection of wheat (60) by *H. sativum* and *Fusarium culmorum* (Table 48). Novogrudsky (646) obtained protection against infection of wheat with *Fusarium* by inoculating the

TABLE 48. EFFECT OF TRICHODERMA LIGNORUM ON GERMINATION AND GROWTH OF BARLEY INFECTED WITH HELMINTHOSPORIUM SATIVUM IN STERILIZED SOIL

STRAIN OF H. SATIVUM	PERCENTAGE OF PLANTS								
	Emerged			Stunted			Contorted leaves		
	H	H+T	H+SI	H	H+T	H+SI	H	H+T	H+SI
21	84	94	94	46	12	6	52	32	15
22	88	94	98	33	8	6	57	27	14
23	86	88	96	25	17	8	78	31	21
24	88	98	94	10	4	3	17	15	10

From Christensen (127).
Notes. Results are based on randomized duplicate pots, each sown with 50 seeds. H, seeds inoculated with a spore suspension of *H. sativum;* H + T, seed inoculated with *H. sativum* plus *T. lignorum;* H + SI, seed inoculated with *H. sativum* and soil with *T. lignorum.*

soil with the bacteria isolated by Chudiakov (130), provided the bacteria were introduced simultaneously with the fungus or preceded it. The role of microbiological antagonism in the natural control of soil-borne fungus diseases of plants has thus been well emphasized (85,

398, 783, 784). Methods for combating plant pathogenic fungi by the use of bacteria and other antagonists have been suggested by various investigators (46, 503, 841).

The principles underlying the biological control of soil-borne plant diseases were outlined by Garrett (312) in terms of the soil population in a state of dynamic equilibrium. When a given crop is grown continuously in the same soil, the parasitic organisms capable of attacking the roots of that crop multiply (72). Organic manures stimulate the development of saprophytic organisms in the soil, and are thus able to check the activity of the pathogens, which are destroyed by the saprophytes. Either the metabolic processes of the saprophytes check the growth of the pathogens, or the saprophytes actually attack and destroy the mycelium of the pathogens. The microbiological control of plant diseases was said to be most effective against those organisms which have become highly adapted to a parasitic form of life. The pathogenic *Ophiobolus*, when present in the form of mycelium inside the infected wheat stubble buried in the soil, is able to tolerate adverse physical soil conditions. Those soil treatments which favor increased activities of the microbiological population, such as addition of organic matter, partial sterilization followed by reinoculation with fresh soil, and improvement in soil aeration, favored loss of viability of the pathogen.

Van Luijk (906) recommended the control of plant parasites by inoculating the soil with specific microorganisms selected for their antagonistic capacity, or by the addition of the growth products of these microorganisms. Living soil fungi, including *Trichoderma viridis* and *Absidia spinosa*, exerted an adverse influence upon *Rhizoctonia* (*Corticium*) *solani* and reduced its pathogenicity to cabbage seedlings (449). Broadfoot (86) and others (248), however, emphasized that the antagonism of a saprophyte to a plant pathogen, determined on artificial culture media, is not a reliable measure of the actual control of the parasite in the soil. A lack of specific microorganisms in the soil is not a sufficient factor limiting biological control under natural conditions. Therefore, no inoculation of soil with an antagonistic organism, such as *T. lignorum*, can have more than a temporary effect in changing the microbiological balance of the soil population. Similar results have been ob-

tained (966) in efforts to control *R. solani*, or the damping-off of citrus seedlings (Figure 34), by the use of *T. lignorum*, and in the action of *B. simplex* upon *Rhizoctonia* in the soil (149).

A number of antagonistic bacteria were found (481) to be able to prevent scab formation by *S. scabies* on potatoes. Daines (161) found that *T. lignorum* produces a diffusible substance which is toxic to *S. scabies* in an artificial liquid medium. However, the toxic principle added to potato soils is rapidly destroyed there by aeration; it can be removed from solution by charcoal and by soil, where it is destroyed. It was suggested, therefore, that it is highly doubtful whether antagonists will be found to be of much assistance in combating potato scab in soil. The physical and biological environments encountered in many cultivated soils offer an important barrier against the establishment of the antagonist. When the latter was added to a 5-day-old culture of *S. scabies*, it was greatly inhibited by the scab organism. Soil bacteria are also able to produce substances toxic to both *Trichoderma* and *Streptomyces* alike. In such a complex physical, chemical, and biological environment as the natural soil, these antagonistic relationships may thus be modified or even entirely destroyed.

