# FUNDAMENTAL PRINCIPLES
*of*
# BACTERIOLOGY

*The quality of the materials used in the manufacture of this book is governed by continued postwar shortages.*

# Fundamental Principles of Bacteriology

By A. J. SALLE, B.S., M.S., PH.D.
*Professor of Bacteriology, University of California at Los Angeles*

THIRD EDITION

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FUNDAMENTAL PRINCIPLES OF BACTERIOLOGY

*To*

CECELIA DAVERSO SALLE

*this book is affectionately dedicated*

# PREFACE TO THE THIRD EDITION

What one man has failed to achieve, another has accomplished, and that what was unknown to one age has been cleared up by the following age, and that the sciences and arts are not cast in a mould, but are formed and shaped by degrees through repeated handling and polishing . . . and in manipulating and kneading this new matter over and over again, stirring and warming it, I open up for him who comes after me some facility for profiting by it more at his ease, and I make it more pliable and manageable for him . . . as much will the second do for the third.

MICHEL DE MONTAIGNE

This book has been thoroughly revised and completely rewritten to bring the contents up to date. Every chapter has been included in the revision. To name the significant changes that have been made would mean the inclusion of almost the entire book.

The author has attempted to include only sound fundamental material to give the beginner a solid foundation for more advanced work on the subject. Explanations of all phenomena are included insofar as it was possible to do so.

Much of the material appears for the first time and may require proper evaluation by others before it can be accepted as fact. The student should be warned against uncritical acceptance of views that may not be solidly established. It is believed that the training afforded by contemporary science and education should prove adequate to develop critical, inquiring students.

Greater emphasis is placed on the use of chemistry for a clearer understanding of the composition of bacteria and the reactions that they produce. The sources of the material are given in the body of the book, and a bibliography is included at the end of each chapter for more detailed information.

The number of illustrations has been greatly increased. Also, most of the old illustrations have been replaced by new and better ones. The illustrative material was prepared and selected with great care and photographed with all the technical skill at our command.

The outline classification of bacteria given in the book is based on the sixth edition of "Bergey's Manual of Determinative Bacteriology." The author is greatly indebted to Dr. Robert S. Breed for making this material available in advance of its date of publication. The names of

the organisms used throughout the book are based on this new classification insofar as it was possible to do so.

The author is greatly indebted to his wife and to Dr. Gregory J. Jann for their aid in reading and checking the proofs; to Dr. B. Wesley Catlin for her technical skill and patience in the preparation and selection of the illustrative materials photographed by the author, and for her aid in various other ways; and to others who offered valuable suggestions and criticisms during the preparation of the manuscript.

The author has attempted to acknowledge the sources of the text material and of the illustrations taken from the literature. Any errors or omissions are entirely unintentional. He alone accepts full responsibility for the content and arrangement of the book and for any errors that may have escaped detection.

A. J. SALLE

LOS ANGELES, CALIF.
*January*, 1948

# PREFACE TO THE FIRST EDITION

This is frankly a plea for the return of the dignity and importance of the preface. Too often the writing of a preface has become a chore, a necessary evil prescribed by custom. But like many practices which have become common through familiarity, the original purpose of the preface has perhaps been obscured by time and by the careless reading habits of the average reader who wishes to get to the meat of the book as quickly as possible. The preface is a vital part of the book and for good reasons. . . .

All of us come to a book loaded with prejudices. We are not as impartial as we think we are. Mention a topic or theme and we can be sure to express a certain point of view—right or wrong. It is the function of the preface to modify these prejudices by suggesting what presumably are new points of view. Thus, the preface is an exercise in persuasion. It must break down "reader resistance"; it must put the reader in the proper frame of mind to approach the reading of the book. If the preface is written with this idea in mind, the reader will come to the book proper already favorably disposed toward the author. If the author is inclined to evade actualities, he must then be prepared for reader apathy and perhaps neglect. . . .

JOHN R. WILBUR

This book has been written for those who are beginning the study of bacteriology and especially for those who plan to specialize in the subject. It is concerned chiefly with a discussion of the important principles and facts of bacteriology which a student should acquire in order to realize to the fullest extent the more advanced work on the subject.

The book is, as its name implies, a textbook on fundamentals. The author has tried at all times to keep this thought in mind in the preparation of the manuscript. The usual textbooks either are too elementary or do not contain sufficient fundamental material to give the beginning student a solid foundation on which to build for more specialized work on the subject. The author has tried to give explanations of all phenomena described in the book insofar as it is possible to do so, a point which has been greatly neglected in most texts. The book is profusely illustrated with chemical formulas because it is believed that no student can intelligently understand bacteriology without first having had at least inorganic and organic chemistry. This statement applies especially to the chapters on Biological Stains, Disinfection and Disinfectants, Enzymes of Bacteria, The Respiration of Bacteria, Protein Decomposition,

Industrial Fermentations, The Bacteriology of Water, and The Bacteriology of Soil.

The book differs in one important respect from practically all texts on fundamental bacteriology in that it is written as a combination textbook and laboratory manual. The experimental portion is not added as an appendix but is woven into the body of the manuscript under the appropriate chapters. The textbook material goes hand in hand with systematically arranged laboratory procedures. It is believed that bacteriology cannot properly be understood or appreciated unless studied in conjunction with experimental laboratory work. The incorporation of laboratory exercises into the body of the book permits the reader better to understand the textbook material, and the addition of text to the laboratory portion aids the student better to understand the experimental procedures.

Sufficient experimental material has been included to meet the fundamental requirements of beginning students in the bacteriology major and of students in the various divisions of agriculture, forestry, home economics, sanitary engineering, physical education, hygiene, public health, etc. The number of experiments should prove ample for a one-semester course. The author has purposely included a large number in order that the instructor may make a selection if desired.

The names of the organisms used are those recommended by the Committee on Classification of the Society of American Bacteriologists. Although the system does not satisfy everyone, it comes nearer to being a standard classification than any that has been used before and it is now in general use in this country.

The author has attempted to indicate in the text the sources of the material and illustrations used. He wishes to thank all who have offered suggestions and have been of assistance in the preparation of the manuscript. He is especially indebted to his wife and to I. L. Shechmeister for their aid in reading and checking the proof. The author alone accepts responsibility for any defects that may be inherent in the plan and scope of the book and for errors that may have escaped detection.

A. J. Salle

Berkeley, Calif.
*December*, 1938

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# CHAPTER I

## INTRODUCTION

Bacteriology is the science that deals with the study of organisms known as bacteria (singular, bacterium). Microbiology in its broadest meaning is the science that deals with the study of all kinds of microorganisms, such as bacteria, yeasts, molds, and algae. The term "microbe" is taken from the French and means a microscopic organism or microorganism. The word "germ," in popular usage, refers to any microorganism, especially to any of the pathogenic or disease-producing bacteria. It is probably synonymous with bacterium. Although this book will include a discussion of microorganisms in general, the major portion of the material will be concerned with the study of bacterial organisms.

Man, who is forever classifying things, has placed living organisms into either the plant or the animal kingdom. Most living organisms possess the characteristics of one kingdom or the other and may be sharply differentiated. However, bacteria and other microorganisms are intermediate or borderline forms and display the characteristics of both plants and animals. For this reason, it is not possible to classify them with either the plant or the animal kingdom.

Haeckel (1884) believed that considerable confusion could be avoided if the bacteria were placed into a new kingdom, which he named the *Protista*. He grouped into this kingdom all microorganisms such as yeasts, molds, bacteria, protozoa, and algae, which were classified with difficulty into the two older kingdoms. His suggestion did not gain wide acceptance, and Haeckel finally abandoned the idea. After all, it makes little difference whether bacteria and other microorganisms are plants or animals so long as their fundamental characteristics have been studied and are understood.

### CLASSIFICATION OF PLANTS

Bacteria are among the simplest forms of life known and, hence, show the characteristics of both plants and animals. They are generally placed in the plant kingdom. This is entirely for the sake of convenience and does not mean necessarily that they are more closely related to plants than to animals.

The plant kingdom may be divided into four main groups or divisions as follows:

1. *Thallophyta.*
2. *Bryophyta.*
3. *Pteridophyta.*
4. *Spermatophyta.*

Division I. *Thallophyta*, a division of the plant kingdom including: (1) the algae, (2) the fungi (bacteria, yeasts, molds), and (3) the lichens. The simpler forms are unicellular; in the higher forms, the plant body is a thallus, being commonly undifferentiated into roots, stems, and leaves.

Class 1. *Schizomycetes*, the bacteria. The organisms are single-celled, do not contain true chlorophyll, and multiply normally by a process of transverse or binary fission. A few species contain bacteriochlorophyll, a photosynthetic pigment that functions in a manner similar to chlorophyll. The cells may be spherical, cylindrical, comma-shaped, spiral, or filamentous, and are often united into chains or into flat or cubical aggregates.

Class 2. *Cyanophyceae*, the blue-green algae. These are unicellular or filamentous plants of primitive organization. They are mostly blue-green in color and autotrophic in their nutrition. They are found on damp earth, rocks, trees, or in fresh or salt water. Reproduction occurs by cell fission.

Class 3. *Flagellatae*, the flagellates. The flagellates are small, unicellular organisms with a true nucleus. The cells are motile by means of one or more cilia (flagella). Multiplication occurs by longitudinal division.

Class 4. *Diatomaceae*, the diatoms. The diatoms comprise a large group of unicellular algae. They are found in large numbers in both fresh and salt water and in damp soil. The cells exhibit great diversity in shape. The cell wall is rich in silica which persists as a skeleton after the death of the organism forming kieselguhr. Each individual cell is composed of two halves (valves) with an overlapping edge (girdle). Multiplication occurs by longitudinal division.

Class 5. *Chlorophyceae*, the green algae. The green algae occur free or attached, in fresh water or in moist places. Some forms are aerophilus and can endure complete desiccation. They are clear green in color, owing to the fact that the green chlorophyll is not masked by other pigments. Multiplication occurs asexually and by means of sexual spores.

Class 6. *Conjugatae*, the pure-green algae with uninucleate cells. They are all conjugate forms. They are a varied group of fresh-water algae, either being unicellular or having the form of unattached, unbranched filaments. The members are remarkable for the absence of any asexual method of spore formation.

Class 7. *Characeae*, the stoneworts. The stoneworts are formed in ponds and ditches, and sometimes form veritable meadows a foot in height. They have jointed stems with whorls of leaves at the nodes, on which the complex reproductive organs of both sexes are borne.

Class 8. *Phaeophyceae*, the brown algae. The brown algae are mostly marine plants, often of gigantic size and very diverse in form. The chlorophyll is masked by the presence of carotene, xanthophyll, and especially by fucoxanthin, a brown pigment allied to xanthophyll.

Class 9. *Rhodophyceae*, the red algae. The chlorophyll is masked by the presence of the red pigment phycoerythrin, and in some cases a blue pigment, phycocyan, in addition. The red algae are marine plants and exhibit great beauty in form and shades of coloring. They are attached by special growths of filaments or by discoid holdfasts. The thallus exhibits a great variety of forms. The sporangia develop usually four asexual, nonmotile spores (tetraspores). Sexual reproduction is oögamous, resulting in the development of asexual spores different from the tetraspores.

Class 10. *Myxomycetes*, the slime molds. The slime molds occur on damp earth and decaying vegetable matter, and consist of naked masses of protoplasm. They are often of considerable size, contain no chlorophyll, and exhibit a slow creeping

motion. Sexual multiplication occurs by the formation of enclosed sporangia which contain asexual spores.

Class 11. *Phycomycetes*, the algal fungi, includes a large class of parasitic or saprophytic organisms. The plant body ranges from an undifferentiated mass of protoplasm to a much-branched and well-developed mycelium. The vegetative mycelium is usually nonseptate. Multiplication is mainly asexual, by the formation of conidia or sporangia. The group shows every form of transition from asexual spore formation through simple conjugation to perfect sexual multiplication by egg and sperm in the higher species.

Class 12. *Ascomycetes*, the sac fungi. This is a large class of higher fungi distinguished by the presence of septate hyphae and by having their spores produced in sacs or asci. It comprises many groups of molds. A group of unicellular fungi, the yeasts, is generally included under the *Ascomycetes*. They reproduce by budding, by fission, by asexual spore formation, and by copulation.

Class 13. *Basidiomycetes*, the basidia fungi. This includes a large class of fungi characterized by having a septate mycelium and bearing the spores on a basidium. Chlamydospores are also formed. The lower *Basidiomycetes* includes many fungi parasitic on plants.

Class 14. *Fungi Imperfecti.* The fungi in this class are separated from the others in not having well-defined fruiting bodies. They produce neither asci nor basidia, but only conidia. The fungi that cannot be classified with the *Phycomycetes*, *Ascomycetes*, and *Basidiomycetes* are placed in this group. Some of the genera of the *Fungi Imperfecti* are *Oidium*, *Monilia*, *Endomyces*, *Torula*, and *Mycoderma.*

Class 15. *Lichenes*, the lichens. A lichen is a composite organism consisting of a mold living symbiotically with an alga. The mold obtains food materials from the alga, while the alga is in turn protected from external injury or exposure. The fructifications of the lichens are produced by the molds, not by the algae, which are always vegetative. The vegetable indicator, litmus, is derived from a lichen.

Division II. *Bryophyta*, a division of the plant kingdom including the liverworts and the mosses. They are characterized by the presence of archegonia of complex structure and by a certain amount of differentiation into stem and leaf. The group shows certain similarities in structure, linking it with the *Pteridophyta* on the one hand and with the *Thallophyta* on the other.

Class 1. *Hepaticae*, the liverworts. This includes the true liverworts and scale mosses, which are small, often moss-like plants. The gametophyte consists of a simple thallus or a thalloid shoot. The liverworts inhabit moist places, old logs, tree trunks, etc., and have a corresponding hygromorphic structure. Only a few true aquatic forms have been found. Some delicate forms grow among the mosses. The antheridia and archegonia are variously developed on the thallus.

Class 2. *Musci*, the mosses. The mosses include a large number of forms found in all parts of the world. They are characterized by small, leafy, often tufted stems bearing sex organs, which give rise to the development of oöspores. The oöspores develop into naked stalked capsules containing asexual spores. The asexual spores germinate and give rise to a structure known as a "protonema" on which the gametophyte, or moss plant proper, originates by budding. The mosses are found on dry soil, on rocks, on tree trunks, in swamps, in tropical forests, and in water.

Division III. *Pteridophyta*, a division of the plant kingdom including the ferns, water ferns, horsetails, and club mosses. They represent the highest type of flowerless plants, having well-developed vascular and tegumentary systems, and displaying

complete differentiation into roots, stems, and leaves. The sporophyte is the conspicuous generation, the gametophyte being reduced to a small thalloid body, the prothallium, bearing archegonia and antheridia. With the exception of a few tree ferns, practically all the *Pteridophyta* are herbaceous.

Class 1. *Psilophytinae.* These are considered the most primitive of the *Pteridophyta.* They are leafless or have small leaves. The sporangia are terminal, not on leaves. They are isosporous.

Class 2. *Lycopodiinae,* the club mosses. The leaves are small. Sporangia are solitary on the upper surface of the sporophyll. They may be isosporous or heterosporous. Spermatozoids are biciliate.

Class 3. *Psilotinae.* Leaves are small. Roots are absent. Sporangia are plurilocular on the upper surface of the sporophyll. They are isosporous. The spermatozoids are multiciliate.

Class 4. *Equisetinae,* the horsetails. The leaves are small and in whorls. The sporangia are borne in numbers on the lower side of the sporophyll. They may be isosporous or heterosporous. The spermatozoids are multiciliate.

Class 5. *Isoetinae.* The leaves are large in proportion to the stem. The sporangia are solitary on the upper surface of the sporophyll. They are heterosporous. The spermatozoids are multiciliate.

Class 6. *Filicinae,* the ferns. The leaves are large. The sporangia are numerous on the lower side of the leaves. They may be isosporous or heterosporous. The spermatozoids are multiciliate.

Class 7. *Pteridospermae,* the seed ferns. The leaves are large. They are heterosporous. The microsporangia are numerous on the lower side of the sporophyll. The macrosporangium contains only one macrospore.

Division IV. *Spermatophyta,* a division of the plant kingdom including the seed plants. This is the most numerous group of plants. The group is characterized by the marked development of the sporophyte, with great differentiation of its parts into roots, stems, leaves, flowers, etc., by the extreme reduction of the gametophyte, and by the development of seeds. All the members are heterosporous. Fertilization of the egg cell occurs either through a pollen tube emitted by the microspore or by spermatozoids.

Class 1. *Gymnospermae,* the cone-bearing plants, pines, hemlocks, etc. This class includes plants having seeds naked or not enclosed in an ovary. In some plants fertilization is accomplished by spermatozoids.

Class 2. *Angiospermae,* the flowering plants. The plants are characterized by having the seeds in a closed ovary. The group contains the vast majority of the seed plants and includes two subclasses: (1) the monocotyledons, or endogenous plants, and (2) the dicotyledons, or exogenous plants.

## DISTRIBUTION OF BACTERIA

Bacteria are widely distributed in nature, being found nearly everywhere. They are found in soil, air, water, petroleum oil from deeply seated regions, foods, decaying organic matter of all kinds, on the body surface, within the intestinal tract of man and animals, etc. The numbers vary from one place to another, depending upon the environmental conditions.

Some bacteria are more commonly distributed in certain places than others. The common occurrence of one or more species in a certain en-

vironment is spoken of as the natural flora of that particular environment. For example, the normal souring of milk is produced by *Streptococcus lactis*, an organism that is present in dung and on the coat of the cow and gains entrance to the milk at the time of collection. Changes in the environmental conditions produce changes in the bacterial flora.

**Soil.**—The numbers and kinds of organisms present in soils depend upon the type of soil, quantity of plant and animal debris (humus), acidity or alkalinity, depth, moisture content, and treatment. The great majority of soil organisms are found in the surface layer. The numbers decrease with depth, owing to lack of oxygen and food materials. A rich garden soil contains many more organisms than a poor uncultivated soil.

**Air.**—Bacteria are found in the atmosphere, being carried there principally by air currents. Organisms do not grow and multiply in air because conditions are not favorable for this to occur. There is no such thing as a normal atmospheric flora. The numbers and kinds depend upon location, amount of moisture, dust particles, wind currents, and the presence of toxic gases. The air over the ocean, far removed from shore, also shows the presence of microorganisms. In general, marine air contains fewer microorganisms than terrestrial air. The air over high mountains is usually free from organisms. The air of the city and country differ as to numbers and kinds of species present. Dusty rooms usually show considerably more organisms than do rooms kept free from dust. Bacteria are found usually adhering to particles of dust. This means that the more particles suspended in air, the greater will be the extent of bacterial contamination. Viable spores of yeasts, molds, and bacteria are commonly found in air, owing to the fact that these bodies are more resistant to the ultraviolet rays of the sun than are the vegetative cells producing them. These bodies are a frequent cause of air contaminations in bacteriological laboratories and, because of their great resistance to heat, require high temperatures to destroy them.

**Water.**—Most waters contain large numbers of bacterial organisms. The numbers vary considerably, depending upon the source of the water; *e.g.*, from deep or shallow wells, springs, rivers, lakes, ponds, streams, etc. Water polluted with sewage may contain thousands, or even millions, of organisms per cubic centimeter. Under some conditions, disease organisms may also be present. Some bacterial species are constantly present and constitute the natural flora of that water. Usually fewer bacteria occur in sea water than in soil. This is probably due to its poorer qualities as a culture medium.

**Foods.**—Foods are rarely free from living organisms. Some organisms are of benefit in producing desirable fermentations, such as occur in the oxidation of alcohol to acetic acid or vinegar, the lactic fermentation of cabbage to sauerkraut, etc. Frequently, undesirable organisms are found

in foods and produce abnormal changes. Certain diseases and intoxications may be produced by the consumption of foods contaminated with the specific organisms or their growth products.

Normal udders of cows are probably never free from bacteria. This means that freshly drawn milk is not sterile. The first milk drawn always contains more organisms than that collected at the close of the milking operation, owing to the fact that the bacteria are washed away from the udders early in the process. However, most of the bacteria found in milk are chiefly those which gain entrance during the operations of milking and handling. Unless the milk is properly stored immediately after collection, these organisms may be capable of producing undesirable changes and make the milk unfit for human consumption.

**Body.**—The outer surface or skin of the body always contains bacteria. The same applies to the alimentary tract and respiratory passages of man and animals. The skin, intestinal tract, and respiratory passages contain normal bacterial floras, which are for the most part harmless. Occasionally, some species penetrate the broken skin and intestinal wall, resulting in the establishment of a disease process. Usually the organisms are destroyed by the defense mechanisms of the host. It has been said that as much as one-fourth of the dry weight of the intestinal contents of man is composed of bacterial cells.