The application to the soil of organic materials which favor the development of antagonists has given much more favorable results than the use of pure cultures. Fellows (251) obtained field control of the take-all disease of wheat in Kansas by the application of chicken and horse manure, alfalfa stems and leaves, boiled oats and barley, as well as potato flour. Garrett believed (313, 314) that the factor chiefly controlling the spread of pathogenic fungus along the roots of the wheat plant was the accumulation of carbon dioxide, with a corresponding lowering of oxygen tension in the microclimate of the root zone. A high rate of soil respiration was, therefore, said to check the growth of *O. graminis*. This can best be maintained, of course, by periodic additions of organic manures. Materials low in nitrogen were found to be more effective than those high in nitrogen. Garrett, therefore, postulated the hypothesis that the soil microflora used the mycelium of the pathogen as a source of nitrogen, in the process of decomposition of the nitrogen-poor materials. The addition of nitrogenous substances, in either an or-

ganic or an inorganic form, was believed to protect the pathogenic organism against attack by the soil microflora, by offering a more readily available source of nitrogen. Tyner (895) suggested that the differences in the microflora associated with the decomposition of different plant residues are largely responsible for differences in persistence and virulence of pathogens causing root rot of cereals.

Against some plant pathogens, however, high nitrogenous materials were found to be very effective. Considerable reduction in the slime-disease of tomato plants resulted from the addition of green manures to the soil before planting (904); organic materials high in nitrogen, as well as the supplementary addition of nitrogenous materials sufficient for complete decomposition of the organic matter, brought about greater reduction of the disease. Organic matter was found to be most effective during the process of decomposition; after it has undergone extensive decomposition and reached a stage of slow decomposition, when it is usually designated as humus, it becomes comparatively inert (878).

The antagonistic action of soil microorganisms has been utilized in several areas of the United States for the control of *P. omnivorum*, the root rot of cotton. It was shown (484–486) that this pathogen can be inactivated when organic manures are added to the soil before the crop-growing season. Eaton and King (223) demonstrated, by the use of the contact slide technique, that microbiological antagonism represents, in this case, the true mechanism of the control process; the development of saprophytic organisms was most profuse in the slides buried in the manured plots, whereas the mycelium of the pathogen was most abundant on the slides kept in the unmanured plots. The conclusion was reached (345) that manuring definitely controls cotton root rot, as a result of the parasitism by bacteria of the fungal strands of the causative agent of the disease. Continuous growth of cotton on certain neutral or alkaline soils in southern United States was believed to bring about an unbalanced soil population in which *P. omnivorum* became a dominant organism; this was accompanied by the absence or only the sporadic presence of *Trichoderma* and other molds (878). The application of organic matter to such soils results in the destruction of most of the sclerotia and mycelium of the pathogen (609). Microbial antagonists

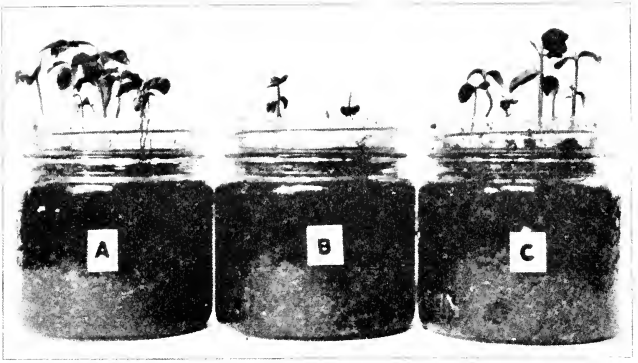

FIGURE 34. Sweet-orange seedlings in nonsterilized soil. A, control; B, *Rhizoctonia* inoculated into soil layer in bottom of jar; C, *Rhizoctonia* as in B, plus *Trichoderma* in top layer of peat. From Weindling and Fawcett (966).

rather than food exhaustion were, therefore, considered to be responsible for the destruction of the pathogen.

The *Sclerotium* rot of sugar beets was found (518) to be controlled by the application of nitrogenous fertilizers. This effect was believed to be due largely to a change in the metabolism of the fungus or of the host. It was also suggested, however, that the possibility exists that the suppression is due to a change in the balance of the soil microbiological population.

The possibility of suppressing the growth and eliminating the infectivity of plant pathogens by utilizing the activities of the soil microflora was demonstrated also for a number of other diseases. It is sufficient to cite the suppression of *Monilia fructigena* on apples by various fungi and bacteria (911), of *F. culmorum* and *H. sativum* on wheat (398, 695), and of species of *Rhizoctonia* on citrus seedlings. These pathogens are markedly influenced by *T. lignorum*, a common soil saprophyte. A species of *Trichoderma* was also found to cause a reduction in the amount of Texas root rot of watermelons caused by *P. omnivorum* (101).

The damping-off of citrus seedlings, caused by a number of fungi, could be suppressed by *T. lignorum*, which parasitizes the fungi (962, 966). The addition of *Trichoderma* spores to acid sterilized soils prevented the damping-off of the seedlings. When *T. lignorum* was inoculated into pots containing *Helminthosporium* sp. and *Fusarium* sp., the pathogenic action of these organisms was checked and rendered harmless to plants (60). *T. lignorum* was also found (17) to be decidedly antagonistic to *Rhizoctonia* and *Pythium*, organisms responsible for seed decay and damping-off of cucumbers.

The presence of *Gibberella* on corn inhibited infection due to *T. viridis;* seed grains inoculated with the former gave more vigorous growth than uninoculated seed grains (226). On the other hand, *T. viridis* was found able to attack and to destroy the sclerotia as well as the mycelium of such pathogenic fungi as *Corticium rolfsii*, *Corticium sadakii*, and *Sclerotinia libertiana* (*S. sclerotiorum*). The utilization of this organism for the biological control of plant diseases has, therefore, been suggested (415).

Henry (398) believed that the biological control by the soil micro-

flora could even be directed against internal seed infection, since appreciable damage to surface-sterilized flax seed was found to occur in sterilized but not in unsterilized soil.

CONTROL OF PATHOGENIC FUNGI IN SOIL BY INOCULATION WITH ANTAGONISTIC MICROORGANISMS

Despite the favorable results obtained from the action, in artificial culture, of antagonistic bacteria and fungi upon plant pathogens, the field results have often been rather disappointing. The soil microflora seems to have no marked effect on certain diseases, such as the seedling blight of barley; the antagonistic action of the soil population appears (127) to be insufficient to suppress the injury caused by diseased seed. The addition of *T. lignorum* and certain other fungi and bacteria to infected seed or to sterilized soil inoculated with *H. sativum* often prevented seedling injury, increased the stand, and decreased the number of deformed seed. Chudiakov (130) suggested inoculation of seed with bacteria, for the control of flax against infection with *Fusarium*. It has been said that wheat seedlings were protected from attack by the simultaneous introduction of lytic bacteria with the pathogenic fungus, but when the bacteria were introduced 24 hours after the fungus, they were unable to protect the wheat sown 3 days later.

On the basis of extensive studies on the control of plant-disease-producing fungi by means of antagonistic soil microorganisms, Novogrudsky (646) came to the following conclusions: The distribution and vigor of parasitic fungi are a result, on the one hand, of resistance and immunity of plants to infection, and, on the other, of the antagonism between soil microorganisms and pathogenic fungi. Among the numerous forms of antagonism existing between soil microorganisms and pathogens, those bacteria which produce lysis of fungi deserve particular attention. The bacteria are widely distributed in nature; they are able to destroy and to dissolve the mycelium and the spores of different fungi, including species of *Fusarium, Colletotrichum,* and other phytopathogenic fungi. The lytic effect takes place not only in artificial media, but also in the soil. The inoculation of sterilized soil with *F. grami-*

nearum led to the inevitable death of wheat plants, but additional inoculation of the soil with lysogenic bacteria protected the wheat from the disease. The addition of bacteria to unsterilized soil which has been made sick by continuous growth of flax markedly lowered the percentage of plants diseased by *F. lini.*

The term "bacterization" was suggested by the Russian investigators (46, 130, 631, 647) to designate the process of treatment of seed with lysogenic bacteria, whereby the plants are protected against pathogenic fungi. The susceptibility of plant seedlings to infection by fungi could be decreased not only by the specific antagonistic bacteria, but also by the presence of other bacteria which are able, in one way or another, to retard the development of the fungi. The conclusion was reached that the effect of bacteria on germinating seeds is due to the metabolic products liberated by the bacteria, which are capable of depressing the development of parasitic fungi. By treating flax seeds with the culture filtrate of the antagonistic bacteria, a similar or even greater decrease in the number of diseased seedlings was obtained. The nature of the active substance produced by the antagonists was not investigated further. It was said to accumulate in 5-to-10-day-old cultures. Heating at 80° C. for 10 minutes had no effect upon the substance, but heating at 100° C. brought about its inactivation.

Jensen (455) concluded that the beneficial result of bacterization is due not to nitrogen-fixation by *Azotobacter* or to production of growth-promoting substances by microorganisms, but to the protection of the seedlings against plant pathogens by specific bacterial antagonists.

Although it has now been definitely established that certain organisms can repress or even destroy disease-producing fungi, the utilization of specific microbial products for the control of plant diseases has made comparatively little progress so far. Leemann (521) tested the action of various secretions and extracts of microorganisms upon *H. sativum.* He concluded that microorganisms, pathogenic or nonpathogenic, can supply substances useful as preventive measures against plant pathogens. No favorable effects upon the development of *Rhizoctonia* disease in plants could be observed, however, from the application to soil of certain bacterial and fungus products.