*Escherichia coli* is found in the large intestine of man. There are other organisms present, but in an adult on a mixed diet this organism predominates. The organism *E. coli*, then, is largely responsible for the natural flora of the large intestine. Changes in the environmental conditions produce changes in the bacterial flora. If the diet of an adult is changed from a high protein to a high carbohydrate diet, the *E. coli* organisms will be gradually reduced in numbers only to be replaced by a much larger organism known as *Lactobacillus acidophilus*. If this particular diet is maintained, *L. acidophilus* will now become the predominating organism of the large intestine.

## FUNCTIONS OF BACTERIA

Those who are not familiar with the activities of bacteria usually believe that the vast majority of them are harmful and that their chief function in this world is to gain entrance to the body and produce various kinds of diseases. This statement is entirely erroneous. Most bacteria are not only harmless but absolutely necessary for the existence of living things. Life could not exist in the complete absence of bacteria. They are necessary for the disposal of human and animal carcasses. The remains of plant crops, plant stubble, leaves, etc., are converted into soluble compounds by the soil organisms and made available to new plants. Some species are capable of taking nitrogen from the air and converting

it into compounds that are utilized by the plants. In the absence of fertilizers, such as animal manures, nitrates, and ammonium salts, there would be no nitrogen in the soil were it not for the activities of these organisms. Sulfur and phosphorus, two elements necessary for plant growth, are also converted into soluble inorganic compounds and absorbed by plant roots.

Fertile soils may always be distinguished from poor soils in containing greater numbers of viable organisms. If the soil is rich in plant remains, contains sufficient moisture, and shows the right temperature and hydrogen-ion concentration (reaction), many organisms will be present to attack the plant and animal residues, converting the insoluble and indiffusible constituents into soluble, diffusible compounds utilizable by the plants.

Bacteria are necessary for the disposal of sewage. They convert the insoluble proteins, fats, and carbohydrates (cellulose) into soluble, odorless compounds which may be disposed of in an inoffensive manner.

The souring of milk is the result of bacterial action. This is the first step in the preparation of butter and various types of cheeses. The ripening of cheese is brought about by the action of bacteria and molds, which are responsible for the odors and flavors imparted to cheeses.

These are only a few examples of the part played by the associated activities of organisms in nature. Many other useful purposes will be discussed under the various chapters in this book.

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## CHAPTER II

## THE MICROSCOPE

A microscope may be defined as an optical instrument, consisting of a lens, or a combination of lenses, for making enlarged or magnified images of minute objects. The term is compounded from the two Greek words *μῑκρός, micro*, small, and *σκοπεῖν, scope*, to view.

Bacteria are so small that they cannot be seen with the naked eye. They must be greatly magnified before they can be clearly seen and studied. The use of a microscope is, therefore, absolutely indispensable to the bacteriologist and to the biologist in general.

A simple microscope, or a single microscope, consists merely of a single lens or magnifying glass held in a frame, usually adjustable, and often provided with a stand for conveniently holding the object to be viewed and a mirror for reflecting the light. A compound microscope differs from a simple one in that it consists of two sets of lenses: one known as an objective and the other as an eyepiece, commonly mounted in a holder known as a body tube (Fig. 1). Accurate focusing is attained by a special screw appliance known as a fine adjustment. Compound microscopes give much greater magnifications than simple microscopes and are necessary for viewing and examining such minute objects as bacteria.

Every user of the microscope should first understand the principles involved in order that the instrument may be employed to the greatest advantage. As Sir A. E. Wright (1907) stated,

> Every one who has to use the microscope must decide for himself the question as to whether he will do so in accordance with a system of rule of thumb, or whether he will seek to supersede this by a system of reasoned action based upon a study of his instrument and a consideration of the scientific principles of microscopical technique.

### GENERAL PRINCIPLES OF OPTICS

The path of light through a compound microscope is illustrated in Fig. 2. The light, in passing through the condenser, object in plane I, and objective lens, would form a real and inverted image in plane II if the ocular or eyepiece were removed. In the presence of the ocular *F*, the rays are intercepted, forming the image in plane III. The real image is then examined, with the eye lens *E* of the ocular acting as a single magnifier and forming a virtual image in plane IV. The distance between the virtual image (plane IV) and the eyepoint is known as the projection dis-

tance. The object is magnified first by the objective lens and second by the ocular or eyepiece. With a tube length of 160 mm. (most microscope

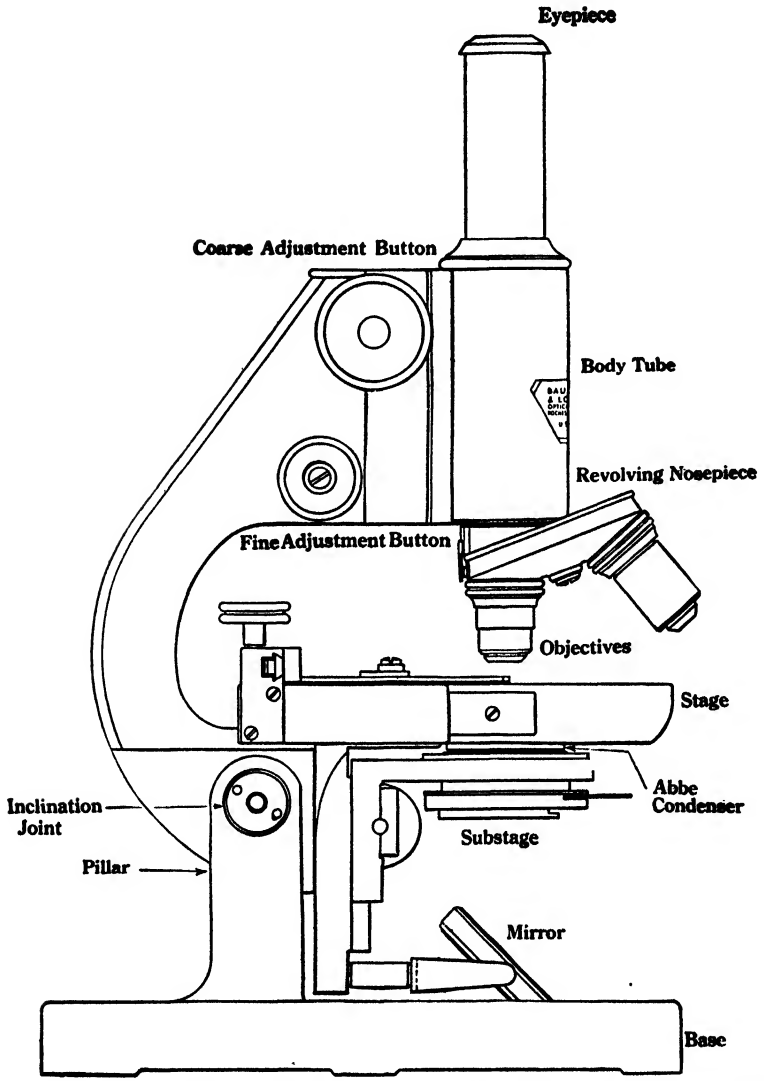



FIG. 1.—Compound microscope and its parts. (*Courtesy of Bausch and Lomb Optical Company.*)

manufacturers have adopted 160 mm. as the standard tube length), the total magnification of the microscope is equal to the magnifying power of the objective lens multiplied by the magnifying power of the ocular.

The above magnifications are obtained on a ground glass placed 10 in.

from the ocular of the microscope. After the microscope has been set at the proper tube length, the total magnification may be computed by

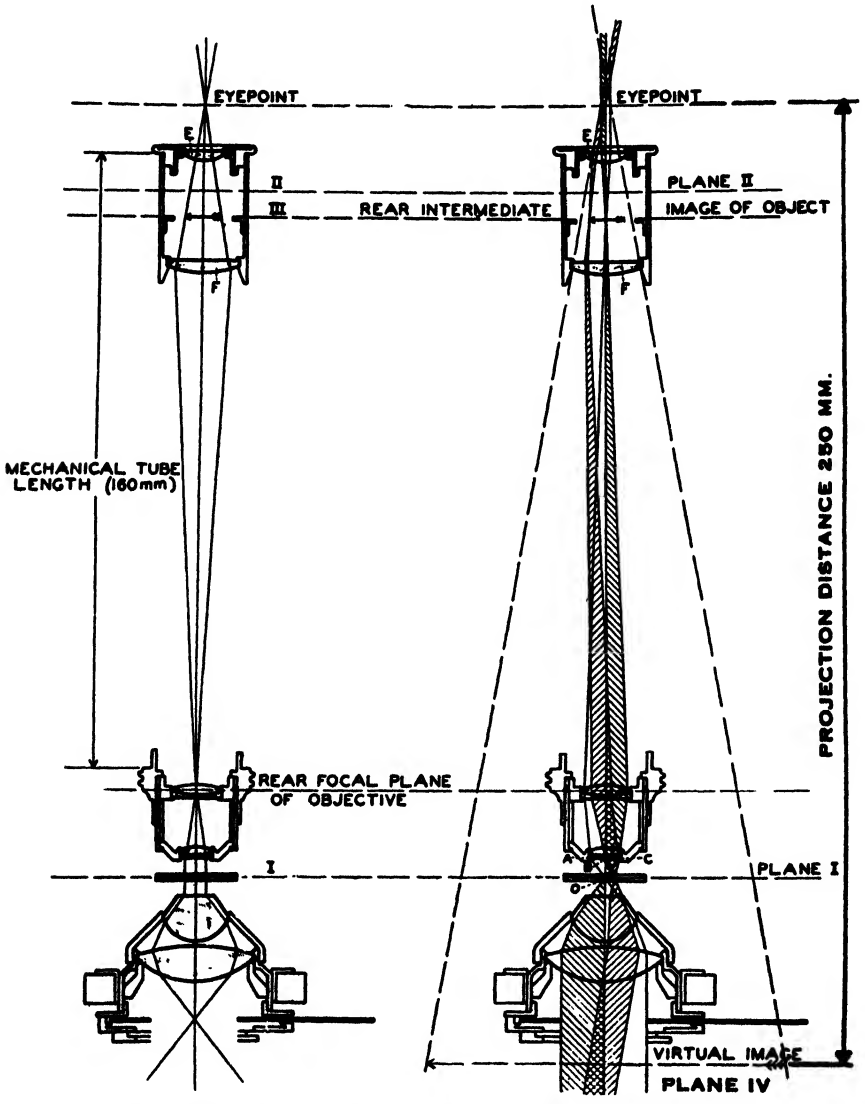



FIG. 2.—Path of light through a microscope. (*From Photomicrography, courtesy of Eastman Kodak Company.*)

multiplying the magnifying power of the objective by that of the eyepiece and by one-tenth of the distance from the eyepiece to the ground glass measured in inches. For example, if the ground glass is placed 10 in.

from the eyepiece of the microscope, the total magnification will be as given on the ocular and objective. If the ground glass is placed 20 in. from the eyepiece, the magnification will be twice as great. If placed 5 in. from the eyepiece, the magnification will be one-half as great. To take a specific example:

| | |
|---|---|
| Magnification of objective..... | 97× |
| Magnification of ocular..... | 10× |
| Distance of ground glass from ocular | 7 in. |
| Total magnification..... | 97 × 10 × 0.7(0.1 × 7) = 679× |

It may be seen that almost any degree of magnification could be obtained by using oculars of different magnifying powers or by varying the length of the draw tube. Even though the magnifying powers of the microscope could be greatly increased in this manner, the amount of detail that can be seen is not improved since this is strictly limited by the structure of light.

**Structure of Light.**—It is generally agreed that light is transmitted from luminous bodies to the eye and other objects by the undulating or vibrational movement of the ether. This is known as the undulatory or wave theory of light. Light waves travel at the rate of about 186,300 miles per second, and the vibrations are transverse to the direction of the propagation of the wave motion.

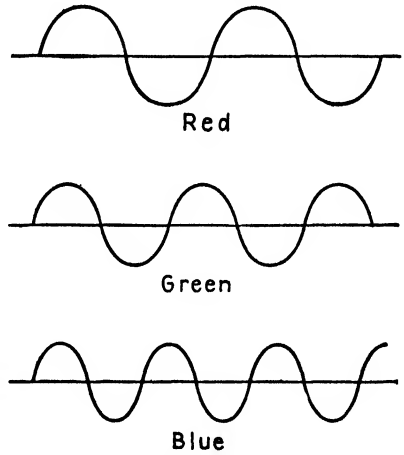



FIG. 3.—Wave lengths of light of different colors.

When a beam of white light is passed through a prism, a spectrum is obtained in which several colors form a series from deep red through orange,

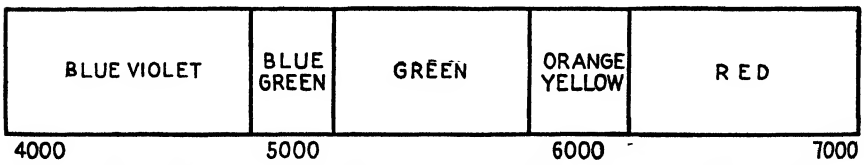



FIG. 4.—Light rays of the visible spectrum and their corresponding wave lengths in angstrom units.

yellow, green, blue, and indigo to deepest violet. It is known that the wave lengths of the various colors are different, that red shows the longest and violet the shortest waves of the visible spectrum.

The length of a light wave is the distance from the crest of one wave to the crest of the next (Fig. 3). The unit of measurement is the angstrom unit (Å.) which is equal to 1/10,000,000 mm. or to approximately 1/250,000,000 in. The visible spectrum, together with the corresponding wave lengths of the light rays in angstrom units, may be represented as shown in Fig. 4. Visible light waves, ranging in length from 4000 to 7000 Å., may be roughly divided into three portions: blue-violet, from 4000 to 5000 Å.; green, from 5000 to 6000 Å.; red, from 6000 to 7000 Å.

## OBJECTIVES

The objective is the most important lens on a microscope because its properties may make or mar the final image. The chief functions of the objective lens are (1) to gather the light rays coming from any point of the object, (2) to unite the light in a point of the image, and (3) to magnify the image.

**Numerical Aperture.**—The resolving power of an objective may be defined as its ability to separate distinctly two small elements in the structure of an object that are a short distance apart. The measure for the resolving powers of an objective is the numerical aperture (N.A.). The larger the numerical aperture, the greater the resolving power of the objective and the finer the detail it can reveal.

Since the limit of detail or resolving power of an objective is fixed by the structure of light, objects smaller than the smallest wave length of visible light cannot be seen. In order to see such minute objects, it would be necessary to use rays of shorter wave length. Invisible rays, such as ultraviolet light, are shorter than visible rays but, since they cannot be used for visual observation (photography only), their usefulness is limited.

The image of an object formed by the passage of light through a microscope will not be a point but, in consequence of the diffraction of the light at the diaphragm, will take the form of a bright disk surrounded by concentric dark and light rings (Fig. 5). The brightness of the central disk will be greatest in the center, diminishing rapidly toward the edge. The image cone of light composed of a bright disk surrounded by concentric dark and light rings is spoken of as the antipoint. If two independent points in the object are equidistant from the microscope lens, each will produce a disk image with its surrounding series of concentric dark and light rings. The disks will be clearly visible if completely separated but, if the images overlap, they will merge into a single bright area the central portion of which appears quite uniform. The two disks will not, therefore, be seen as separate images. It is not definitely known just how close the centers of the images can be and still allow them to be seen as separate antipoints.

The minimum distance between the images of two distinct object points decreases as the angle of light $AOC$ (Fig. 2), coming from the object $O$, increases. The angle formed by the extreme rays is known as the aperture of the objective. The ability of the objective lens system

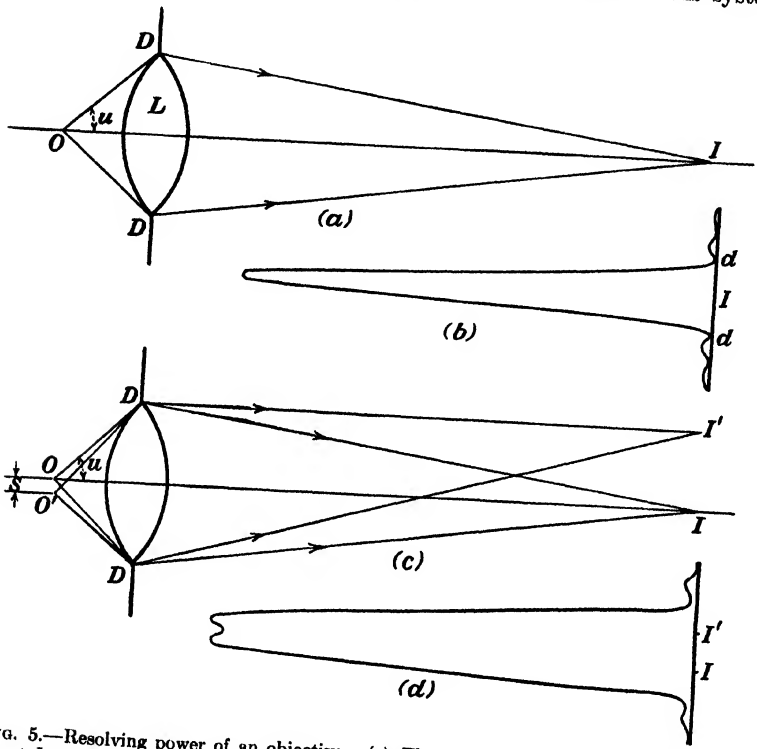



FIG. 5.—Resolving power of an objective. (*a*) The rays from the object at $O$ form an image at $I$. (*b*) Distribution of light in the image at $I$. The bright disk, *dd*, is surrounded by concentric dark and light rings. (*c*) Two independent points in the object $O$ and $O'$, form two images at $I$ and $I'$. (*d*) The two independent object points $O$ and $O'$ are so close together that their images overlap at $I$ and $I'$ and merge into a single bright area, the central portion of which appears quite uniform. (*From Sir Herbert Jackson and H. Moore, Microscope, courtesy of the Encyclopaedia Britannica, Inc.*)

to form distinct images of two separate object points is proportional to the trigonometric sine of the angle. The latter, then, is a measure of the resolving power of the objective. Actually, however, the sine of angle $AOB$ is used, which is just one-half of angle $AOC$. This is usually referred to as sin $u$. Since the sine of an angle may be defined as the ratio of the side opposite the angle in a right-angled triangle to the hypotenuse then,

$$\sin u = \frac{AB}{AO}$$

The light, in passing through the objective, is influenced by the refractive index $n$ of the space directly in front of the lens. This is another factor that affects the resolving power of an objective. The two factors, refractive index $n$ and sin $u$, may be combined into a single expression, the numerical aperture, which may be expressed as follows:

$$\text{N.A.} = n \sin u$$

**Importance of N.A.**—If a very narrow pencil of light is used for illumination, the finest detail that can be revealed by a microscope with sufficient magnification is equal to

$$\frac{\text{w.l.}}{\text{N.A.}}$$

where w.l. is the wave length of the light used for illumination and N.A. is the numerical aperture of the objective. The resolving power of the objective is proportional to the width of the pencil of light used for illumination. This means that the wider the pencil of light, the greater the resolving power. The maximum is reached when the whole aperture of the objective is filled with light. In this instance, the resolving power is twice as great. The finest detail that the objective can reveal is now equal to

$$\frac{\text{w.l.}}{2\text{N.A.}}$$

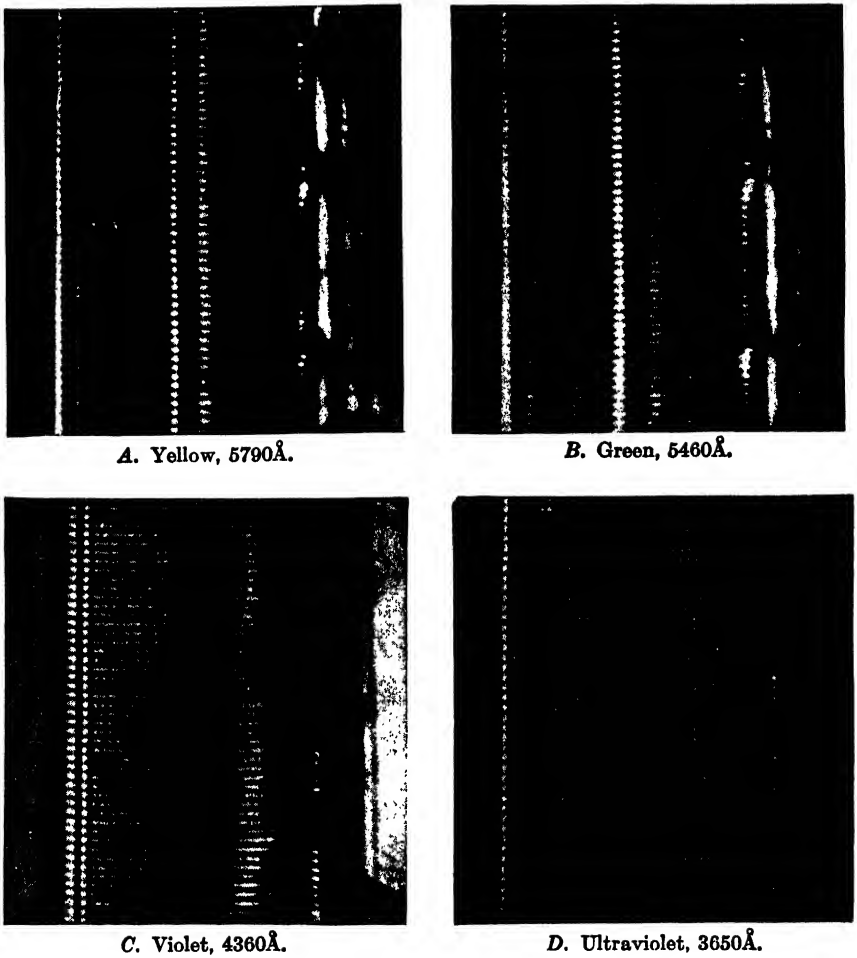
For example, the brightest part of the spectrum shows a wave length of 5300 Å. An objective having a numerical aperture equal to 1.00 will resolve two lines separated by a distance of 5300 Å./1.00 = 5300 Å. (48,000 lines to the inch) if a very narrow pencil of light is used, and 5300 Å./(2 × 1.00) = 2650 Å. (95,000 lines to the inch) if the whole aperture of the objective is filled with light.

From the above, it is evident that the maximum efficiency of an objective is not reached unless the back lens is filled with light. This may be ascertained by removing the eyepiece from the microscope and viewing the back lens of the objective with the naked eye. If the back lens is completely filled with light, the efficiency will then be according to the numbers engraved on the objective.

**Resolving Power.**—The relation between wave length and resolving power is illustrated in Fig. 6. The shorter the wave length of light, the finer the detail revealed by the objective. With an objective having a N.A. of 1.00 and a yellow filter (light transmission of 5790 to 5770 Å.), it is possible to see about 88,000 lines to an inch; with a green filter (light transmission of 5460 Å.) about 95,000 lines to an inch; with a violet filter (light transmission of 4360 Å.) about 115,000 lines to an inch; and with

ultraviolet light (light transmission of 3650 Å.) about 140,000 lines to an inch.

**Electron Microscope.**—Within the past few years, a new type of instrument has been developed having a higher resolving power than the usual



*A.* Yellow, 5790Å. *B.* Green, 5460Å.

*C.* Violet, 4360Å. *D.* Ultraviolet, 3650Å.

FIG. 6.—*Amphipleura pellucida*, a diatom. Effect of light of different wave lengths on the resolving power of the objective. (*From Photomicrography, courtesy of the Eastman Kodak Company.*)

microscope. This instrument employs a beam of electrons instead of visible light rays. It is known as the electron microscope (Fig. 7).

Beams of electrons or cathode rays (1) travel in a straight line in the absence of any matter or field, (2) can be concentrated in certain cases, and (3) can be deflected in an electrostatic or magnetic field. A close analogy exists between the action of a magnetic or electric field of rota-

tional symmetry on an electron beam and the action of a glass lens on a light beam (Fig. 8).

In the electron microscope, it is possible to make highly magnified images with electron optical systems by combining two or more electronic "lenses." The wave length of the electron is considerably shorter than that of visible light, being a function of the electron velocity. For velocities between 30,000 and 100,000 volts, the wave length is only 1/100,000 of the wave length of visible light. The high-speed electron waves are far too short to be "seen" in the usual sense, but they do cause microorganisms and other tiny particles under observation to produce images on a fluorescent screen, where they are visible and can be photographed.

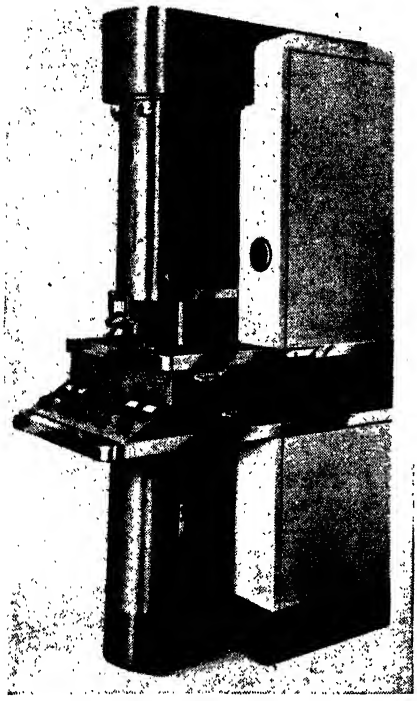


FIG. 7.—RCA electron microscope and its associated power supply. (*From RCA Electron Microscope, courtesy of Radio Corporation of America.*)

An optical-lens microscope magnifies about 2000 diameters. If ultra-violet light is used, this magnification can be increased to about 3000 diameters. With the electron microscope, direct magnifications of from 10,000 to 30,000 diameters are obtainable, and with such delicate detail that photographic enlargements, called "photomicrographs," are possible to 100,000 and even to 200,000 times natural size (Fig. 9). The resolving power is so much greater than that of the ordinary optical microscope that it is now possible to obtain images of protein molecules, virus crystals, bacteriophages, unstained flagella, internal structures of bacteria, etc.

For additional information on the electron microscope, see Burton and Kohl (1946) and Hillier and Vance (1941).

**Immersion Objectives.**—When a dry objective is used, an air space is present on both sides of the microscope slide and cover slip. The largest cone of light coming from *O* (Fig. 10) that could possibly be used is 180° in air, which is equal to an angle of about 82° in the glass. This corresponds to a numerical aperture of 1.0. In actual practice, however, these figures become 143 and 77° respectively, owing to the fact that the air space must

be wide enough to correspond to a practical working distance of the objective. Rays of greater angular aperture than 82° in glass, which originate at the object point *O* by diffraction, will be completely reflected at the upper surface of the cover slip *t*.

The refractive index *n* of the air is equal to 1.0. If the air space between the cover slip and the objective is filled with a fluid having a higher refractive index, such as water ($n = 1.33$), or, what is still better, a liquid having a refractive index approaching that of glass, such as cedarwood oil ($n = 1.51$), angles greater than 82° are obtained. Numerical apertures greater than 1.0 are realized by this method. Cedarwood oil causes the light ray to pass right through the homogeneous medium, with the result that a cone of light of about 134° is obtained, which corresponds to a numerical aperture of 1.4. Finer detail can, therefore, be resolved by this procedure. With an oil-immersion objective and a numerical aperture of 1.4, two lines as close together as 1/100,000 in. (0.2 $\mu$) can be separated. This means, then, that the greater the numerical aperture of the objective, the greater will be its resolving power or ability to record fine detail.

The refractive indexes of a number of media that have been employed for immersion objectives are given in Table 1.

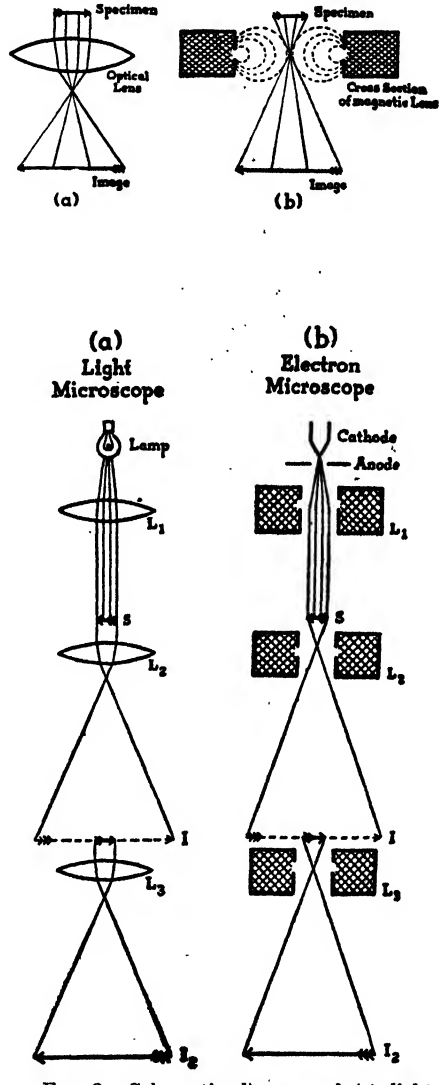



FIG. 8.—Schematic diagram of (*a*) light microscope, and (*b*) magnetic electron microscope. (*From RCA Electron Microscope, courtesy of Radio Corporation of America.*)

**Depth of Focus.**—The depth of focus is known also as the depth of sharpness or penetration. The depth of focus of an objective depends upon the N.A. and the magnification, and is inversely proportional to both. This means that the higher the N.A. and the magnification, the

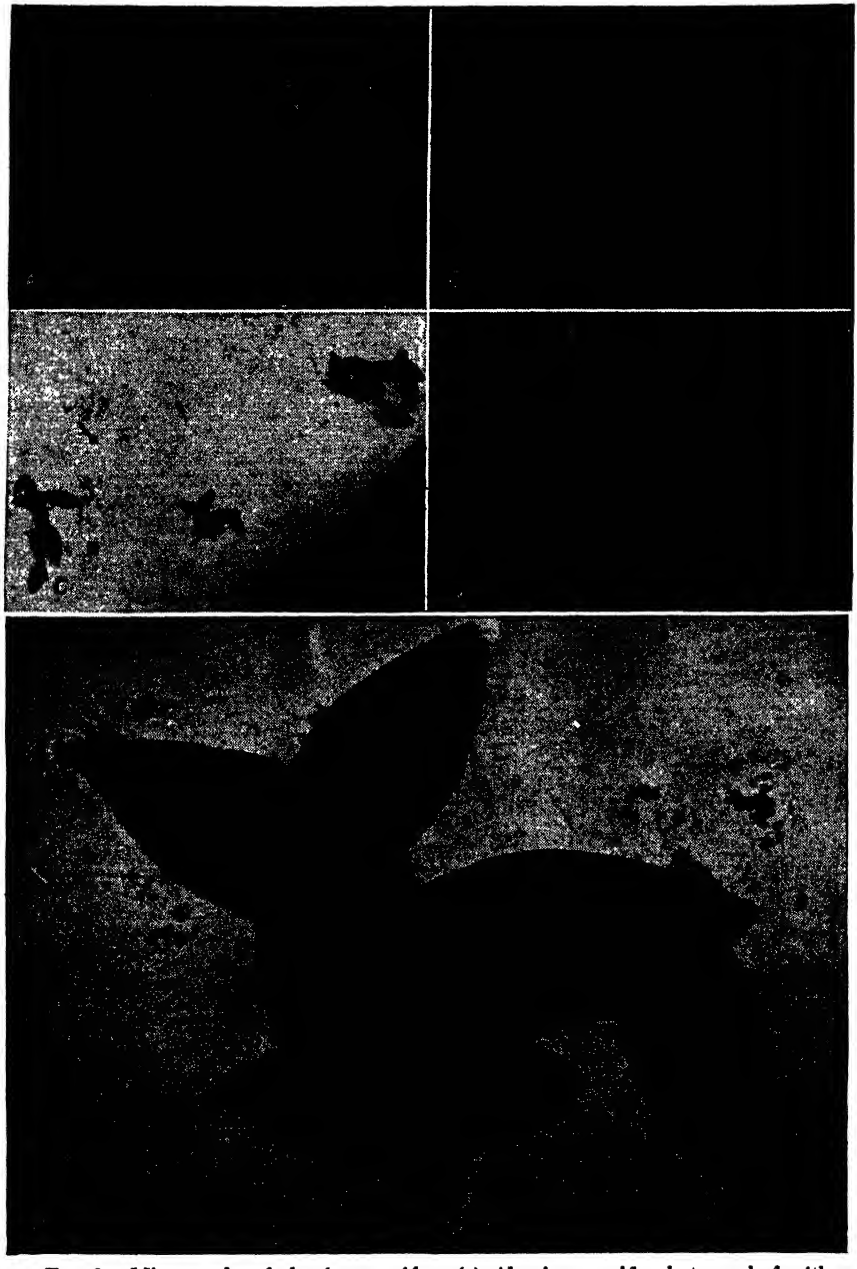


FIG. 9.—Micrographs of aluminum oxide. (*a*) Aluminum oxide photographed with a standard light microscope. Magnification 1000×. (*b*) A part of the light micrograph shown in (*a*) enlarged photographically to 5000×. (*c*) Aluminum oxide photographed with the electron microscope. Magnification 1000×. (*d*) A part of the electron micrograph shown in (*c*) enlarged photographically to 5000×. (*e*) A part of the electron micrograph shown in (*c*) magnified photographically to 13,000×. Total magnifications up to 200,000× are practical. (*From the RCA Electron Microscope, courtesy of Radio Corporation of America.*)

less the depth of focus. Therefore, high-power objectives must be more carefully focused than low-power objectives. These conditions cannot be changed by the optician.

**Equivalent Focus.**—Objectives are sometimes designated by their equivalent focal lengths measured in either inches or millimeters. An objective designated by an equivalent focus of 1/12 in., or 2 mm., means that the lens system produces a real image of the object of the same size as is produced by a simple biconvex or converging lens having a focal distance of 1/12 in., or 2 mm. An objective designated 1.3 in., or 33 mm.,

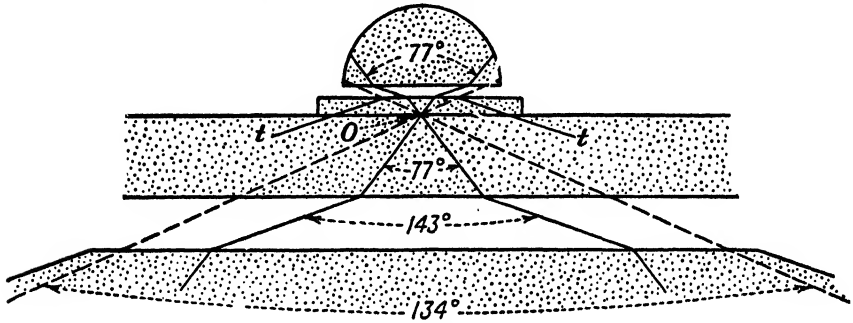



FIG. 10.—Passage of light through an object on a glass slide using dry and immersion objectives. See text for details. (*Redrawn from Photomicrography, courtesy of Eastman Kodak Company.*)

produces a real image of the same size as is produced by a simple biconvex or converging lens having a focal distance of 1.3 in., or 33 mm.

TABLE 1

| Medium | Refractive Index at 25°C. |
|---|---|
| Water | 1.33 |
| Glycerol | 1.46 |
| Mineral (paraffin) oil | 1.47 |
| Cedarwood oil | 1.51 |
| Sandalwood oil | 1.51 |
| Balsam | 1.53 |
| Crown oil | 1.55 |

**Working Distance of Uncovered Objects.** — If the object on a glass slide is not covered with a cover slip, the working distance may be defined as the distance between the front lens of the objective and the object on the slide when in sharp focus. The working distance is always less than the equivalent focus of the objective. This is illustrated in Fig. 11*A*. The working distance may be determined easily by noting the number of complete turns of the micrometer screw (fine adjustment) required to raise the objective from the surface of the slide, where the object is located, to a point where the microscope is in sharp focus.

To take a specific example:

Each turn of the micrometer screw = 0.1 mm.

Number of turns required to bring object in sharp focus = 6

Then,

$$\text{Working distance} = 6 \times 0.1 = 0.6 \text{ mm.}$$

**Working Distance of Covered Objects.** — If the object is covered with a cover slip, the free distance from the upper surface of the cover slip to the front of the objective will be less than in the case of an uncovered

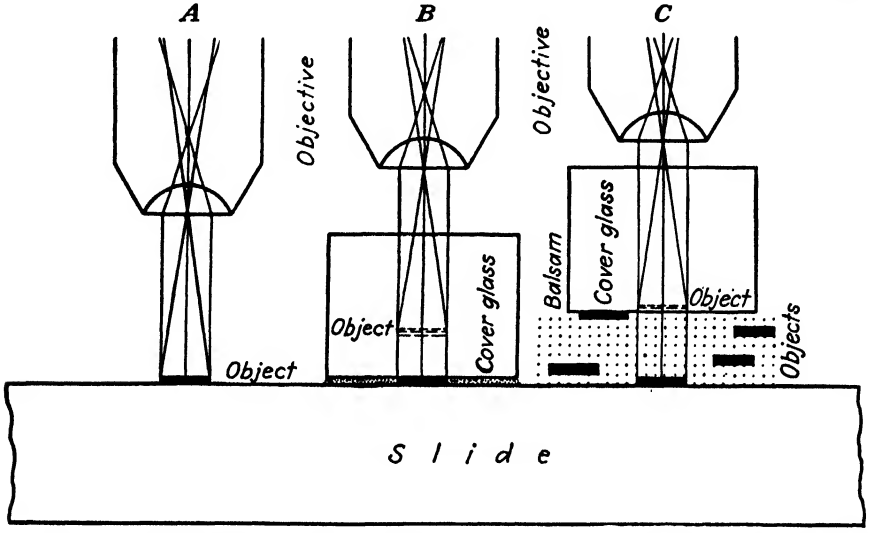



FIG. 11.—Working distance of an objective. *A*, object not covered with a cover glass. *B*, *C*, object covered with a cover glass. (*Redrawn from Gage, The Microscope, The Comstock Publishing Company, Inc.*)

object. It is obvious from this that, if the cover glass is thicker than the working distance of the objective, it will be impossible to get the object in focus. On the other hand, if the glass is thin it will be possible to get the object in focus, but the focus of the microscope on a covered object will be different from that on an uncovered object. It follows from this that an object covered with a glass cover slip or other highly refractive body will appear as if raised, and the amount of elevation will depend upon the refractive index of the glass or other medium covering the object. Also, the greater the refraction of the covering body, the more will be the apparent elevation. This is shown in Fig. 11*B*, *C*. The apparent depth of the object below the surface of the covering medium may be calculated by taking the reciprocal of its index of refraction. For example, if a glass cover slip is used, it will have an index of refraction of 1.52. The reciprocal of this figure is $1/1.52 = \frac{2}{3}$, approximately. This means that the apparent depth of the object is only two-thirds its actual depth.

The working distance of covered objects may be determined by noting the number of complete turns of the micrometer screw (fine adjustment) required to raise the objective from the surface of the cover slip to a point where the objective is in sharp focus.

To take a specific example:

Each turn of micrometer screw = 0.1 mm

Number of turns required to bring object in sharp focus = 3.5

Then,

$$\text{Working distance} = 3.5 \times 0.1 = 0.35 \text{ mm.}$$

**Chromatic Aberrations in Objectives.** — As has already been stated (page 11), white light, in passing through a prism, is broken up into its

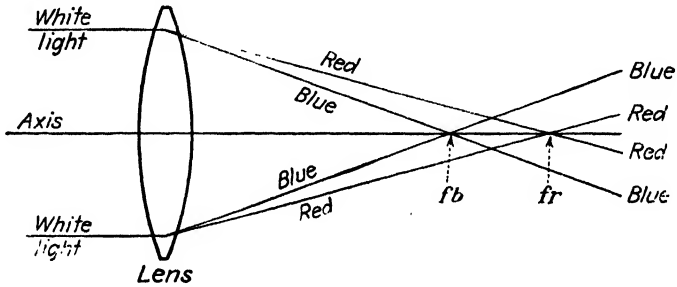



FIG. 12.—Chromatic aberration with white light. White light, in passing through a lens, is dispersed into its constituent colors. The red or long waves are refracted less than the blue or short waves. The blue rays (*fb*) cross the optical axis of the lens before the red rays (*fr*). The blue light will focus nearer the lens than the red light.

constituent colors, the wave lengths of which are different. A simple or compound lens, composed of only one material, will exhibit different focal lengths for the various constituents of white light. This is due to the dispersive power of the lens. Every wave length is differently refracted, the shortest waves most and the longest waves least. The blue-violet rays cross the lens axis first and the red rays last. There will be a series of colored foci of the various constituents of white light extending along the axis. This is shown in Fig. 12. The lens will not, therefore, produce a sharp image with white light. Instead, the image will be surrounded by colored zones or halos, which interfere with the visual observation of its true color. This is spoken of as "chromatic aberration." It may be lessened by reducing the aperture of the lens or, better still, by using a lens composed of more than one material (compound lens). Two or more different glasses or minerals are necessary for correcting the chromatic aberration of an objective, and the amount of correction depends upon the dispersive powers of the components of the objective.

If two optical glasses are carefully selected to image light of two different wave lengths at the same focal point, the lens is said to be "achromatic" and an objective containing such a lens system is spoken of as an

achromatic objective. The remaining rays of the white light will be imaged at approximately the same point. An achromatic objective will yield images free from pronounced color halos. If the focus is shifted slightly, faint green and pink halos may be observed. The slight residual color will not prove objectionable for the usual microscopic work. Achromats are the universal objectives for visual work and are very satisfactory in photomicrography when used in monochromatic light (obtained by the use of filters).