The only important procedure which has thus far found a practical

application is the treatment of the soil in such a manner as to modify its microbiological population, which in its turn can destroy or in other ways control the activities of plant pathogens. Many such instances can be cited for illustrative purposes. In the case of root rots of the strawberry, it was found that carbohydrate decomposition induces a favorable change in the soil microflora from pathogenic or potentially harmful organisms to beneficial or rather innocuous types (413). Studies of the eradication of cotton root rot led to recommendations of treatments of soil with organic materials, especially during the period of increased microbial activity (610). The survival of the fungus is considered to be limited by microbial interrelationships rather than by food exhaustion. During the early stages of incubation, viable sclerotia are destroyed more rapidly than dead sclerotia in soils treated with organic matter. It was suggested, therefore, that the germination of the sclerotia is an important factor for their elimination from the soil. Field application of organic materials, accompanied by early October plowing, results in an increase in microbial activity. This brings about a reduction of the incidence of dead cotton in the succeeding crop and greater difficulty of finding sclerotia.

It was reported recently (63) that *R. solani* is able to cause 100 per cent damping-off of radish seedlings planted at a distance of 4 cm. from the inoculum; when the seeds were planted at a distance of 9 cm. the damage was 40 per cent. The addition of 1 per cent ground wheat straw or dried grass to the soil had a marked depressing effect upon the growth of the fungus. This was ascribed to the nitrogen starvation of the mycelium, accompanied by the rapid utilization of the available soil nitrogen by the cellulose-decomposing microorganisms multiplying at the expense of the fresh organic material; carbon dioxide produced by the cellulose-decomposers was believed to cause a marked fungistatic action on the *Rhizoctonia*.

Treatment of the soil with organic materials, which results in the destruction of certain plant pathogens by stimulating the development of saprophytic microorganisms, may be called "partial disinfection."

The possible injury caused to leguminous plants through the antagonistic action of soil bacteria upon the root nodule bacteria has been but little investigated (495).

CHAPTER 14

THE OUTLOOK FOR THE FUTURE

THE production of antibiotic substances by microorganisms under controlled laboratory or factory conditions and the utilization of these substances for disease control are of very recent origin. It has been known for more than half a century that certain microbes are capable of combating others, and it has even been suggested that saprophytic microorganisms may in time be utilized for controlling the growth of pathogenic forms. Until recently, however, these suggestions were largely speculative. Such positive facts as were available were merely isolated items, the full significance of which was not sufficiently well recognized. This is true, for example, of the claims and counterclaims concerning pyocyanase and pyocyanin, two antibiotic substances of bacterial origin, the antibacterial properties of which have long been known. It is true also of certain antibiotic substances produced by fungi, such as gliotoxin. The existing confusion is due largely to the fact that the utilization of these antibiotic substances for disease control gave results that were rather disappointing.

In 1929, Fleming observed that the growth of a mold, which was later identified as *P. notatum*, on a plate seeded with staphylococci prevented the growth of these bacteria. In liquid media, this mold produced a soluble substance, designated as penicillin, that inhibited pyogenic cocci and members of the diphtheria group but not gram-negative rods. Fleming, however, did not go beyond the suggestion that this substance be utilized for disease control or beyond the statement that it might prove useful because of these antibacterial properties. This contribution received no further attention for more than a decade, with the exception of very few experiments that served to prove Fleming's original observations. The full realization of the fact that we are dealing here with an entirely new field of biology and chemotherapy that is bound to enrich the subject of control of disease came only about half a decade ago.

In 1939, Dubos announced that new antibacterial agents can be iso-

lated from soil microorganisms that are active not only *in vitro* but also *in vivo*. He proceeded with the isolation of such substances in a novel and unique manner, which consisted in enriching the soil with pathogenic bacteria, isolating from such enriched soil specific antagonistic organisms capable of destroying the pathogens, and finally isolating the antibiotic substances from the bacteria. This work pointed to a new method of approach to the isolation of antibiotic substances and attracted the immediate attention of bacteriologists, chemists, and medical investigators. One no longer had to depend for the isolation of antagonistic organisms upon mere air contaminations of exposed bacterial plates, or upon accidental observations of the destructive action of one microbe upon another. One could now proceed systematically with the isolation of microorganisms capable of destroying *specific* disease-producing bacteria. The mechanisms whereby such destruction was brought about were found to be due to the production of characteristic substances, known as antibiotic agents. The isolation of each required special methods, because of its specific chemical nature. Since most of the antibiotic substances are selective in their action upon different bacteria, affecting some and not others, the method proved to be of great importance in pointing a way to the isolation of a variety of agents active upon gram-positive bacteria as well as gram-negative forms.