Lens systems corrected for light of three different wave lengths are called "apochromatic" objectives. These objectives are composed of fluorite in combination with lenses of optical glass. The images produced by objectives in this group exhibit only a faint blue or yellow residual color. Since these objectives are corrected for three colors instead of for two, they are superior to the achromats. Their finer color correction makes possible a greater usable numerical aperture. The violet rays are brought to the same focus as visual rays. This fact makes these objectives excellent for photographic use for both white and monochromatic light.

Another group of objectives exhibit qualities intermediate between the achromats and the apochromats. They are called "semiapochromats." If the mineral fluorite is used in their construction, they are termed "fluorite objectives." These objectives also yield excellent results when used for photomicrography.

## OCULARS

The chief functions of the ocular or eyepiece are the following:

1. It magnifies the real image of the object as formed by the objective.
2. It corrects some of the defects of the objective.
3. It images cross hairs, scales, or other objects located in the eyepiece.

Several types of eyepieces are employed, depending upon the type of objective located on the microscope. Those most commonly used are known as Huygenian, compensating, and hyperplane oculars.

**Huygens Eyepiece.**—In this type of eyepiece, two simple plano-convex lenses are employed, one of which is below the image plane (Fig. 13). The convex surfaces of both lenses face downward. Oculars in this group are sometimes spoken of as negative eyepieces. This type of ocular is made with a large field lens, which bends the pencils of light coming from the objective toward the axis without altering to any great extent the convergence or divergence of the rays in the individual pencils. Above the field lens and at some distance from it, is a smaller lens known as the eye lens, the function of which is to convert each pencil of light into a parallel or only slightly diverging ray system capable of being focused by the eye. The rays, after emerging through this lens, then pass through a small circular area known as the Ramsden disk, or eyepoint. It may be seen that the real image of the object is formed between the two eyepiece lenses. In an eyepiece of this type, the distance separating the two lenses

is always a little greater than the focal length of the eye lens. The reason for this is to prevent any dirt on the field lens from being seen sharply focused by the eye. An image should be viewed with the eye placed at

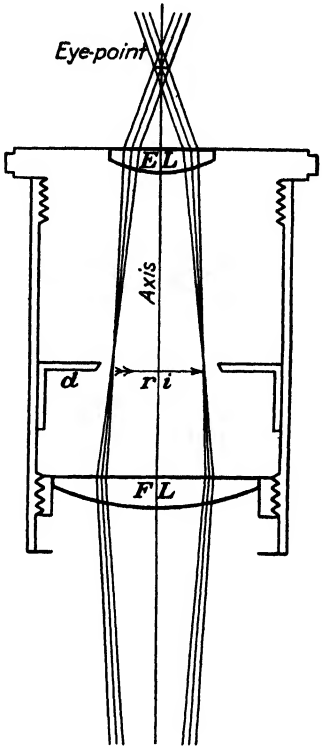



FIG. 13.—Huygens eyepiece. *EL*, eye lens. *FL*, field lens. *ri*, real image formed between the ocular lenses and the diaphragm *d*. (*Redrawn from Gage, The Microscope, The Comstock Publishing Company, Inc.*)

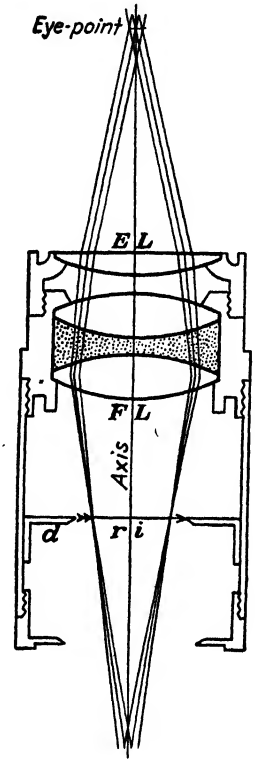



FIG. 14.—Compensating eyepiece. *EL*, eye lens. *FL*, field lens composed of three components. *ri*, real image formed below the lenses at the diaphragm *d*. (*Redrawn from Gage, The Microscope, The Comstock Publishing Company, Inc.*)

the Ramsden disk in order to obtain the largest field of view and, also, to obtain the maximum brightness over the field.

A Huygenian eyepiece should give equal magnification of light of different colors even though the images given by the various rays do not lie in the same plane. This is due to the fact that the eye is not very sensitive to off-focus effects. The eyepiece is practically achromatic. Huygenian eyepieces are intended to be used primarily with achromatic objectives.

**Compensating Eyepiece.**—Oculars of this type consist of an achromatic triplet combination of lenses (Fig. 14). These eyepieces are more

perfectly corrected than are those of the Huygenian type. A compensating eyepiece is corrected to neutralize the chromatic difference of magnification of the apochromatic objectives. Such eyepieces are intended, therefore, to be used primarily with apochromatic objectives, although they may be employed successfully with the higher power achromats and fluorite objectives with good results.

**Flat-field Eyepiece.**—Apochromatic objectives, when used with compensating eyepieces, give fields that are not flat. Flat-field eyepieces have been designed to correct this defect. They give much flatter fields than do the other types already discussed, but they are less perfectly corrected chromatically. Oculars of this type are referred to as hyperplane, planoscopic, periplane, etc. They may be employed with the higher power achromatic, fluorite, and apochromatic objectives without introducing chromatic aberrations into the image. Their color compensation falls about midway between the Huygenian and the compensating eyepieces.

FIG. 15. — Substage condenser. N.A. = 1.4. *(Courtesy of Carl Zeiss, Inc.)*

## CONDENSERS

Several methods are employed for illuminating the object under examination. In bacteriology, the two methods commonly used are (1) illumination by transmitted light and (2) dark-field illumination.

**Illumination by Transmitted Light.**—A condenser may be defined as a series of lenses for illuminating, with transmitted light, an object to be studied on the stage of the microscope. It is located under the stage of the microscope between the mirror and the object, whereas the objective and ocular lenses are located above the stage. It is sometimes referred to as a substage condenser (Fig. 15).

A condenser is necessary for the examination of an object with an oil-immersion objective in order to obtain adequate illumination. A condenser is also preferable when working with high-power dry objectives. Probably the most commonly employed condenser has a numerical aperture of 1.2. If an immersion objective having a numerical aperture of 1.3 is used, the condenser should have a numerical aperture of 1.4. As the numerical aperture of a condenser increases, the working distance and also the area illuminated decrease.

A good condenser sends light through the object under an angle sufficiently large to fill the aperture of the back lens of the objective. When this is accomplished, the objective will show its highest numerical aperture. This may be determined by first focusing the oil-immersion objective on the object. The eyepiece is then removed from the ocular tube. The back lens of the objective is observed by looking down the microscope tube, care being taken not to disturb the focus. The back lens of the

objective should be evenly illuminated. If it is not, the mirror should be properly centered. If the condenser has a smaller numerical aperture than the objective, the peripheral portion of the back lens of the objective will not be illuminated, even though the condenser iris diaphragm is wide open. If the condenser has a greater numerical aperture than that of the objective, the back lens of the objective may receive too much light, resulting in a decrease in contrast. The smaller the aperture, the greater the depth of focus and the greater the contrast of the components of the image. The lowest permissible aperture is reached when diffraction bands become evident about the border of the object imaged. This difficulty may be largely overcome by closing the iris diaphragm of the condenser until the leaves of the iris appear around the edges of the back lens of the objective. The diaphragm is then said to be properly set. The setting of the iris diaphragm will vary with different objectives.

**Dark-field Illumination.**—The microscope is most commonly employed by allowing the light to pass through the object. This is called "microscopy in transmitted light," or "bright-field microscopy." An object cannot be seen in bright-field microscopy unless it absorbs or refracts the light passing through it. Contrast is thus set up between the object and the surrounding medium. Objects that display feeble contrast with the background are difficult to see in bright-field illumination.

If the aperture of the condenser is opened completely and a dark-field stop inserted below the condenser, the light rays reaching the object form a hollow cone. If a stop of suitable size is selected, all the direct rays from the condenser can be made to pass outside of the objective. Any object within this beam of light will reflect some light into the objective and be visible. This method of illuminating an object, where the object appears self-luminous against a dark field, is known as dark-field illumination.

There are three types of condensers employed for dark-field illumination: (1) the Abbe, (2) the paraboloid, and (3) the cardioid.

The Abbe condenser is probably more commonly employed than the other two because it is especially suitable for objects that do not require the highest magnifications to make them visible. It may be employed either by inserting a dark-field stop below the condenser (Fig. 16), or by unscrewing the top part of the condenser and substituting for it a dark-field element (Fig. 17).

The paraboloid condenser is designed to be used with high-power oil-immersion objectives and an intense source of light (Fig. 18). In using this condenser, it is necessary to place cedar oil or glycerin between the condenser and the slide. Also, the specimen must be mounted in a liquid or cement and protected with a cover slip. The numerical aperture of the objective must not be greater than that of the condenser.

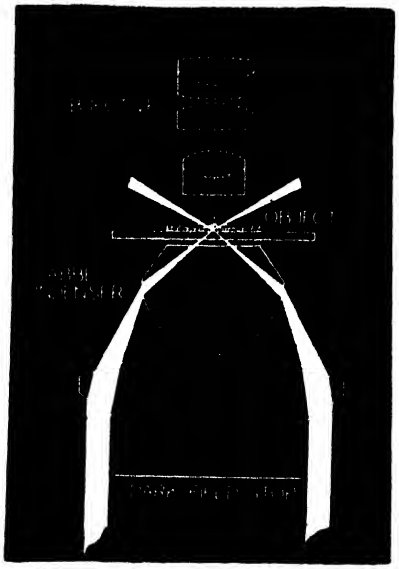


FIG. 16.—Abbe condenser with dark-field stop inserted below the condenser. (*From Dark Field Optical Systems, courtesy of Bausch & Lomb Optical Company.*)


FIG. 17.—Abbe condenser. (*From Dark Field Optical Systems, courtesy of Bausch & Lomb Optical Company.*)

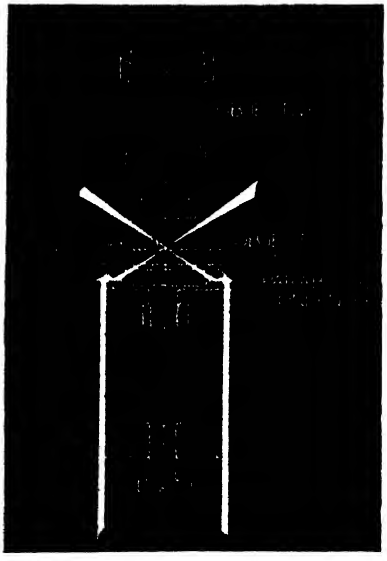


FIG. 18.—Paraboloid condenser. (*From Dark Field Optical Systems, courtesy of Bausch & Lomb Optical Company.*)

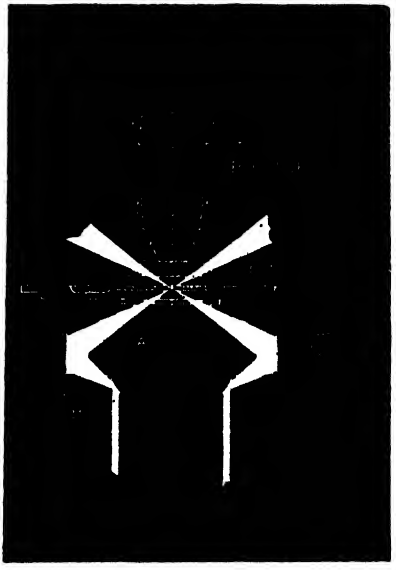


FIG. 19.—Cardioid condenser. (*From Dark Field Optical Systems, courtesy of Bausch & Lomb Optical Company.*)

The cardioid condenser is the most refined type of dark-field illuminator (Fig. 19). It is especially designed to be used for the examination of colloidal solutions or suspensions, *i.e.*, particles measuring less than 0.25 $\mu$ in diameter.

The cardioid condenser is best employed with a strong arc lamp. Since the concentration of light is so great, ordinary glass slides and cover slips

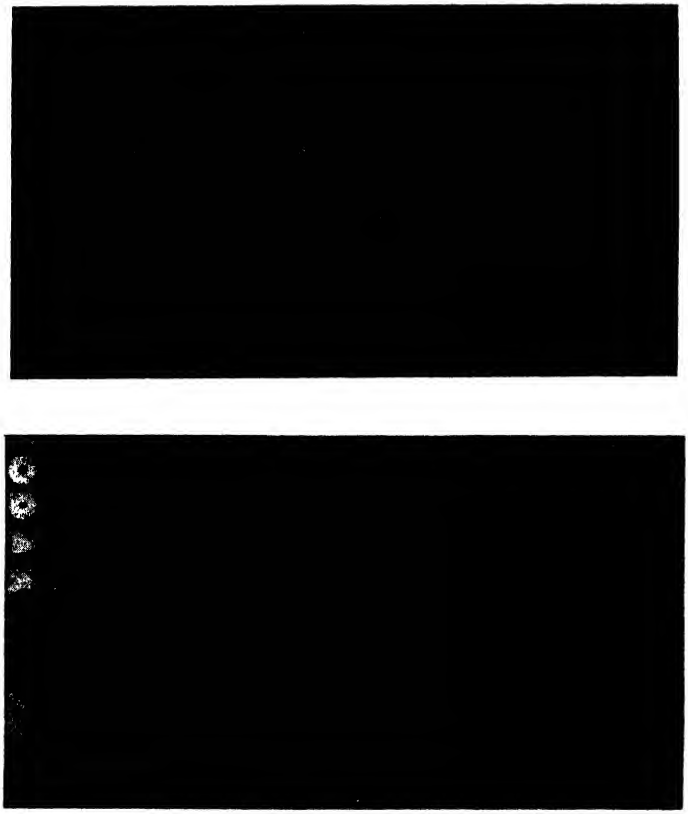


FIG. 20.—Motion photomicrograph of living cells of *Proteus vulgaris*, taken by dark-field illumination.

should not be used. Visible defects and the difficulty of removing foreign objects from the glass ruin the visibility of ultramicroscopic particles. It is better to employ fused-quartz object slides and fused-quartz cover slips. They are free from bubbles and other imperfections and can be heated in a flame to drive off all dirt, after being chemically cleaned.

A motion photomicrograph of living cells of *Proteus vulgaris*, taken by dark-field illumination, is shown in Fig. 20.

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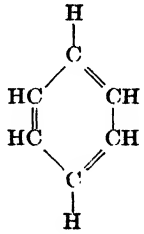
## CHAPTER III

## BIOLOGICAL STAINS

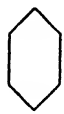
Bacteria are difficult to see under a light microscope unless they are first stained. Staining bacteria is also necessary to reveal their internal structure. The presence of certain structures in the protoplasm of some bacteria furnishes the basis for their identification and classification.

Natural dyes predominated during the early years of bacteriology, but at present only a few of them are being used. They have been gradually discarded in favor of the artificial or synthetic dyes. Since the first artificial dyes were produced from aniline, they are generally referred to as aniline dyes. However, there are a large number of them that are not derived from aniline and bear no relation to the compound. Since all of them are derived from one or more substances found in coal tar, they are more correctly referred to as coal-tar dyes.

The coal-tar dyes may be considered as derivatives of the cyclic compound benzene or benzole:




The empirical formula is $C_6H_6$. It is customary to write the structural formula by omitting the double bonds and the hydrogen atoms, abbreviating it to a hexagon, each corner of which represents an atom of carbon and one of hydrogen:

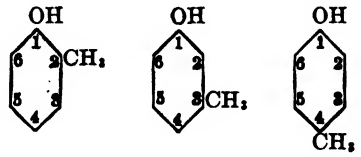


This hexagon is known as the benzene ring.

One or more hydrogen atoms may be replaced by some element or radical. For example, if one hydrogen atom is replaced by a hydroxyl

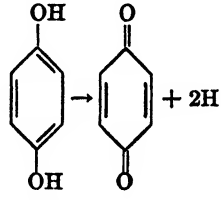
(OH) group, the compound phenol or carbolic acid ($C_6H_5OH$) is produced:


If another hydrogen atom is replaced by a methyl group ($CH_3$), the compound known as cresol is produced. Three different cresols are possible, depending upon which hydrogen atoms are substituted:




The substituted radicals are in the 1–2 or ortho, 1–3 or meta, and 1–4 or para positions. The compounds are named orthocresol, metacresol, and paracresol, respectively. The prefixes are usually abbreviated to the letters *o*-, *m*-, and *p*-.

The quinones are compounds derived by the elimination of two hydroxyl-hydrogen atoms from aromatic dihydroxy derivatives. The simplest quinone is benzoquinone. It is also called "quinone."



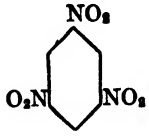

The benzene ring of the quinones contains two double bonds instead of three as in cresol. The formula of benzoquinone shows that it is not a true benzene derivative but the diketone of a *p*-hydrobenzene. Substances containing the quinone ring are called "quinoid compounds." The double bonds in the quinoid compounds are supposed to be fixed, not mobile as in benzene. A large number of dyes contain the quinone ring.

## DYES

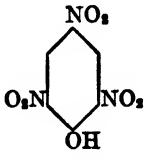
**Definition of a Dye.**—A dye may be defined as an organic compound containing both chromophore and auxochrome groups linked to benzene rings. A chromophore group imparts to the compound the property of color. Compounds of benzene containing chromophore radicals have been called "chromogens." Such a compound, even though colored, is not a

dye. It possesses no affinity for, or ability to unite with, fibers and tissues. The color may be easily removed by mechanical methods. In order for a compound to be a dye it must contain not only a chromophore group but also another group that imparts to the chemical the property of electrolytic dissociation. Such groups are known as auxochromes. Auxochrome groups furnish salt-forming properties to the compound.

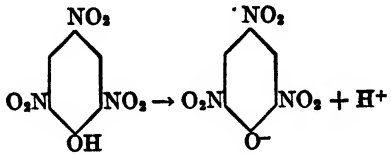
This may be illustrated by the following example: The nitro group ($NO_2$) may be considered a chromophore. When three hydrogen atoms in the benzene molecule are replaced by three nitro groups, the compound trinitrobenzene is formed:




This compound is yellow in color and is a chromogen but not a dye. It does not dissociate electrolytically and, therefore, is unable to form salts with either acids or bases. If, however, another hydrogen atom is replaced by an auxochrome group, such as (OH), the compound known as picric acid is formed:




This compound is also yellow in color and is capable of dissociating as follows:

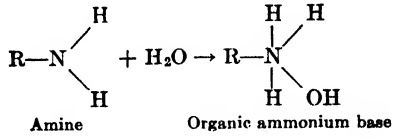



The dye portion of the molecule has a negative electrical charge. Therefore, it is an acid dye, being capable of forming salts with bases. The color of picric acid is due to the chromophore groups ($NO_2$), and its dyeing properties are due to the auxochromic hydroxyl group (OH), which imparts to the compound the property of electrolytic dissociation.

**Acidic and Basic Dyes.**—Auxochrome groups are either acidic (OH) or basic ($NH_2$). Acid dyes ionize to give the dye portion of the molecule or anion a negative electrical charge. Basic dyes ionize to give the dye portion of the molecule or cation a positive electrical charge.

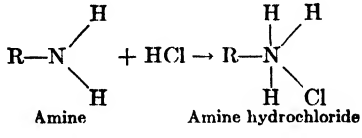
The amino group is basic by virtue of the ability of its nitrogen (N) atom to become pentavalent on the addition of water or of an acid.

With water:




Amine — Organic ammonium base

With an acid:




Amine — Amine hydrochloride

The hydroxyl group is acid by virtue of its power to furnish hydrogen ions by dissociation. The amino ($NH_2$) group is a stronger base than the hydroxyl group (OH) is an acid. If one of each of these radicals is present, the basic character of the amino radical predominates.

Some dyes have the sulfonic group ($SO_2OH$) attached to the benzene ring. It is a strongly acid group, possessing salt-forming properties. The radical is only weakly auxochromic. It serves two very important purposes in the dye molecule: (1) It renders insoluble dyes soluble and (2) a basic dye is changed to one acidic in character by the introduction of the sulfonic group in the benzene ring. Since the radical is only weakly auxochromic, a compound containing a chromophore and a sulfonic acid group is not a dye unless an auxochrome radical is also present.

The dyes of commerce are not acids or bases in the true sense. They are ordinarily salts. Basic dyes are salts of color bases, usually the chloride, sometimes the sulfate or acetate; acid dyes are salts of color acids, usually the sodium salts, sometimes the potassium, calcium, or ammonium salts.

Both acid and basic dyes are used in bacteriology. The acid dyes are used chiefly to stain cytoplasm. The basic dyes, on the other hand, stain nuclear material more intensely than cytoplasm.

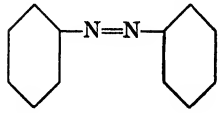
**Chromophores.**—In order that a compound be a dye, it must contain at least one chromophore group that imparts to the substance the property of color. Some of the chromophore groups are basic in character; others are acidic. The basic chromophores include (1) the azo group, (2) the azine group, and (3) the indamine group.

1. The azo group

$$—N{=}N—$$

is found in all azo dyes. In these compounds a benzene group is attached

to each atom of nitrogen. The dyes of this group may be considered as derivatives of azobenzene:



Examples of dyes containing this chromophore are methyl orange and Bismarck brown.

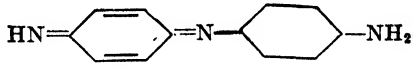
2. The azine group

is found in the phenazines. Neutral red and the safranines are examples of azine dyes.

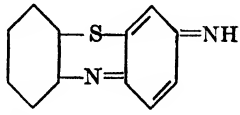
3. The indamine group



is found in the indamines, the thiazines, and others. Many of the dyes have two benzene rings attached to a nitrogen atom. One of the rings shows the quinoid structure:



The thiazines have the two benzene rings further joined together by an atom of sulfur. The simplest thiazine nucleus has the following structure:



The best known dye having the thiazine base is methylene blue.

The acid chromophores include (1) the nitro group and (2) the quinoid ring.

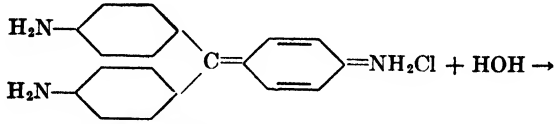
1. The nitro group ($NO_2$) is found in many compounds, an example of which is picric acid.

2. The quinoid ring

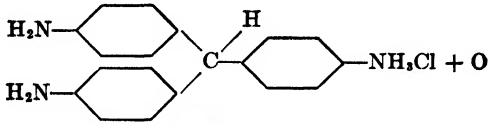
occurs in many dyes such as the indamines, the xanthenes, and the di- and triphenylmethanes. Some of the well-known dyes in this group are rosolic

acid, fuchsin, the methyl violets, methyl green, crystal violet, and pararosaniline.

The chromophores are easily reduced by combining with hydrogen at the double bonds. The nitro group may be reduced to an amino radical; the double bonds of the quinoid ring may break and one atom of hydrogen be taken up by each valence set free. A reduction of the chromophore group results in a loss of color. These decolorized dyes are known as "leuco compounds." Dyes may be used as indicators of oxidation and reduction. The decolorization of pararosaniline may be represented by the following equation:




Pararosaniline




Leucopararosaniline

## CLASSIFICATION OF BIOLOGICAL DYES

The most important dyes used in bacteriology are given in Table 2. Some of them are acidic and others are basic. The basic dyes, for reasons already given, are the most important ones from the standpoint of the bacteriologist. The dyes are classified according to the chromophore groups they contain.

## THEORY OF STAINING

Many theories have been advanced to explain the phenomenon of staining. All of them attempt to explain the process on a purely physical or chemical basis.

**Physical.**—A physical process may be defined as a reaction between two substances in which no new compound is formed. The proponents of the physical theory claim that all staining reactions can be explained on the basis of capillarity, osmosis, adsorption, and absorption. There does not appear to be any general agreement on the amount of weight that should be given to each force, although all agree that they occur in the process of staining.

**Chemical.**—In a chemical reaction, a new compound is formed having physical and chemical properties different from the original reacting substances. Furthermore, it is impossible to recover the original reactants by means of simple solvents. When bacteria are stained, there is no evi-

dence that the dye has been changed chemically to form a new compound. It is usually possible to extract all or nearly all of the dye from the bacterial cells by sufficiently long immersion in water, alcohol, or other solvents. The bacterial protoplasm never completely removes all of the dye from solution. This is contrary to a chemical reaction, which tends to continue until one of the components of the reaction is exhausted.

TABLE 2.—CLASSIFICATION OF BIOLOGICAL DYES

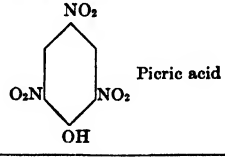
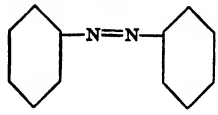
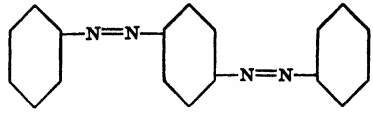
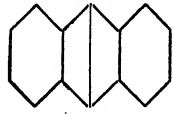
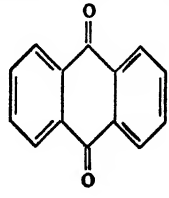
| Dye | Chromophore |
|---|---|
| I. The nitro dyes<br>1. Picric acid<br>2. Aurantia<br>3. Martius yellow | The chromophore is —$NO_2$<br>The dyes are all acid:<br>  <br><br>Picric acid |
| II. The azo dyes<br>1. Bismarck brown *Y*<br>2. Bordeaux red<br>3. Brilliant yellow *S*<br>4. Chrysoidin *Y*<br>5. Congo red<br>6. Janus green *B*<br>7. Methyl orange<br>8. Methyl red<br>9. Orange *I*<br>10. Orange *G*<br>11. Ponceau 2*R*<br>12. Sudan *R* | The chromophore —N=N— joins together benzene or naphthalene rings:<br>  <br><br>Sometimes the chromophore occurs more than once:<br>  <br><br>Groups in the benzene ring are usually in the para position to each other. |
| III. The anthraquinone dyes<br>1. Alizarin<br>2. Alizarin red *S*<br>3. Purpurin | The dyes are derivatives of anthracene,<br>  <br>through the oxidized compound anthraquinone:<br>  <br> |

TABLE 2.—(*Continued*)

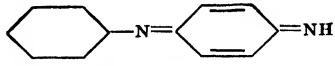
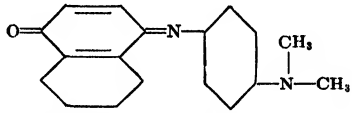
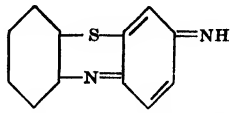
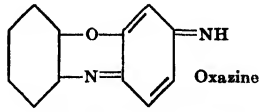
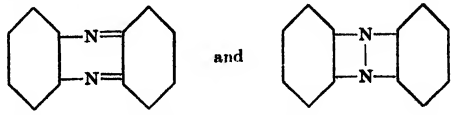
| Dye | Chromophore |
|---|---|
| IV. The quinonimine dyes<br>1. The indamines<br>No members of any biological importance | The dyes of this group contain two chromophores, the indamine group —N= and the quinoid ring:<br>  <br>They are derivatives of the theoretical compound paraquinone-diimine:<br>  <br>In a typical formula one of the hydrogen atoms is replaced by a phenyl group:<br>  |
| 2. The indophenols | The indophenols are closely related to the indamines. The most common member is indophenol blue:<br>  |
| 3. The thiazines<br>*a.* Methylene azure<br>*b.* Methylene blue<br>*c.* Methylene green<br>*d.* Methylene violet<br>*e.* Thionine<br>*f.* Toluidine blue *O* | The thiazines have a sulfur atom attached to both the phenyl and the quinone groups to form a third closed ring:<br>  |
| 4. The oxazines<br>*a.* Brilliant cresyl blue<br>*b.* Cresyl violet<br>*c.* Nile blue sulfate | In the oxazines, the sulfur of the thiazines is replaced by an atom of oxygen:<br>  <br>Oxazine |
| 5. The azines<br>*a.* The amidoazines<br>(1) Neutral red<br>(2) Neutral violet | The dyes are derivatives of phenazine. Two formulas are possible:<br>  |

TABLE 2.—(*Continued*)

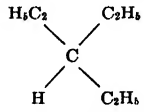
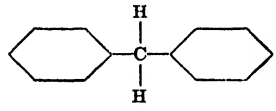
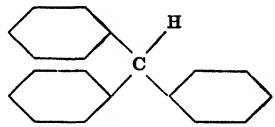
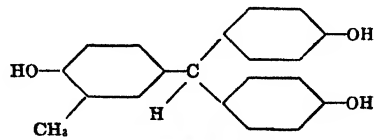
| Dye | Chromophore |
|---|---|
| *b.* The safranines<br>(1) Safranine *O*<br>*c.* The indulines<br>(1) Nigrosine | In the first formula the quinoid ring is the chromophore; in the second formula, the azine group<br>  <br>is the chromophore. |
| V. The phenylmethane dyes<br>1. The diamino triphenylmethane dyes<br>*a.* Brilliant green<br>*b.* Light green *SF* yellowish<br>*c.* Malachite green | This group comprises the most important dyes used in bacteriology. The compounds are substituted methanes. One or more hydrogen atoms may be replaced. If three hydrogen atoms are replaced by ethyl groups, the compound triethylmethane is formed:<br>  <br>If two hydrogens of methane are replaced by phenyl groups, diphenylmethane is formed:<br>  |
| 2. The triamino triphenylmethane dyes<br>*a.* Acid fuchsin<br>*b.* Basic fuchsin<br>*c.* Crystal violet<br>*d.* Ethyl violet<br>*e.* Hofmann's violet<br>*f.* Methyl blue<br>*g.* Methyl green<br>*h.* Methyl violet<br>*i.* New fuchsin<br>*j.* Pararosaniline<br>*k.* Rosaniline | If three hydrogens are replaced, triphenylmethane is produced:<br>  <br>The introduction of amino and other groups and substituted amino groups accounts for the large number of compounds possible. |
| 3. Hydroxy triphenylmethane dyes<br>*a.* Rosolic acid | These are triphenylmethane derivatives in which the amino groups of the rosanilines are replaced with hydroxyl groups:<br>  <br>Leucorosolic acid |

TABLE 2.—(*Continued*)

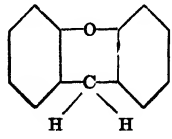
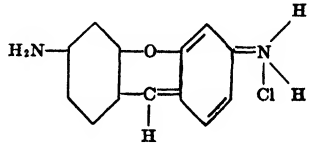
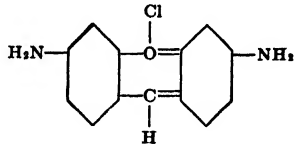
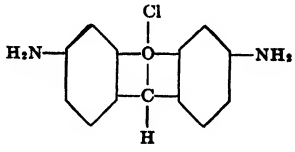
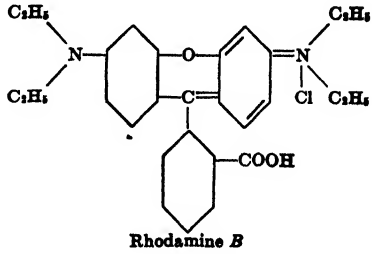
| Dye | Chromophore |
|---|---|
| VI. The xanthene dyes<br>1. The pyronine dyes<br>*a.* Pyronine *B*<br>*b.* Pyronine *Y* | The xanthenes are derivatives of the compound xanthene:<br>  <br>The pyronines are methylated diamino derivatives of xanthene. They are closely related to the diphenylmethanes:<br>  <br>or<br>  <br>or<br>  |
| 2. The rhodamine dyes<br>*a.* Fast acid blue *R*<br>*b.* Rhodamine *B* | The rhodamines are similar to the pyronines except that they contain another benzene ring with a carboxyl group in the ortho position:<br>  <br>Rhodamine *B* |

TABLE 2.—(*Continued*)

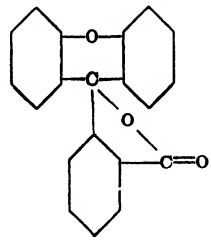
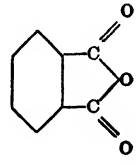
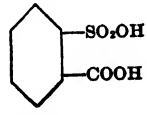
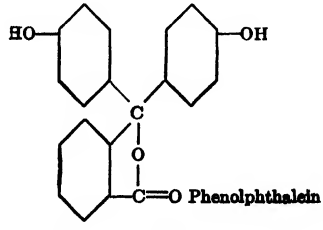
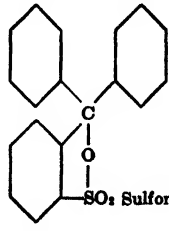
| Dye | Chromophore |
|---|---|
| 3. The fluorane dyes<br>*a.* Eosin *B*<br>*b.* Eosin *Y*<br>*c.* Erythrosin bluish<br>*d.* Erythrosin yellowish<br>*e.* Ethyl eosin<br>*f.* Fluorescein<br>*g.* Methyleosin<br>*h.* Phloxine *B*<br>*i.* Rose bengal | The fluorane dyes are derivatives of fluorane:<br>  |
| 4. The phenolphthalein and the sulfonephthalein dyes<br>*a.* Bromochlorophenol blue<br>*b.* Bromocresol green<br>*c.* Bromocresol purple<br>*d.* Bromophenol blue<br>*e.* Bromophenol red<br>*f.* Bromothymol blue<br>*g.* Chlorophenol red<br>*h.* Cresolphthalein<br>*i.* Cresol red<br>*j.* Metacresol purple<br>*k.* Phenolphthalein<br>*l.* Phenol red<br>*m.* Thymol blue | A phthalein is a compound of phthalic anhydride,<br>  <br>with phenol or a derivative of phenol. A sulfonephthalein is a compound of orthosulfobenzoic acid,<br>  <br>with phenol or a phenol derivative:<br>  <br>Phenolphthalein<br>  <br>Sulfonephthalein |

TABLE 2.—(*Continued*)

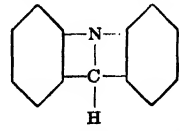
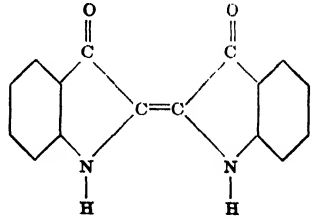
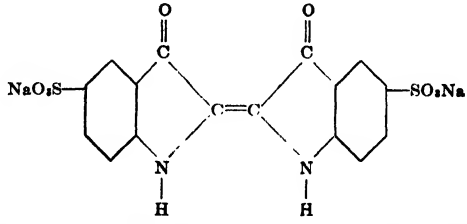
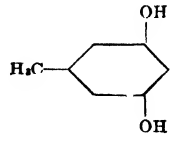
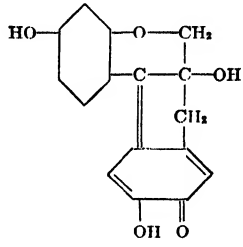
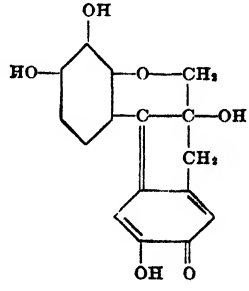
| Dye | Chromophore |
|---|---|
| 5. The acridine dyes<br>*a.* Acriflavine<br>*b.* Phosphine | The acridines are derived from acridine:<br>  <br>a compound closely related to xanthene. |
| VII. The natural dyes. These are not so important as the artificial dyes. Also, their chemistry is not well understood. Only a few of them are of any importance in bacteriology. They are | |
| 1. Indigo | Several species of plants of the genus *Indigofera* contain a glucoside, indican, which on fermentation yields the dye indigo:<br>  |
| 2. Indigo-carmine |  |
| 3. Carmine | This dye is prepared by treating the dried bodies of the female insect, *Coccus cacti*, with water. A red dye, cochineal, is extracted which is converted into carmine by treatment with alum. The dye principle is known as carminic acid. The exact formula is not known. |
| 4. Orcein and litmus. Both dyes are obtained from the lichens, *Lecanora tinctoria* and *Rocella tinctoria* | When these lichens are treated with ammonia and exposed to air, blue or violet colors develop. The colors are due to acids, one of which is known as orcinol: |

TABLE 2.—(*Continued*)

| Dye | Chromophore |
|---|---|
| |  <br>When orcinol is treated with ammonia and exposed to air, the violet dye orcein develops. The formula is not known.<br>Litmus is obtained from the same lichens as orcein. The lichens are treated with lime and potassium or sodium hydroxide. Ammonia is then added and the lichens are exposed to air for the color to develop. Its colored principle is known as "azolitmin". The formula is not known. |
| 5. Brazilin | Brazilin is obtained from the bark of brazil wood. It is colorless but on exposure to air it is oxidized to the red dye brazilein:<br>  |
| 6. Hematoxylin | Hematoxylin is obtained from the bark of logwood by extraction. It is not a dye but, on standing in air, the hematoxylin is oxidized to the dye hematein, which is similar to brazilein in composition:<br>  |

It is well established that some parts of a cell are acidic in reaction; others are alkaline. This fact led chemists to explain the phenomenon of staining on a purely chemical basis. The coal-tar dyes are either anionic (acid) or cationic (basic); *i.e.*, the dye portion of the molecule is either the negative or the positive ion. The proponents of the theory state that the acid constituents of the cell (nucleuses, chromatin) react with basic dyes and the basic constituents (cytoplasm) react with acid dyes. The process is not so simple as this, however, and probably does not explain all the facts.

It is well known that bacteria possess a negative electrical charge. McCalla (1940*a*, *b*) showed that bacteria attract positively charged ions, according to the equation:

$$(n \text{ bases}^+) + (B^{n-}) \rightleftharpoons (n \text{ bases}^+)(B^{n-})$$

where B represents the bacterial cell and $n$, an unknown number of negative ionic charges.

McCalla (1941) found that, when a negatively charged bacterial cell was treated with magnesium, the base was adsorbed to the cell until a neutral system was produced. In other words, the positively charged magnesium ions were attracted to the negative valences of the bacterial cell. If a stain, such as methylene blue, was added to the bacteria saturated with magnesium, the base was displaced by the stain, according to the equation:

$$(\tfrac{1}{2}nMg^{++})(B^{n-}) + nS^+Cl^- \rightleftharpoons (nS^+B^{n-}) + \tfrac{1}{2}nMgCl_2$$

where S represents the stain (methylene blue ion). The magnesium was displaced by the methylene blue in stoichiometrical proportions.

McCalla concluded that the reaction of stains with bacteria is an adsorption exchange process, chemical in nature. Basic stains act as cations, replacing similarly charged ions from the bacterial system. These results are in support of the chemical nature of staining.

Summarizing, it may be stated that more evidence is needed before it can be stated definitely that staining is physical or chemical. It is highly probable that staining is neither entirely physical nor entirely chemical, but a combination of both processes.

## STAINING SOLUTIONS

Preparations employed for staining bacteria are aqueous solutions. In most cases, the dyes are first dissolved in alcohol and the staining solutions prepared by diluting the alcoholic solutions with distilled water. Since alcohol removes dyes from stained cells, pure alcoholic solutions of dyes should not be employed.

Staining solutions generally contain low concentrations of dyes. Rarely do the concentrations amount to more than 1 per cent. A very dilute

staining solution acting for a relatively long period of time will, in general, produce much better results than a more concentrated solution acting for a short time interval. This is the method followed where it is desired to reveal the internal structure of bacteria. In actual practice, however, the more concentrated staining solutions are used because of the greater saving in time. Where time is not a factor, the more dilute preparations should be employed.

**Mordants.**—In some staining solutions, substances are added that have a strong chemical affinity for substrate and dye. They are chemicals that have the ability to make certain structures take up more stain than would occur in their absence. These substances are called "mordants." Tannic acid is an example of a mordant. Mordants are of special importance in the staining of flagella.

**Simple Stains.**—Many different kinds of staining solutions are employed in the various bacteriological procedures. Some are for general use; others are designed for specific purposes. A simple staining solution is one that contains only a single dye dissolved in a solvent. It is applied to the bacteria in one application. The simple staining solutions that are employed probably more than any of the others for routine purposes are carbolfuchsin, crystal violet, and methylene blue.

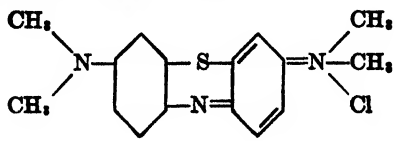
*Carbolfuchsin Stain.*—This stain is prepared by dissolving about 0.3 per cent of basic fuchsin (a triamino triphenylmethane dye) in a 5 per cent solution of phenol. For use as a simple stain, it is usually advisable to dilute the solution about ten times with distilled water.

For information on the composition of the various fuchsins of commerce, see pages 50 and 51.

**Crystal Violet Stain.**—Crystal violet is also a member of the triamino triphenylmethane dyes. Chemically it is hexamethyl pararosaniline (see page 45).

The dye is also known as methyl violet 10*B*, gentian violet, hexamethyl violet, and violet *C*, *G*, or 7*B*. It produces the deepest shade of the pararosanilines and is considered the most satisfactory of all the violet compounds as a simple bacterial stain.

*Methylene Blue Stain.*—Methylene blue is tetramethyl thionine, a basic dye, having the following formula:




The methylene blue of commerce is generally the double salt of the dye and zinc chloride. Since the zinc is toxic, this metal is not used in preparations of methylene blue intended for medicinal purposes.