It was not sufficient, however, merely to isolate these protoplasmic poisons. It was essential to determine the effect of animal tissues upon the action of these agents and to establish their selective activity upon the bacteria in the animal body as compared to the test tube. However, once it was recognized that some of these antibiotic substances act *in vivo* against a variety of diseases, that branch of medical science known as chemotherapy acquired a new group of tools. This work led to a re-examination of the potentialities of penicillin, which in turn led to the second important contribution, namely, the work of Chain and Florey and their co-workers. These investigators succeeded in obtaining crude active preparations of penicillin, which, they demonstrated, had a marked effect upon various gram-positive bacteria in the animal body. The importance of this discovery was accentuated by the great need of the moment and the urgency for new methods of combating infections resulting from World War II. These investigations immediately at-

tracted universal attention, and were soon followed by remarkable development in the manufacture, isolation, and study of the chemical nature of this highly important antibiotic agent. Penicillin fully deserves the designation "Wonder Drug" given to it by the popular press.

These two epoch-making contributions were rapidly followed by a series of investigations that resulted in the isolation of a number of other antibiotic substances. It was soon recognized that one is not dealing here with only two types of chemical compounds capable of destroying various pathogenic bacteria and fungi, but that a new field of science bordering on microbiology, chemistry, and pathology was being opened that was bound to result in many chemotherapeutic applications. The fact that many of these agents, including penicillin, are produced by several different organisms and, further, the fact that many of these compounds are produced in different chemical modifications open to the chemist new fields for the synthesis of types of compounds heretofore unknown, and point out to the medical world new ways of combating infections and epidemics.

The rapid progress made in the utilization of antibiotic substances in so brief a period of time can best be illustrated by the following two citations:

On May 4, 1940, Garrod (315), in discussing the use of antiseptics in wounds, wrote: "Only a few years ago it was thought impossible to kill bacteria within the body with chemicals and likely always to remain so. This belief was shaken by the discovery of a urinary antiseptic which really worked, and it was shattered by the introduction of Prontosil, with all its manifold consequences. Are we still to deny the possibility of killing bacteria which are merely lying on a body surface?"

Less than four years later, Florey (275) summarized the value of penicillin: "1. As a preventive of infection in wounds, enabling a potentially septic wound to be treated in much the same way as an aseptic one, 2. in the promotion of healing in burns and for ensuring the success of skin grafts, 3. in infections (due to sensitive organisms) either (a) chronic, or (b) of such severity as to render the prospect of death likely, which have not responded to other forms of treatment, 4. in acute infections due to sensitive organisms, 5. in the rapid curing of gonorrhoea including sulphonamide-resistant cases, 6. in pneumonia,

7. probably in gas gangrene, but here numbers have been few and methods not fully tried out."*

It appears, therefore, that certain generalizations concerning possible future developments in the field of antibiotic substances are justified.

A SEARCH FOR NEW ANTIBIOTIC AGENTS: A PROBLEM FOR THE MICROBIOLOGIST

Although some fifty compounds or preparations possessing bacteriostatic and fungistatic properties have already been isolated from microorganisms, there is sufficient evidence that many more can be obtained without too great difficulty, if enough organisms are studied in greater detail. In this connection, three methods of approach have been followed: (a) testing organisms found in culture collections for antibacterial activity in general, followed by a detailed study of one or more substances produced by one or more organisms; (b) isolating specific organisms, such as members of the *P. notatum–P. chrysogenum* groups, from different soils and from moldy food materials and testing them for the production of penicillin, in the hope of finding more active organisms than those now known to exist; (c) enriching the soil with specific bacteria, followed by the isolation of organisms capable of inhibiting the growth of or of destroying such bacteria.

Several surveys (26, 282, 504, 628, 644, 934, 936, 986) have already been made concerning the distribution of organisms capable of producing antibiotic substances among certain groups of bacteria and fungi. Only very few such organisms were selected for more detailed investigation. The reasons for this are quite obvious and are based largely upon the great amount of time and experimentation required for the isolation of any one substance. The selection of a particular substance was largely governed by its specific antibiotic spectrum, or its activity upon particular bacteria, its toxicity to animals, and its activity *in vivo*. The following illustrations will suffice:

Of all the aerobic spore-forming bacteria known to produce antibiotic substances, only *B. brevis* has been utilized for the isolation of

* Further information on this subject is found in the various reports listed in Chapter 12 of this book and in a group of papers presented before a symposium on antibiotic agents (65, 163, 189, 399a).

tyrothricin. It is known, for example, that various strains of *B. mesentericus*, *B. mycoides*, *B. subtilis*, and *B. simplex* are capable of producing antibiotic substances, some of which are markedly different chemically, biologically, or in selective activity. A more detailed study of these organisms and the substances produced by them is bound to enlarge greatly our knowledge of this group of chemical compounds and their therapeutic potentialities.