Methylene blue is used perhaps more than any other dye in biological work. Because of its strongly basic nature, it stains nucleuses and nucleic acid granules very intensely. It is very useful in making a rapid survey of the bacterial population of milk (page 501). The dye is usually preferred in staining smears for the diagnosis of diphtheria. It is used in combination with eosin for staining blood films. Methylene blue is incorporated with eosin in a lactose agar base for distinguishing typical *Escherichia coli* from typical *Aerobacter aerogenes* (page 479). These are only a few of its many uses in bacteriology.

**Differential Stains.**—Differential stains are composed of more than one dye. In some of the staining techniques, the dyes are applied separately; in others, they are mixed and applied in one solution. The two most important differential stains used in bacteriology are the Gram stain and the acid-fast stain.

*Gram Stain.*—This is probably the most important differential stain employed by the bacteriologist.

Christian Gram (1884) found that, when histological sections were stained with gentian violet by the method of Ehrlich (1882) and then treated with an aqueous solution of iodine, the stain could be easily removed from the tissue sections by alcohol but not from the bacteria embedded therein. He was working on a new method of staining bacteria in tissues but discovered a new differential stain.

In this method of staining, the bacterial film is covered with a solution of one of the methyl violet dyes and allowed to act for a definite period of time. The stain is poured off and a solution of iodine added. This is allowed to remain for the same period of time. Next, the slide is treated with alcohol until no more of the dye is removed from the film. Finally a counterstain, such as carbolfuchsin or safranine, is added.

Some organisms retain the violet stain whereas others are readily decolorized by the alcohol and take the counterstain. Those retaining the first stain are called "Gram-positive" organisms; those failing to retain the primary stain but taking the counterstain are called "Gram-negative" organisms. Organisms can be placed into either of two groups on the basis of the Gram stain.

The pararosaniline dyes give the best results in the Gram stain. The two most important members are methyl violet and crystal violet.

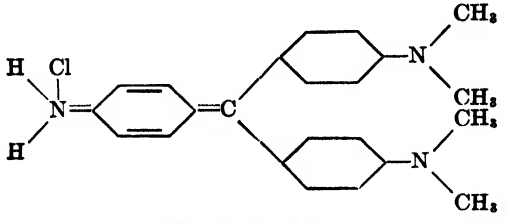
Strictly speaking, methyl violet is the name given to the tetramethyl pararosaniline compound. Commercially, the name is usually applied to various mixtures of the tetra-, penta-, and hexamethyl pararosanilines.

Their structural formulas are given on page 45.

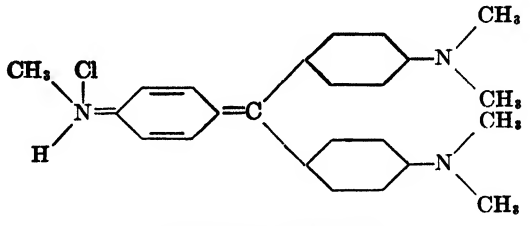
The shade of color of pararosaniline is deepened by increasing the number of methyl groups in the molecule. Hence, hexamethyl pararosaniline is deepest in shade and tetramethyl pararosaniline lightest of the three

compounds. The names methyl violet 3*R*, 2*R*, *R*, *B*, 2*B*, 3*B*, etc., refer to the number of methyl groups present. The letter *R* denotes the red shades; the letter *B* refers to the blue shades. The most completely methylated pararosaniline is the hexamethyl compound (crystal violet).

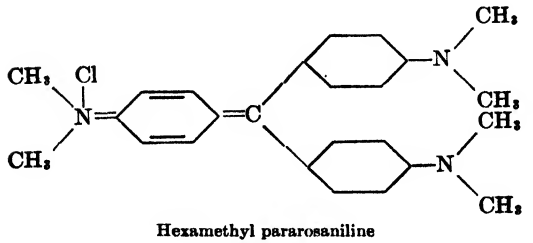
The ability of cells to retain the Gram stain is not a property applicable to all living matter in general, but is confined almost entirely to the yeasts



Tetramethyl pararosaniline
(methyl violet)



Pentamethyl pararosaniline



Hexamethyl pararosaniline
(crystal violet)

and bacteria. The cells of higher plants and animals do not retain the primary stain. Molds stain somewhat irregularly. Granules present in the mycelia tend to retain the stain. The Gram reaction is not a hard and fast one. It may change with the age of the culture, with the pH of the medium, and perhaps in other ways. The change of Gram character with age is especially true of those organisms which are only weakly Gram-positive and are cultivated in media containing fermentable substances that become acid in reaction as growth proceeds. The Gram reaction is of value only when the various factors are controlled.

A number of theories have been advanced to explain the mechanism of

the Gram stain. Stearn and Stearn (1923, 1924*a*, *b*) based their theory on a chemical combination between dye and bacterial protein. Proteins and amino acids are amphoteric compounds, *i.e.*, have the power to react with both acids and bases by virtue of their carboxyl (COOH) and amino ($NH_2$) groups. In acid solutions, they react with acids; in alkaline solutions, they react with bases.

*Isoelectric Point.*—According to the classical theory, the isoelectric point may be defined as the pH where an amphoteric compound shows the least amount of dissociation; stated differently, it is that point (pH) where the maximum amount of the compound is present in the un-ionized or molecular state. Opposed to this theory is the newer concept known as the "zwitter ion" hypothesis, which states that the isoelectric point is that pH where the acid and basic groups of the amphoteric compound are completely ionized (see page 193). On the acid side of the isoelectric point, the compound behaves as a base; on the basic side, it behaves as an acid.

Basic and acid dyes also combine with proteins. The basic dyes react on the basic side of the isoelectric points and the acid dyes on the acid side of the isoelectric points. The amount of combination in either case is proportional to the degree of alkalinity or acidity of the solutions. At the isoelectric points, proteins combine with neither basic nor acid dyes. With the protein casein as an example, the action of acid and basic dyes may be schematically represented as shown in Fig. 21.

It has been shown that the staining reactions of bacteria are due largely to their protein content. This means that bacteria behave as amphoteric compounds combining with acid dyes in acid solution and basic dyes in basic solution. Combination with either acid or basic dyes does not occur at the isoelectric "range." Since organisms contain more than one protein, the isoelectric point does not have a fairly definite value but rather a series of points extending over two or three pH units. An isoelectric range or zone rather than a point is found. Stearn and Stearn found that Gram-positive organisms have an isoelectric zone at a lower pH range than Gram-negative bacteria.

On the basis of their experimental data Stearn and Stearn concluded as follows:

1. Gram-positive organisms can be rendered Gram-negative by increasing acidity.
2. Gram-negative organisms can be rendered Gram-positive by increasing alkalinity.
3. Acid dye-positive organisms can be rendered Gram-negative by increasing alkalinity.
4. Basic dye-positive organisms can be rendered Gram-negative by increasing acidity.

5. At the isoelectric range, there is little tendency for any stain to be retained. This range is characteristic of each species.

6. There appears to be good evidence that the proteins of bacteria are not simple proteins but a loose combination of proteins with lipoidal or fatty substances. An example of such a fatty substance is lecithin, and a combination of lecithin and protein is known as lecithoprotein.

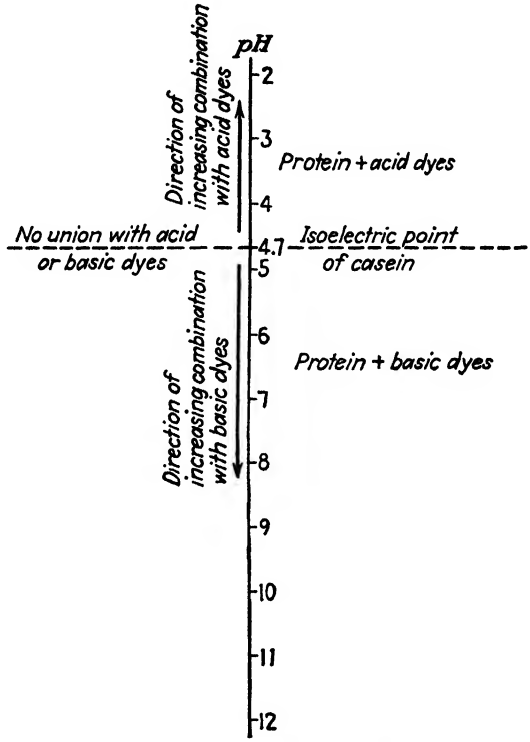



FIG. 21.—Combination of acid and basic dyes with casein, an amphoteric compound.

7. The lipoidal material extracted from Gram-positive organisms differs from that extracted from Gram-negative organisms in that the former contains a much larger proportion of unsaturated acids that have a great affinity for oxidizing agents. All mordants (such as iodine) used in the Gram stain are oxidizing agents. Their effect is in general to render the substance oxidized more acid in character. This increases the affinity of an organism for basic dyes.

8. The change of Gram character with age is especially true of those organisms which are only weakly Gram-positive and are cultivated in media containing fermentable substances that become acid in reaction as growth proceeds.

Another approach to the explanation of the Gram reaction depends upon evidence suggesting the existence of an outer layer surrounding a Gram-negative core.

Henry and Stacy (1943) treated heat-killed Gram-positive organisms with a 2 per cent bile solution and were able to dissolve away an outer surface layer. The Gram-positive organisms so treated became Gram-negative. The extracted material was composed of polysaccharides, protein, and the magnesium salt of ribonucleic acid. The extracted material could be "replated" back on the Gram-negative "skeleton" forms, restoring their Gram reaction, provided the skeleton was maintained in a suitable state of reduction by the presence of formalin. Neither the skeleton nor the extracted material was Gram-positive except in combination, and recombination of these materials was not possible unless the skeleton was reduced by the presence of formalin.

Bartholomew and Umbreit (1944) used a 2 per cent solution of sodium cholèate or of bile salts instead of whole bile and obtained similar results (Fig. 22). They also observed that a solution of pure crystalline ribonuclease (see page 283) was capable of destroying the Gram-positive character of heat-killed cells. Since ribonuclease is a specific enzyme for ribonucleic acid, there is little doubt that this acid is the important factor in the Gram-positive character of the organisms employed.

On the basis of their results, Bartholomew and Umbreit came to the following conclusions:

When ribonucleic acid is removed by bile salts, it will no longer unite with the cell protein if the latter has been oxidized with air. Sulfhydryl groups are autoxidizable with air when exposed on the protein surface.

The recombination is possible, however, even on exposure to air, if the "skeletons" are treated with formaldehyde. The latter unites in low concentration with sulfhydryl groups and prevents their autoxidation.

Iodine is essential to the Gram reaction, and it is the only known material which reacts with sulfhydryl groups of proteins even when these are located deep within the protein molecule. Furthermore, while the relations (if any) between hydroquinone, stannous chloride, or pyrogallol and sulfhydryl groups are not known, mercuric chloride, which can partially replace iodine, does react with sulfhydryl (Fildes, 1940).

In considering the data as a whole one can scarcely fail to conclude that the Gram-positive character of a cell resides in an outer layer of material surrounding a Gram-negative core. The important material in this outer layer is magnesium ribonucleate. The ribonucleate in combination with the cell protein is responsible for the Gram-positive characteristics, and crystal violet and iodine react chemically with this combination.

*Acid-fast Stain.*—The great majority of bacteria are easily stained by the usual bacteriological stains. However, there are a few notable exceptions. Some bacteria are said to be surrounded by a covering composed of fatty and waxy materials. These organisms are not penetrated readily by stains but, when once stained, retain the color even

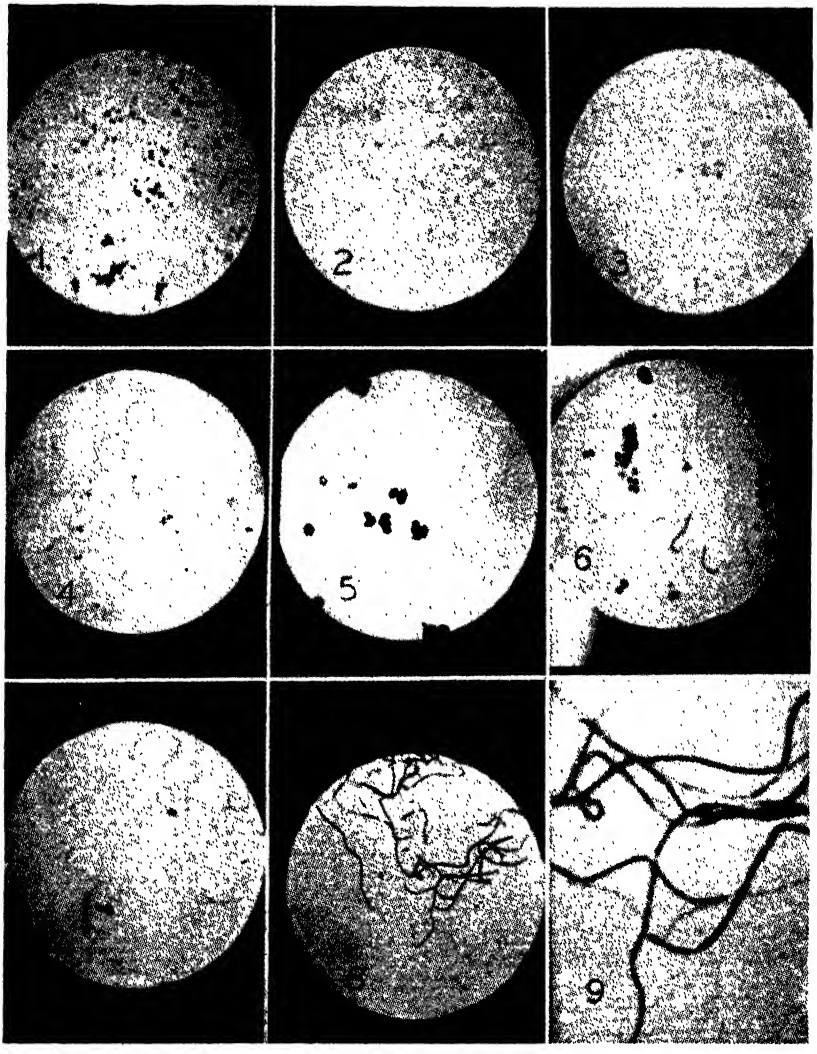



FIG. 22.—*Staphylococcus aureus.* 1, Gram stain; 2, as in 1, except smear treated with sodium choleate for 30 min. at 60°C., cells largely Gram-negative; 3, as in 2, except smear treated with sodium choleate for 60 min. at 60°C., cells completely Gram-negative; 4, as in 3, but "replated" with magnesium ribonucleate, cells again Gram-positive.

*Saccharomyces cerevisiae.* 5, Gram stain; 6, as in 5, but exposed to ribonuclease (enzyme) for 30 min., Gram-positive characteristics less pronounced; 7, as in 6, but exposed to ribonuclease for 60 min., Gram stain largely negative.

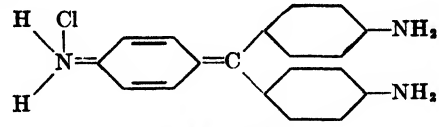
*Bacillus cereus.* 8, treated with ribonuclease for 60 min., Gram stain variable; 9, enlargement of central portion of 8. Note Gram-negative cells among the Gram-positive. (*After Bartholomew and Umbreit.*)

though treated with alcohol containing acid. The organisms are called "acid-fast" because they are resistant to decolorization with acid alcohol. The two best known members of the acid-fast group are the organisms of tuberculosis (*Mycobacterium tuberculosis*) and of leprosy (*M. leprae*).

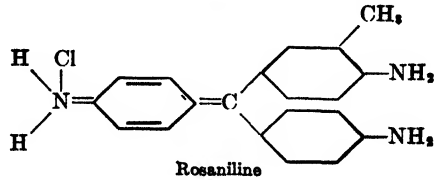
The acid-fast method of staining the organism of tuberculosis was first announced by Ehrlich (1882). He stained smears with aniline oil methyl violet. The organisms may be stained either by long exposure to the dye in the cold, or by gently steaming the dye on the slide for a shorter period. Ehrlich found that the tubercle bacillus was not decolorized when treated with 30 per cent nitric acid but that tissue and other bacteria lost their color. He used vesuvin as a counterstain for the aniline oil methyl violet. By this method the acid-fast organisms appeared purple; everything else on the slide stained brown. Later Ziehl (1882) improved the keeping qualities of the stain by substituting phenol for the aniline oil. Neelsen (1883) used carbolfuchsin in place of the aniline oil methyl violet and decolorized the smears with sulfuric acid instead of nitric acid. This modification, with some improvements, is now known as the Ziehl-Neelsen method, but is essentially the same as that of Ehrlich.

The basic fuchsin of commerce is usually a mixture of pararosaniline, rosaniline, and magenta II. Another compound, new fuchsin, may be purchased in pure form and is frequently employed in the acid-fast method of staining.

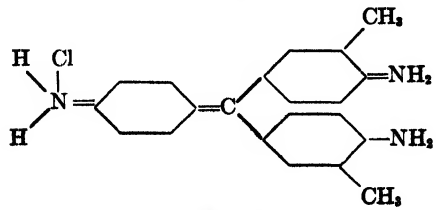
The formulas are as follows:



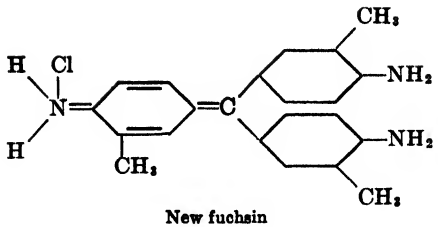

Pararosaniline




Rosaniline




Magenta II

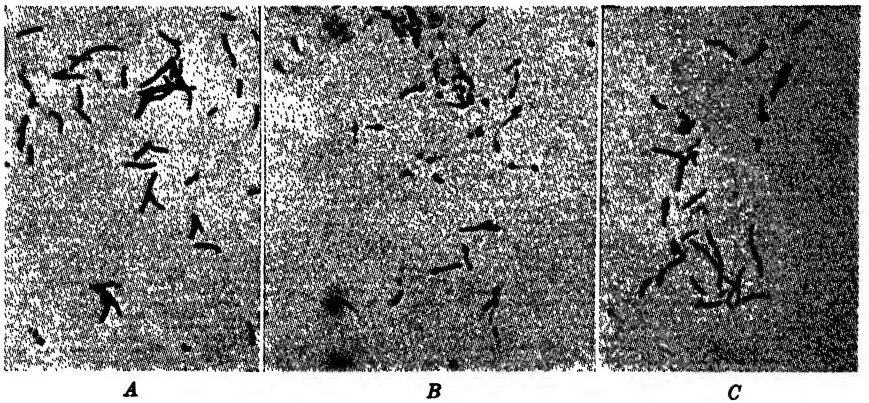



New fuchsin

The more methyl groups present in the molecule, the deeper will be the shade of red. New fuchsin (3 methyl groups) is the deepest in shade, and pararosaniline (no methyl group) the least so of the above compounds. For staining acid-fast organisms, the deeper the shade of dye used, the better will be the degree of differentiation.

Yegian and Baisden (1942) found that they could make tubercle bacilli stain solid or beaded by varying the staining technique (Fig. 23). To quote,

The presence of beads in films of tubercle bacilli stained by the Ziehl-Neelsen technique depends not only upon the existence of structures of the bacterial cell, but to a large extent upon the conditions under which the staining reaction is



*A* *B* *C*

FIG. 23.—*A*, Smear of *Mycobacterium tuberculosis* stained with carbolfuchsin. No electrolyte added to the staining solution; bacilli solidly stained. *B*, Smear of *M. tuberculosis* stained with carbolfuchsin, but with added electrolyte; bacilli show intense beading. *C*, Smear of beaded organisms in *B* washed with 95 per cent ethyl alcohol. Beaded organisms have become solidly stained. (*After Yegian and Baisden.*)

carried out. Films prepared from the same suspension of tubercle bacilli and stained under the same conditions, may present uniformly, solidly stained bacilli, or bacilli exhibiting beads in varying number, depending on the preparation of carbolfuchsin used. Addition of small amounts of electrolytes to the dye solution greatly increases the number of beads with all dyes, even with those which never produce any beads in the absence of salt. In preparations stained in the presence

of salt, the bacilli often appear as chains of heavily stained purple bodies, larger than the diameter of the cell proper, whereas the body is stained only faintly pale pink. The production of beads is much decreased even in the presence of salt, when the dyes are used in lower concentrations than is the usual practice. In all cases washing of the stained preparations with neutral alcohol (following decolorization with acid alcohol) removes practically all the beads and leaves most of the bacillary bodies as evenly stained rods of a pink tinge.

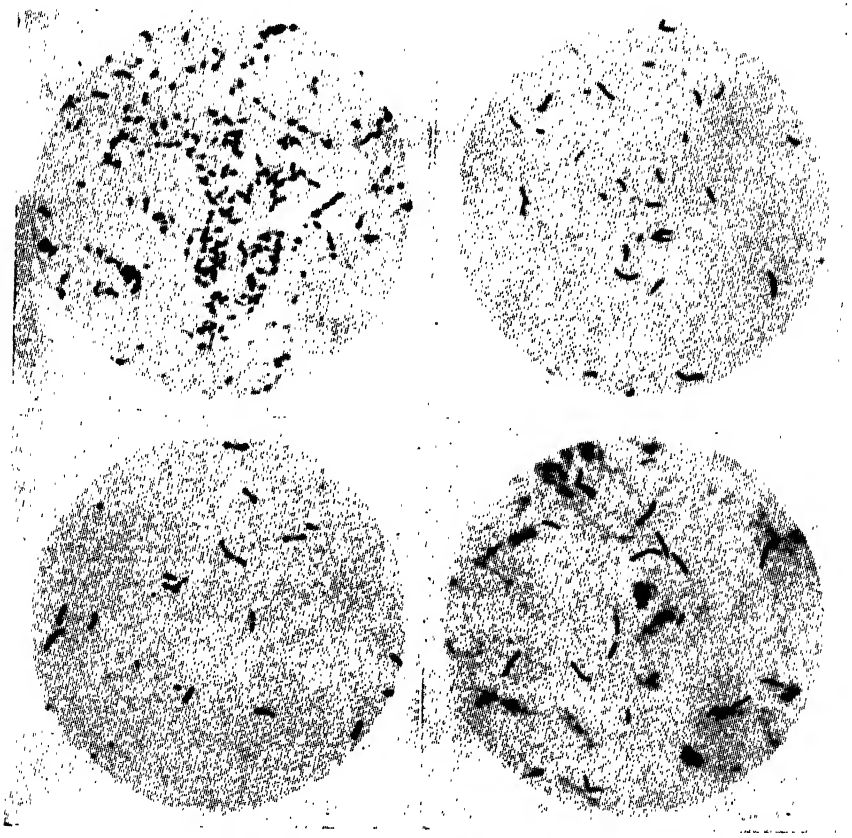


FIG. 24.—(Upper left) Virulent culture of *Mycobacterium tuberculosis* stained with rosaniline acetate; tubercle bacilli beaded.

(Upper right) Virulent culture of *M. tuberculosis* stained with rosaniline chloride; tubercle bacilli solidly stained.

(Lower left) *M. tuberculosis* in sputum stained with rosaniline acetate; tubercle bacilli beaded.

(Lower right) *M. tuberculosis* in sputum stained with rosaniline chloride; tubercle bacilli solidly stained. (*After Yegian and Budd.*)

In a later report Yegian and Budd (1943) found that the composition of the dye markedly affected the appearance of the tubercle bacilli (Fig. 24). Smears stained with rosaniline or pararosaniline acetate showed from 50 to

100 per cent beaded organisms. On the other hand, smears stained with rosaniline or pararosaniline chloride showed, without exception, solidly stained forms. For best results the chloride salts of the dyes should be used in the Ziehl-Neelsen technique.

For additional information on the subject consult the report by Lamanna (1946).

## STAINING OF BACTERIA

**Preparation of Smears.**—A bacterial smear is prepared by removing a loopful of a liquid culture from a tube, by means of a sterile wire loop, and spreading the liquid on a glass slide over an area of about ½ sq. in. If a solid culture is used, a minute amount of the growth is first emulsified in a drop of distilled water, previously placed in the center of a glass slide, then spread out over an area of about ½ sq. in. The smear is carefully dried by holding the slide high over a low gas flame to avoid steaming. The dried smear is fixed by quickly passing the slide five or six times through the upper portion of the Bunsen flame. This prevents the film from being washed off during the staining process. The dried and fixed smear is then covered with the staining solution and allowed to stand for a definite period of time. This will vary depending upon the staining solution used. Finally the slide is washed in water, dried by blotting, and examined under the microscope.

**Viability of Fixed and Stained Organisms.**—It is generally stated that bacteria in dried, fixed, and stained smears are no longer viable and that danger from infections is not possible if pathogenic organisms are so treated. Thurn (1914) reported that organisms in fixed and stained preparations are not necessarily killed. He found that heat-fixed, but unstained, smears of *Micrococcus pyogenes* var. *aureus*, *Salmonella typhosa*, *Escherichia coli*, *Bacillus anthracis*, *Vibrio comma*, *Corynebacterium diphtheriae*, and *Saccharomyces cerevisiae*, still contained viable organisms. Eighteen preparations of pathogenic and nonpathogenic organisms failed to show viable organisms after being stained by the Gram method. On the other hand, *B. anthracis* survived 1 min. and *B. mesentericus* 3 min. of treatment with carbolfuchsin, and both organisms survived 5 min. of treatment with methylene blue. Morton (1939) showed that certain organisms are capable of surviving treatment with basic fuchsin, Hucker's crystal violet, aqueous safranine, and methylene blue stains.

More care should be used in the handling of stained preparations in the laboratory, especially if pathogenic organisms are employed, since the process of staining is no indication that the organisms are necessarily killed.

For excellent discussions on dyes and staining solutions, see Committee on Bacteriological Technic (1946) and Conn (1947).

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## CHAPTER IV

## MORPHOLOGY OF BACTERIA

Bacteria belong to the class of organisms known as the *Schizomycetes* (*schizo*, fission, and *mycetes*, fungi). The organisms grouped in this class are so named because they reproduce typically by transverse or binary fission (see page 2).

The class *Schizomycetes* is divided into five orders. One of these orders, the *Eubacteriales*, includes all the common or true bacteria. The other four orders embrace those organisms possessing characteristics intermediate between the true bacteria and other plants or animals.

Bacteria are characterized as typically unicellular plants, the cells being usually small and sometimes ultramicroscopic. They lack the definitely organized nucleus found in the cells of higher plants and animals. However, bodies containing chromatin, which may represent simple nucleuses, are demonstrable in some cases. Individual cells may be spherical, rod-shaped, or spiral, and may occur in regular or irregular masses, or even in cysts. When they remain attached to each other after cell division, they may form chains or even definite filaments. The latter may show some differentiation into holdfast cells and into motile or nonmotile reproductive cells (conidia). Some grow as branching mycelial threads whose diameter is not greater than that of ordinary bacterial cells, *i.e.*, about 1 $\mu$. The cells may be motile by means of long, whip-like processes known as flagella (singular, flagellum). Some species produce pigments. The sulfur purple and green bacteria possess pigments much like or related to the true chlorophylls of higher plants. These pigments have photosynthetic properties. Multiplication is typically by cell division. Endospores are formed by some species included in the *Eubacteriales*. Sporocysts are formed in the *Myxobacteriales*. The bacteria are free-living, saprophytic, parasitic, or even pathogenic. The latter types cause diseases of either plants or animals.

**Shape of Bacteria.**—Bacterial cells exhibit three fundamental shapes: (1) the spherical, (2) the rod, and (3) the spiral. All bacteria exhibit pleomorphism, in more or less degree, under normal or other conditions, but a bacterial species is still generally associated with a definite cell form when grown on standard media under certain specified conditions.

Some of the spherical or coccus forms (singular, coccus; plural, cocci) are apparently perfect spheres; others are slightly elongated or ellipsoidal in shape. Spherical forms that grow normally in pairs (diplococci), fours

(tetrads), or chains (streptococci), are usually slightly flattened at their adjacent surfaces. A pair of such organisms is usually referred to as coffee bean-shaped.

The rod forms also show considerable variation. A rod is usually considered to be a cylinder with the ends more or less rounded. Some rod forms are definitely ellipsoidal in shape. The ends of rods also show considerable variation. Some species are markedly rounded; others exhibit flat ends perpendicular to the sides. Gradations between these two forms may be seen.

Rods may show marked variation in their length/width ratio. Some rods are very long in comparison to their width; others are so short they may be confused with the coccus forms.

The shape of an organism may also vary depending upon certain environmental factors, such as temperature of incubation, age of the culture, concentration of the substrate, and composition of the medium. Bacteria usually exhibit their characteristic morphology in young cultures and on media possessing favorable conditions for growth. Henrici (1928) distinguished three forms of bacteria: (1) the embryonic, (2) the mature, and (3) the senescent. The embryonic forms corresponded to the growth phase and appeared as long, slender, and uniform cells. The mature forms corresponded to the resting phase and were characterized as short cells of small size but more viable in form. The senescent forms corresponded to the death phase and showed great variation in both form and size.

Those forms which depart widely from the standard morphological picture, when one or more environmental factors are changed, have been called "involution forms" and "forms of degeneration." Henrici called them "senescent forms," because he believed that an organism may change morphologically as it becomes older just as a multicellular organism changes with age. Some morphologists have considered them as definite stages in an orderly life cycle of an organism.

Many experiments may be cited to show the effect of environmental changes on the growth of bacteria. It is well known that *Escherichia coli*, when inoculated into a medium containing soap (low surface tension) grows in the form of long filaments, whereas the same organism inoculated into a medium containing calcium chloride (high surface tension) grows as very short rods almost spherical in form. Metchnikoff (1888) inoculated the organism causing avian tuberculosis into a medium containing 12 per cent glycerol and incubated the culture at 44°C. He reported extensive branching of the organisms. Such forms are very rarely, if ever, encountered on the usual media incubated at 37°C.

Young cells are, in general, larger than old organisms of the same species. As a culture ages, the cells become progressively larger until a maximum is reached after which the reverse effect occurs. Bacterial varia-

tions resulting from changes in age are only temporary; the original forms reappear when the organisms are transferred to fresh medium.

**Size of Bacteria.**—Bacteria vary greatly in size according to the species. Some are so small that they approach the limit of visibility when viewed with the light microscope. Others are so large that they are almost visible with the normal eye. Regardless of their size, none can be clearly seen without the aid of a microscope. However, the sizes of the majority of bacteria occupy a range intermediate between these two extremes.

A spherical or coccus form is measured by the size of its diameter; a rod or spiral form by its length and width. Calculation of the length of a spiral organism by this method gives only the apparent length, not the true length. The true length may be computed by actually measuring the length of each turn of the spiral. Mathematical expressions have been formulated for making such computations.

The method employed for fixing and staining bacteria may make a difference in their size. The bacterial cell shrinks considerably during drying and fixing. This will vary somewhat depending upon the type of medium employed for the cultivation of the organisms. The magnitude of the shrinkage will average about one-third the length of the cell, when compared to an unstained hanging-drop preparation. Knaysi (1938, 1941) reported a shrinkage of from 15 to 25 per cent when young cells of *Bacillus megatherium* were transferred from nutrient broth to a similar medium containing sodium chloride in $2M$ concentration. The usual culture media contain sodium chloride in about a $0.1M$ concentration.

The measurements will show some variation depending upon the staining solution used and the mode of application. In dried and fixed smears, the cell wall and slime layer do not stain with weakly staining dyes such as methylene blue but do stain with intensely staining dyes such as pararosaniline, new fuchsin, crystal violet, and methyl violet. The cell wall and slime layer are included in negatively stained preparations, but the organisms measure larger than their true size owing to the fact that the colloidal dye film retracts on drying. In the living condition, the cell wall and the slime layer cannot be seen, and measurements of such organisms include only the cytoplasm.

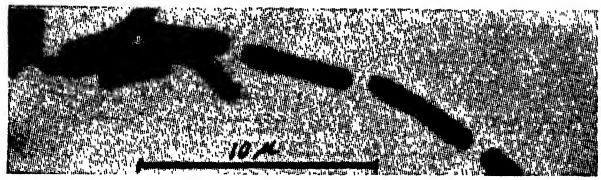
Organisms that have been studied and classified have also been measured. Most of the measurements have been made on fixed and stained preparations. In some instances dried, negatively stained smears have been used; in other instances living material has been employed. It follows from this that the method employed should be specified when measurements of bacteria are reported; otherwise, the figures will not have much significance.

Knaysi (1945) made a comparative study of the cell width of *Bacillus cereus* and *B. mycoides*. Measurements of the living cells in the medium

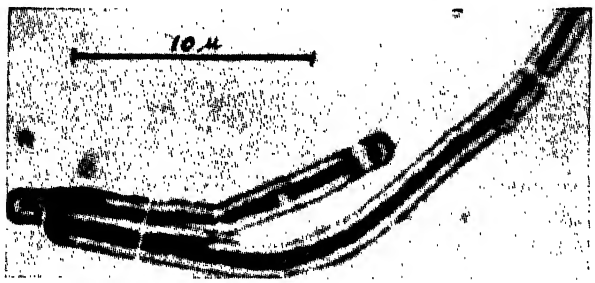
in which they were grown agreed with those made on similar cells stained by a method showing the cell wall. In stained smears where the cell wall was not visible, the cells appeared much smaller than they really were and represented the shrunken masses of protoplasm (Fig. 25).

The unit of measurement for bacteria is the micron, expressed by the symbol $\mu$. It is 0.001 mm. or 0.0001 cm. A millimicron is 0.001 $\mu$ or 0.000001 mm. It is expressed by the symbol m$\mu$.

Some bacteria measure as large as 80 $\mu$ in length; others as small as




*A*




*B*

FIG. 25.—*Bacillus cereus*. *A*, Cells from a 6-hr.-old agar slant culture incubated at 33°C. and stained with methylene blue. *B*, Cells from the same culture as in *A* but stained by a different procedure to reveal the cell wall. (*After Knaysi.*)

0.1 $\mu$. The large forms are members of the sulfur and iron bacteria, which show characteristics intermediate between the true bacteria and the higher plants. However, a majority of the commonly encountered organisms, including the pathogenic bacteria, measure about 0.5 $\mu$ in diameter for the cocci and 0.5 by 2 to 3 $\mu$ for the rod forms. Organisms producing spores are generally larger than those which are unable to produce spores. The dimensions of some common bacteria in dried and stained preparations are as follows: *Escherichia coli*, 0.5 by 1.0 to 2.0 $\mu$; *Proteus vulgaris*, 0.5 to 1.0 by 1.0 to 3.0 $\mu$; *Salmonella typhosa*, 0.6 to 0.7 by 2.0 to 3.0 $\mu$; *Streptococcus lactis*, 0.5 to 1.0 $\mu$ in diameter; *Streptococcus pyogenes*, 0.6 to 1.0 $\mu$ in diameter; *Micrococcus pyogenes* var. *aureus*, 0.8 to 1.0 $\mu$ in diameter; *Lactobacillus acidophilus*, 0.6 to 0.9 by 1.5 to 6.0 $\mu$; *Bacillus subtilis*, 0.5 to 0.8 by 1.6 to 4.0 $\mu$; *B. megatherium*, 1.0 to 1.5 by 3.0 to 6.0 $\mu$; *B. anthracis*, 1.0 to 1.5 by 5.0 to 10.0 $\mu$.

The most commonly employed method for measuring bacteria is by means of an ocular micrometer. Measurements may also be made by using a camera lucida attachment and drawing oculars, or by projecting the real image on a screen and measuring the bacteria.

The same factors that cause variations in the shape of bacteria also produce alterations in their size. With a few exceptions, young cells are much larger than old or mature ones. Knaysi (1938) showed that cells of *B. subtilis* from a 4-hr. culture measured from five to seven times longer than cells from a 24-hr. culture. Variations in width are much less pronounced. The organism, *Corynebacterium diphtheriae*, is a notable exception to the rule of decreasing cell size with age.

A decrease in cell length and width appears to be due to a variety of factors. The major causes appear to be changes in the environment with the accumulation of waste products. An increase in the osmotic pressure of the medium will also cause a decrease in cell size and may possibly be the most important factor.

The interrelation of particle size of bacterial cells, viruses, molecules, atoms, radiations, and basic units, is given in Table 3.

## STRUCTURE OF THE CELL

Bacteria, as a group, do not show the same morphological picture. Differences in structure do exist between species. It is generally agreed that a bacterial cell consists of a compound membrane enclosing cytoplasm, nuclear material, and often containing various granules, and one or more vacuoles. The term "protoplasm" is commonly used to denote both cytoplasm and nucleus. In addition, some species contain resistant bodies known as spores, and some have one or more organs of locomotion called "flagella."

**Presence of a Nucleus.**—The question of the presence of a well-defined nucleus in a bacterial cell has been a subject fraught with considerable controversy since the beginning of the science of bacteriology. Nuclear studies have been concerned mainly with organisms classified with the higher bacteria, having characteristics intermediate between the true bacteria and plants or animals. The organisms studied are for the most part very large. Some of these do appear to have discrete nucleuses. However, there is still some doubt that any worker has conclusively demonstrated the presence of a well-defined nucleus in an organism classified in the order *Eubacteriales*, or true bacteria. Cells of the true bacteria are exceedingly small. If the ratio between the size of a bacterial cell and that of its nucleus is the same as for other organisms, the dimensions of the nucleus in bacteria would approach the limit of the resolving power of the light microscope. This undoubtedly explains why the larger organisms have been used.

Some of the earlier cytologists maintained that bacteria were very primitive organisms, devoid of nucleuses and consisting simply of cytoplasm, granules, and vacuoles. This view was based on their failure to observe a nucleus in a bacterial cell. Others held the view that the whole bacterial body should be regarded as a "naked nucleus" corresponding to the nucleus of higher organisms. The naked nucleus is regarded as a primitive form of living matter. Since bacteria have the structural and physiological attributes of true cells, this concept cannot apply to such organisms.

TABLE 3.—INTERRELATION OF PARTICLE SIZE (DIAMETERS) OF BACTERIA, VIRUSES, MOLECULES, ATOMS, RADIATIONS, AND BASIC UNITS

| 0.0001 to 0.000001 mμ (basic units) | 0.001 mμ (radiations) | 0.1 to 0.01 mμ (atoms) | 0.01 μ to 0.1 mμ (molecules) | 1 to 0.01 μ (viruses) | Up to 1 μ (bacteria) |
|---|---|---|---|---|---|
| Neutron | Gamma rays from radium | Diameter of H atom, 0.1 mμ | Proteins, 4 to 9 mμ | Psittacosis, 0.25 μ | Micrococcus, 1 μ |
| Electron | | X rays | Small colloid particles | Variola, 0.2 μ | |
| Cosmic rays | | | | Herpes, 0.12 μ | |
| Alpha rays | | | | Poliomyelitis, 0.01 μ | |
| Proton | | | | | |
| True solutions < 1 mμ | | Colloid solutions 1 to 200 mμ | | Emulsions and suspensions 200 mμ to 1000 μ (1 mm.) | |

In the great majority of bacteria, nothing can be demonstrated other than chromatin granules scattered throughout the cytoplasmic material. The chromatin is identified by its behavior toward dyes and by other physical and chemical tests. Considerable confusion is caused by the fact that a reserve food material known as volutin is chemically similar to chromatin (see page 63). Lindegren (1942) believed that a nucleus could not perform its special function if present in a diffuse form. Genes scattered throughout the cytoplasm would be unable to transmit hereditary characters with any degree of constancy.

Knaysi and Mudd (1943) and Knaysi (1946*b*), by means of electron micrographs, reported that the cell of *Micrococcus flavocyaneus* contained one or more granules which had solubilities similar to nucleoproteins and which often appeared constricted or in pairs. The granules gave tests for both protein and nucleic acid. The granules were separated from the cells by sonic vibration and were shown to contain desoxyribonucleic acid. In young, actively growing cells, the granules were reduced in size, and

there was evidence that the nuclear material was then partly in solution. The cells of *Neisseria meningitidis* also showed granules which were insoluble in hot water and which were likely to be nuclear material (Fig. 26). On the other hand, studies on *Streptococcus pyogenes*, *Micrococcus pyogenes* var. *aureus*, and *N. gonorrhoeae* failed to reveal the presence of any cytoplasmic structures.

Knaysi and Mudd concluded from these observations that (1) bacteria

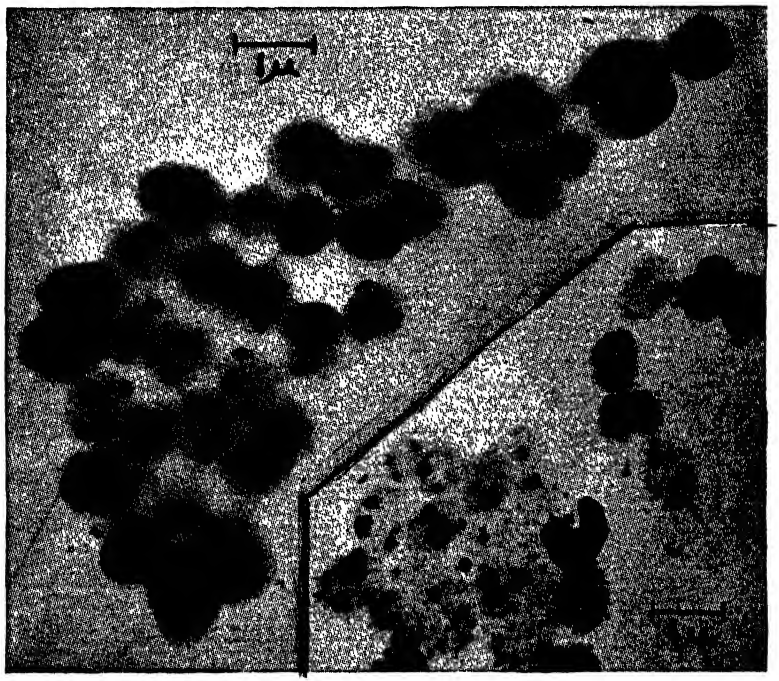



FIG. 26.—*A*, *Staphylococcus flavo-cyaneus*, 48-hr. culture at room temperature. Electron micrograph, ×11,000. *B*, *Neisseria meningitidis*, 22-hr. culture at 37°C., heat-fixed and exposed to distilled water at 80°C. for 10 min. Electron micrograph, ×10,000. (*After Knaysi and Mudd.*)

contain nuclear material which may be partly or totally differentiated into a nucleus, or (2) cells contain the nuclear material in solution or in very fine dispersion. This latter conclusion is in support of the older hypothesis that nuclear material is present as chromatin granules scattered throughout the cytoplasm.