Of all the nonspore-forming bacteria possessing antagonistic properties found in soils and water basins, only two have been studied in detail: *Ps. aeruginosa* has been utilized for the production of pyocyanase and pyocyanin, and *C. iodinum* for the production of iodinin. It is known, however, that a large number of other nonspore-forming bacteria are capable of producing a variety of antibiotic substances, the chemical nature and biological activities of which are still but little understood.

Only very few of the antibiotic substances produced by actinomycetes have so far been investigated, isolated, or concentrated; namely, actinomycetin, actinomycin, streptothricin, and proactinomycin. Even these few substances, however, differ markedly in chemical nature and in biological activity. In view of the fact that as many as 20 to 40 per cent of all the actinomycetes are known to be capable of producing antibiotic substances, many of which undoubtedly differ from those that have already been isolated, the wealth of material that is awaiting investigation can only be surmised. Some of these possibilities have been definitely indicated. Here belong the lysozyme-like agents discussed by Russian investigators (507), micromonosporin which is active largely against gram-positive bacteria, and streptomycin (795). The latter was found, on the one hand, to resemble streptothricin in its chemical properties and activity *in vivo*, and, on the other hand, to differ from it in its antibacterial spectrum.

The production of antibiotic agents by fungi likewise has been but insufficiently studied. The following pertinent facts may direct attention to the many problems still awaiting investigation:

(a) Some antibiotic substances, like penicillin, clavacin, and gliotoxin, are produced by a number of different organisms; the nature of the or-

ganism often influences not only the yield of the substance but its chemical nature and its biological activity.

(b) The mode of nutrition and the manner of growth of a single organism have often been found to influence the concentration and the nature of the antibiotic substance, which may be formed in one medium and not at all or in much lower amounts in another medium. Some organisms are greatly favored in the production of antibiotic substances by the presence in the medium of certain vitamin-like complexes. Moreover, the formation of a substance is usually associated with a certain stage of growth of the organism, since the substance is produced at one time and then rapidly destroyed, the range of its accumulation often being very narrow. These facts point to certain fundamental aspects in the physiology of the organism producing the antibiotic substance that are still little understood.

(c) Certain organisms, such as members of the *Fusarium* group, produce bacteriostatic substances, the action of which, however, is rapidly overcome by the bacteria. This points to problems on the stability of the antibiotic substance and on the adaptation of bacteria to the substance.

(d) Although it is known that certain yeasts produce antibiotic substances, either of an adaptive or of a nonadaptive kind, very little is known concerning the nature and mode of action of such substances.

These and many other problems are awaiting solution. The microbiologist is faced with a new field of research second only to that of the very discovery of the causation of disease by microorganisms.

THE OPPORTUNITY FOR THE CHEMIST

The chemist has been searching far and wide for new chemotherapeutic agents. He has synthesized many thousands of compounds, only very few of which have proved to be of practical chemotherapeutic value. The chemist has started from a certain lead, such as the arsenical group in the salvarsan type of compounds and the sulfa-radical in the sulfanilamides. The discovery of new chemical agents possessing antibacterial or antifungal properties offers the chemist many new models to draw upon for varied types of syntheses.

Although only very few antibiotic agents have so far been isolated,

and even fewer crystallized, it is already well established that we are dealing here with a great variety of chemical compounds. It is sufficient to mention the polypeptides (tyrothricin), oxidation-reduction systems (pyocyanin, actinomycin), sulfur compounds (gliotoxin), quinones (citrinin), various other non-nitrogenous simple (clavacin) and more complex (fumigacin) compounds, a variety of nitrogenous compounds comprising both bases (streptothricin, proactinomycin) and acids (penicillin). Compounds, like actinomycin, that are highly active against bacteria but also highly toxic to animals, may possibly be modified in such a manner as to reduce their toxicity without impairing their activity. This is also true of simpler compounds, such as the less toxic but also less active clavacin and gliotoxin. Many a chemist is awaiting the solution of the problem of the chemical nature of penicillin before beginning new syntheses.

Doubtless most of the compounds that prove to be useful as chemotherapeutic agents will sooner or later be synthesized. The contribution of the bacteriologist may be all but forgotten in the light of the forthcoming chemical developments, but even the bacteriologist will be grateful for new tools to help combat disease-producing agents.

THE FIELD OF CHEMOTHERAPY

The utilization of the activities of antagonistic microorganisms for the control of human and animal diseases has only begun. The same may be said of the control of plant diseases. Many practices in surgery and many old-time remedies are based on the creation of conditions favorable to the development of antagonistic microbes. Consider, for example, the method of cast surgery developed during the Spanish Civil War. To what extent the application of pure cultures of antagonists may improve these and similar practices still remains to be determined. Plaster treatment of wounds, without the use of antiseptics, has often given marvelous results. Such wounds have been found to contain aerobic bacteria with no one group predominating, except that *Ps. aeruginosa* tends to become more numerous when the healing process has been established (889). It still remains to be determined whether this organism exerts a favorable effect due to its antagonistic properties or is only another wound-infecting agent.

Of particular importance is the development of the manufacture of antibiotic substances. Largely because of the stimulus given by World War II when the need for new antibacterial agents became very acute, an intensive study was made of the practicability of utilizing some of the agents already known, and search was made for new ones. Among these, penicillin occupies a leading place. As these lines are written, a large number of great concerns in this country, in Great Britain, and elsewhere are engaged in the manufacture of this drug by utilizing several strains of *P. notatum* and *P. chrysogenum*. An intensive search is being made for new agents capable of inhibiting the growth of and destroying other pathogens resistant to the action of penicillin.

The progress made in the isolation of antibiotic substances from many microorganisms has not kept pace with their evaluation as chemotherapeutic agents. In discussing antimicrobial agents of biological origin, Dubos (189) emphasizes that students of infectious diseases are primarily concerned with the action of these substances upon certain strains and stages of the parasites, with the mechanism of their action upon the susceptible cells, and with physiologic and pathologic effects on the host. McIlwain (560), on the other hand, believes that animal testing in chemotherapy is not necessarily much nearer to the conditions under which the drug will be finally used than are properly chosen *in vitro* conditions; although *in vitro* testing does not reproduce all the conditions of the normal environment of the parasite, it is less likely, under present conditions of testing, to introduce new and unknown factors than is testing in another host. The *in vitro* and *in vivo* studies of an agent are considered as complementary.

The utilization of fungi and bacteria against plant diseases has also been variously attempted (472). The main difficulty involved is to establish the antagonist in the soil. This can be done by modifying soil conditions, as by the addition of stable manure or other plant and animal residues, in order to favor the development of the antagonist.

The activities of antagonistic microorganisms are also utilized for combating injurious insects and other lower animal forms destructive to plants and to animals. Among the insects, the Japanese and other Asiatic beetles have been treated rather successfully by the use of nematodes and certain specific bacteria. Extensive use has already been made

of these bacteria, by inoculating the soil with grubs heavily infected with them.

Comparatively little is yet known of the ability to control, by means of antibiotic agents, diseases caused by protozoa, such as malaria and trypanosomes, virus infections, and certain bacterial diseases such as tuberculosis.

These instances suffice to arouse hope that even greater progress can be expected in the control of disease by utilization of the activities of antagonistic microorganisms. So far, most energies have been directed to the treatment of acute infections caused by bacteria. Less is known of chronic infections. Whether or not man will ever be able to control all diseases caused by the numerous microscopic and ultramicroscopic forms of life through the utilization of the activities of antagonistic microorganisms, he will have gained sufficient knowledge from the mode of action of these organisms, and of the substances produced by them, to justify further hope in the possibilities thus opened.

MODE OF ACTION OF ANTIBIOTIC SUBSTANCES: A FIELD FOR THE PHYSIOLOGIST

Finally, there remains the fourth important group of problems involved in the study of antibiotic substances, namely, the mode of action of these substances upon bacteria. The fact that different agents vary greatly in their bacteriostatic and bactericidal action upon different bacteria is well established. A number of mechanisms have been propounded, some of which hold true for one substance and some for more than one. Each of these mechanisms involves some extremely puzzling physiological problems. To take only two illustrations:

(a) If a given substance interferes with the utilization by the bacteria of a certain metabolite in the medium, as in the relation of sulfa-drugs to *p*-amino-benzoic acid, one must assume that the sensitive bacteria require the metabolite in question and the resistant forms do not, or that the resistant bacteria synthesize larger concentrations of the particular metabolite than the sensitive forms. Since the sensitivity of the bacteria to an antibiotic substance is often more of degree than of kind, as in the case of actinomycin, the assumption would be that the metabolite is

either required in different concentrations by the various organisms or is synthesized to a different extent.

(b) The adsorption of the antibiotic substance by the bacterial cell, rendering the cell incapable of multiplying or dividing, points to another type of mechanism that may be rather common. This may often express itself in the abnormal enlargement of the cell. A clear understanding of this phenomenon will have to await a better knowledge of the mechanism of cell fission. Should one assume that the resistant cells and the sensitive cells divide by different mechanisms?

All these and many other problems point directly to the fact that a better understanding of the physiology of the microbial cell will be gained from a clearer appreciation of the mode of action of antibiotic substances upon the bacterial cell.

It is thus to the smallest of living systems, the microbe, that we must look for the solution of some of the most important problems that have faced man as well as his domesticated and friendly animals and plants.

CLASSIFICATION OF ANTIBIOTIC SUBSTANCES

PRODUCED BY ACTINOMYCETES

Actinomyces lysozyme (*Streptomyces* sp.)
Actinomycetin (*S. albus*)
Actinomycin (*S. antibioticus*)
Micromonosporin (*Micromonospora* sp.)
Proactinomycin (*N. gardneri*)
Streptomycin (*S. griseus*)
Streptothricin (*S. lavendulae*)

PRODUCED BY ALGAE

Chlorellin (*Chlorella* sp.)

PRODUCED BY BACTERIA

B. simplex factor (*B. simplex*)
Diplococcin (Streptococci)
Gramicidin (*B. brevis*)
Iodinin (*Ch. iodinum*)
Pyocyanase (*Ps. aeruginosa*)
Pyocyanin (*Ps. aeruginosa*)
Subtilin (*B. subtilis*)
Toxoflavin (*B. cocovenenans*)
Tyrocidine (*B. brevis*)
Tyrothricin (*B. brevis*)
Violacein (*B. violaceum*)

PRODUCED BY FUNGI

Aspergillic acid (*A. flavus*)
Chaetomin (*Ch. cochliodes*)
Citrinin (*P. citrinum*, *A. candidus*)
*Clavacin (*A. clavatus*, etc.)
*Clavatin (*A. clavatus*)
*Claviformin (*P. claviforme*)
†*E. coli* factor (*P. notatum*)
‡Flavicin (*A. flavus*)
§Fumigacin (*A. fumigatus*)
Fumigatin (*A. fumigatus*)
‡Gigantic acid (*A. giganteus*)
Gliotoxin (*Trichoderma*, *Gliocladium*, *A. fumigatus*)
§Helvolic acid (*A. fumigatus*)
Kojic acid (*A. oryzae*)
†Notatin (*P. notatum*)
‡Parasiticin (*A. parasiticus*)
*Patulin (*P. patulum*)
†Penatin (*P. notatum*)
Penicidin (*Penicillium* sp.)
Penicillic acid (*P. puberulum*, *P. cyclopium*)
Penicillin (*P. notatum*, *P. chrysogenum*)
†Penicillin B (*P. notatum*)
Puberulic acid (*P. puberulum*)
Spinulosin (*A. spinulosum*, *A. fumigatus*)

NOTE. Terms marked with the same symbol are synonyms.

GLOSSARY

Antagonism. The phenomenon of a living organism inhibiting the growth or interfering with the activities of another living organism as a result of the creation of unfavorable conditions in the medium or the production of a specific antimicrobial substance.

Antagonist. An organism having the capacity to inhibit the growth or interfere with the activity of another organism.

Antagonistic substance. A term frequently used to designate a substance that neutralizes the bacteriostatic action of an antibiotic substance.

Antibiosis. The inhibition of growth of one organism by another.

Antibiotic. Inhibiting the growth or the metabolic activities of bacteria and other microorganisms by a chemical substance of microbial origin.

Antibiotic substance, antibiotic. A chemical substance, of microbial origin, that possesses antibiotic properties.

Anti-inhibitor, inhibitor, suppressor are terms of similar significance to *antagonistic substance.*

Bactericidal. Causing the death of bacteria.

Bacterioantagonistic. Inhibiting the growth of bacteria.

Bacteriolytic. Causing not only the death of bacteria but also their lysis or disintegration.

Bacteriostatic. Inhibiting the growth of bacteria.

Bacteriostatic or *antibiotic spectrum.* A range of inhibition of growth of different bacteria by different concentrations of an antibiotic substance. It may be expressed graphically, the bands of the spectrum representing the concentrations of the substance.

Biostatic complex. The sum total of factors that limit microbial development in a medium. The absence of such factors may result in the formation of toxic products.

Fungicidal. Causing the death of fungi.

Fungistatic. Inhibiting the growth of fungi.

Inactivator, nontoxic. A substance that inactivates plant viruses and is not detrimental to most forms of life.

Inhibitor or *inhibitive substance*. A term variously applied, but usually used to designate a substance that inhibits the growth of bacteria and other microorganisms.

Lysogenesis. The production by an organism of substances that cause the lysis of bacterial cells.

Lysozyme. A substance produced by living tissues (white of egg, tears, and also certain microorganisms) that is capable of dissolving living bacterial cells, especially certain micrococci.

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