Robinow (1944) brought forth additional evidence in favor of the nuclear hypothesis. The organisms were stained by the Feulgen technique. Wet preparations of *Escherichia coli* were prepared and fixed in osmic acid vapor for 3 to 5 min. After drying, the slides were immersed in normal HCl for about 9 min. at a temperature of 53 to 55°C., then washed

and stained in 1:20 Giemsa solution for 10 to 60 min., according to the staining properties of the specimen. The preparations were examined under a light microscope at a magnification of 1350 X.

The chromatinic structures in *E. coli* from old cultures, although

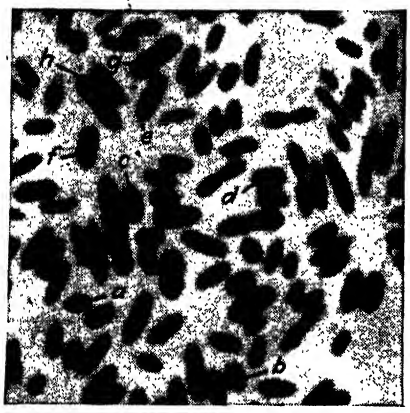



FIG. 27.—*Escherichia coli*, grown on an agar slant for 45 to 90 min. at 37°C. *a–h*, successive stages in the development of a coccoid element with a central pair of closely contiguous chromatinic bodies into a typical rod-shaped bacterium with four chromatinic structures. Note the V-shaped division stage at *d*. The dumbbell body forming the left limb of the V is much broader than the right one, and it seems plausible to assume that it is preparing for the next division. (*After Robinow.*)

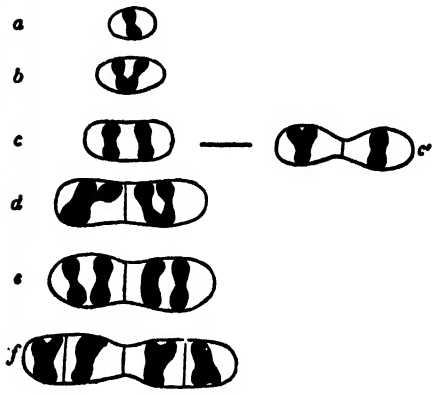



FIG. 28.—*Escherichia coli*. Diagram of successive division stages of the chromatinic bodies from the beginning of the lag phase, after transfer to a fresh nutrient medium, to the first division of the growing organism. *c–c'* and *c–f* are alternative modes of development, *c–f* being that most commonly followed. (*After Robinow.*)

usually distinguishable from the cytoplasm, were too small to be resolved accurately. After transfer to a fresh medium, the chromatinic structures increased in size and gave rise to short, often dumbbell-shaped rods or chromosomes, which multiplied by splitting lengthwise in a plane more or less parallel with the short axis of the cell (Figs. 27 and 28). A single cell of *E. coli* contained one chromatinic body or one or two pairs of these representing primary and secondary division products. Few bacteria from young cultures were single cells.

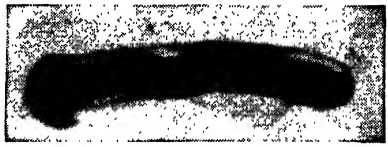


FIG. 29.—*Bacillus megatherium*. All four component cells of a young organism have retracted independently from the outer cell wall. The third cell from the left has also retracted from the transverse partition separating it from the fourth cell. The left end of the cell is still contained in the spore case. (*After Robinow.*)

Bacteria from young cultures of *Bacillus megatherium* assumed a banded appearance indicating that each bacterium consisted of two, three, or four separate cells (Fig. 29).

For more information see Knaysi and Baker (1947).

**Cell Inclusions.**—Vacuoles have been identified in young bacteria. They are cavities in the protoplasm and contain a fluid known as cell sap. As the cells approach maturity, some of the water-soluble reserve food materials manufactured by the cell dissolve in the vacuoles. Insoluble constituents precipitate out as cytoplasmic inclusion bodies.

The best known inclusion bodies identified in bacterial cells are known as volutin or metachromatic granules (Fig. 30). The granules are believed to originate in the cytoplasm of young bacteria and to localize in the vacuoles as the cells become mature. The granules show a great affinity for basic dyes, indicating that they are acid in character.

FIG. 30.—Metachromatic granules in *Bacillus subtilis*. Culture 18 hr. old and stained with a 1:5000 solution of crystal violet.

Metachromatic granules are composed of nucleic acid combined with basic proteins or organic bases. It is fairly well established that the granules contain ribonucleic acid as distinguished from desoxyribonucleic acid of the nucleus (see page 586). Chemically, nucleuses and metachromatic granules are very similar.

Metachromatic granules are believed not to be living constituents of either cytoplasm or nucleus, but merely stored-up reserve food material of a nitrogenous nature. Usually granules are not seen in young cells, *i.e.*, organisms in the active growth phase. They are formed in old cells after growth has ceased. As a general rule, young cells stain solid whereas old cells display a very granular appearance.

Burdon, Stokes, and Kimbrough (1942) showed that certain organisms of the *Bacillus cereus–B. megatherium* group, when grown on glycerol or carbohydrate agar, were capable of storing fat in the form of globules. Fat globules were demonstrated within 24 hr. and usually reached a maximum after about 48 hr. (Fig. 31). In some types, the fat was present in large globules; in others, it appeared in small, scattered granules. They noted that structures that appeared to be vacuoles in smears stained with methylene blue were, in reality, fat globules when treated with Sudan black *B*, a fat-staining dye.

FIG. 31.—*Bacillus cereus*, with fat globules. From a 48-hr. glycerin agar culture stained with Sudan black B and safranine. Fat droplets appear bluish-black; cytoplasm stains pink. (*After Burdon, Stokes, and Kimbrough.*)

For more information on the staining of fat globules in bacteria see Burdon (1946).

**Cell Membrane.**—According to Knaysi (1938, 1944, 1946*a*), the bacterial cell is surrounded by three membranes: (1) the cytoplasmic membrane, (2) the cell wall, and (3) the slime layer.

The cytoplasmic membrane first appears in young cells as an interfacial fluid film, becoming thicker and denser as surface active material accumulates. It is finally converted into a firm structure composed sometimes of several layers. Knaysi believes it is composed of lipoids, lipoproteins, and other materials in a highly stable chemical combination. It is the membrane principally responsible for the Gram and the acid-fast reactions. When a cell is plasmolyzed by immersion in a hypertonic solution, this membrane is drawn in with the cytoplasmic constituents. The thickness of the cytoplasmic membrane varies even in a single cell. In young cells of *Bacillus cereus* it is usually from 0.21 to 0.35 $\mu$ in thickness.

The cell wall is a more rigid structure and is responsible for the form of the bacterial body. It behaves as a selectively permeable membrane and apparently plays a fundamental role in the life activities of the cell. The cell wall has a low affinity for dyes. This means that it is probably not stained by the usual procedures followed for staining bacterial smears. Chemically, it appears to be composed of a complex carbohydrate of unknown nature and usually referred to as a hemicellulose. This carbohydrate is believed to be impregnated with other substances, some of which contain nitrogen. A polymerized, acetylated glucosamine known as chitin has been reported to be present in the walls of some bacteria. This compound has been definitely identified in the cell walls of molds.

The slime layer is considered a modified outer layer of the cell wall. The two structures give, in many instances, the same microchemical tests. The soluble carbohydrates elaborated by many bacteria, such as the various

types of pneumococci and certain streptococci, have their origin in the slime layer. The slime layer, like the cell wall, has also a low affinity for dyes. When the slime layer is large and remains fixed around the cell, it is called a "capsule."

**Capsules of Bacteria.**—Capsules are mucilaginous or gummy envelopes of a carbohydrate nature, formed from the external or slime layer of bacteria. Some believe that all organisms produce small amounts of capsular material. A few species are surrounded by relatively large capsules, which can be readily seen by appropriate staining methods and which may be used to identify the bacteria (Figs. 32 and 33).

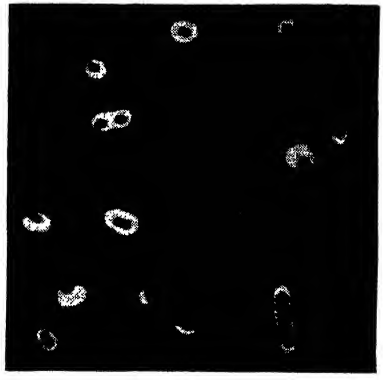
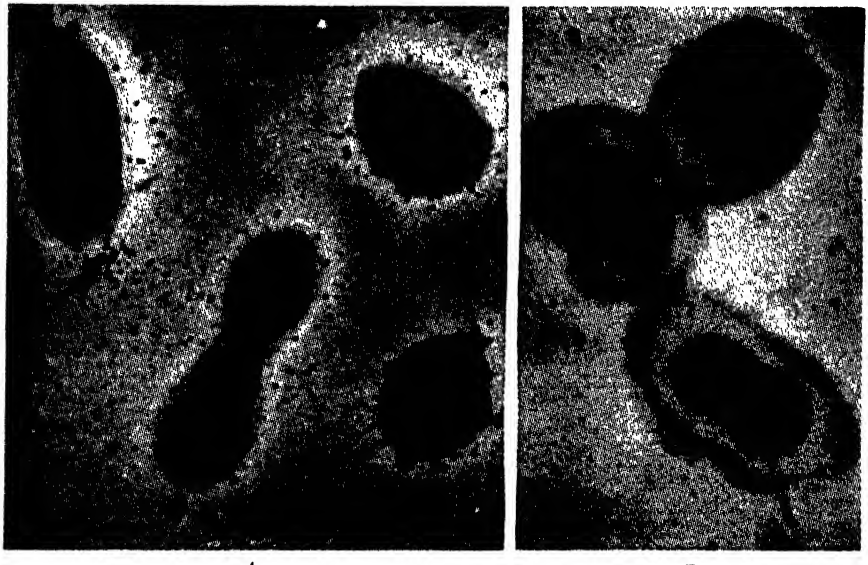


FIG. 32.—*Klebsiella pneumoniae.* Capsule stain by Gins' India-ink method.

Broth cultures of capsule-producing organisms are usually stringy in texture, and agar colonies exhibit a very moist, glistening surface which is



*A* *B*

FIG. 33.—*Diplococcus pneumoniae*, electron micrographs. *A*, Capsulated cells of Type III organisms, ×14,000. *B*, Capsules of Type I organisms increased in density and thickness by treatment with specific immune serum, ×10,500. (*After Mudd, Heinmets, and Anderson.*)

described as mucoid. Capsule formation depends upon the composition of the culture medium, especially upon the dissociation phase of the

organism. Some disease-producing organisms produce large capsules in culture media rich in animal fluids. Others produce prominent capsules when cultures are incubated at low temperature (10 to 20°C.).

Hoogerheide (1939) in his studies on *Klebsiella pneumoniae* believed that capsule formation occurred when conditions were unfavorable for growth and not during active multiplication. Freshly isolated mucoid strains of the organism should not be transferred to fresh medium at short intervals but should be stored as long as possible before making new cultures. He believed that a medium low in nutrients was more favorable to capsule formation than one containing an abundance of nutrient substances.

In a later report, Hoogerheide (1940) stated that inhibition of capsulation of *K. pneumoniae* could be brought about by a great variety of electrolytes. The adsorption of electrolytes on surfaces, in general, follows the lyotropic series and increases in the direction Li < Na < K, etc. Hoogerheide concluded that, inasmuch as the inhibition of encapsulation also followed the lyotropic series, it might be possible that physical adsorption of an electrolyte on the bacterial cell, and perhaps on the enzyme systems responsible for polysaccharide synthesis, was sufficient to inhibit these enzymes.

Chemical analyses of capsular material recovered from a number of bacteria have shown them to be complex carbohydrates known as polysaccharides. They are sometimes referred to as carbohydrate gums.

Polysaccharides that are morphologically evident as well-defined capsules are difficult to distinguish from those gums which flow away from the cells as they are formed. Gums of the latter type are not considered to be true cellular constituents. Organisms producing gums do so when grown in certain sugar solutions. Some organisms produce gums only in the presence of a specific sugar; others produce them in the presence of any one of several sugars. In the absence of sugar, very little, if any, gum is formed. Organisms producing gums of this type are the cause of considerable loss in the sugar industry. The increased viscosity due to the presence of the gums interferes with the filtration of the sugar solution.

Alford and McCleskey (1942), in a study of the slime in cane juice from Louisiana sugar mills, concluded that the most important organism was *Leuconostoc dextranicum*. Other organisms which have been encountered in cane sugar solutions and which are also active slime producers are *L. mesenteroides* and *L. citrovorum*.

Gums are classified as levulans, arabans, dextrans, galactans, etc., depending upon the sugars released when the polysaccharides are hydrolyzed. Some organisms produce gums that yield only one sugar on hydrolysis; others yield more than one kind of sugar. The names of a few

organisms producing soluble gums and their hydrolytic products are given in Table 4.

**Motility of Bacteria.**—Bacterial motion is generally believed to be due to the presence of organs of locomotion known as flagella. They were first observed in stained specimens by Cohn (1875). A few of the sulfur bacteria

TABLE 4

| Organism | Gum | Products of hydrolysis |
|---|---|---|
| *Leuconostoc mesenteroides* | Dextran | Glucose |
| *Azotobacter chroococcum* | Araban | Arabinose |
| *Bacillus subtilis* | Levulan | Levulose |
| *B. mesentericus* | Levulan | Levulose |
| *Diplococcus pneumoniae*, Type I | Soluble specific substance | Amino sugar, galacturonic acid |
| *D. pneumoniae*, Type II | Soluble specific substance | Glucose, aldobionic acid (glucose + glycuronic acid) |
| *D. pneumoniae*, Type III | Soluble specific substance | Glycuronic acid, galactose |
| *D. pneumoniae*, Type IV | Soluble specific substance | Acetic acid, amino sugar, glucose |
| *D. pneumoniae*, Type VIII | Soluble specific substance | Glucose, aldobionic acid |
| *D. pneumoniae*, Type XIV | Soluble specific substance | Glucosamine, galactose, acetic acid |

are exceptions in that they do not possess flagella but exhibit a slow, creeping motion caused probably by a contraction of their protoplasm. The presence of flagella does not mean that the organisms are always motile, but indicates a potential power to move.

Independent bacterial motion is a true movement of translation and must be distinguished from the quivering or back-and-forth motion exhibited by very small particles suspended in a liquid. This latter type of motion is named Brownian movement after Robert Brown (1828, 1829, 1830). It is caused by the bombardment of the bacteria by the molecules of the suspending fluid.

Flagella are very delicate organs and easily destroyed in the usual methods of preparing smears. In the stained state, they are long, slender, undulating organs with ends in some cases blunt and in others slightly thickened. The flagella are always directed backward to the direction of motion at an angle of about 45°. Reversal of direction occurs by swinging the flagella through an angle of about 90°. Turning movements take place by swinging the flagella forward on one side only. They propel the organisms by a spiral or corkscrew motion.

Flagella show considerable variation in length, depending upon age and changes in the environment. There appears to be a certain inverse

relation between the length of flagella and that of the organism. This means that, as cell size decreases, the length of flagella increases.

Some believe that flagella originate from the cell wall; others that they have their origin in the cytoplasm and extend through pores in the cell

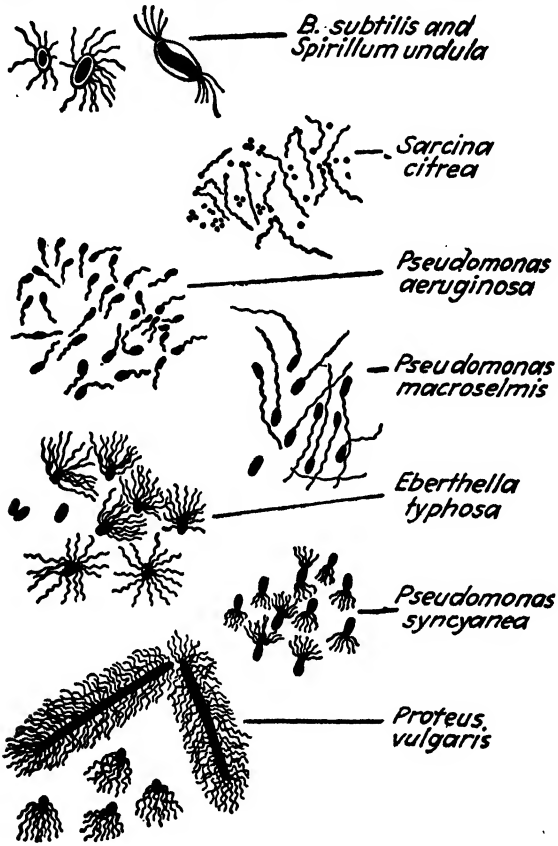



FIG. 34.—Various types of flagellated organisms. (*Courtesy of Encyclopaedia Britannica, Inc.*)

wall. Electron micrographs of a number of organisms show the flagella penetrating the faint outer zones and extending into the cytoplasm. If the outer zone is the cell wall, then the flagella have their origin in the cytoplasm.

The number and arrangement of flagella vary with different bacteria, but they are generally constant for each species. Some have only one flagellum; others have two or more. Also, the arrangement about the organisms varies considerably (Figs. 34 and 35).

Organisms may be classified on the basis of the number and arrangement of flagella as follows:

Monotrichous—a single flagellum at one end of the cell.
Lophotrichous—two or more flagella at one end or both ends of the cell.
Amphitrichous—one flagellum at each end.
Peritrichous—flagella surrounding the cell.

Knaysi (1942) measured the width of unstained flagella of *Aerobacter cloacae* from an electron micrograph and estimated them to be about 0.03 $\mu$ in diameter. Hofer (1944) measured the flagella of a number of

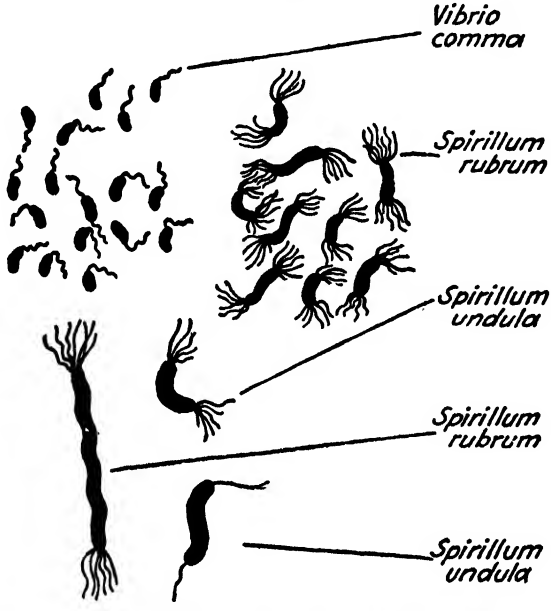



FIG. 35.—Various types of flagellated organisms. (*Courtesy of Encyclopaedia Britannica, Inc.*)

species of *Azotobacter* (Fig. 36) and obtained essentially the same results. This figure is about one-tenth the wave length of visible light and, if true, undoubtedly explains why flagella cannot be seen in hanging-drop preparations or in smears stained by the usual simple procedures (see page 12). When special staining methods are employed, sufficient dye becomes deposited on the flagella to make their diameters greater than that of visible light. They may then be seen under a light microscope.

On the other hand, Pijper (1946) questioned the belief that flagella are responsible for motility in bacteria. He added methyl cellulose to a culture of a motile organism to increase the viscosity of the medium and found that it caused a marked decrease in the rate of motility of the cells. Under these conditions the organisms exhibited a gyratory undulating movement, like other aquatic creatures. To quote,

That motile bacteria always exhibit a gyratory undulating movement was confirmed by making a slow motion cinemicrographic film of fast-swimming bacteria in

broth, and also by examining the same bacteria at lower temperatures, which reduced their speed.

This spirillar motion of bacteria is sufficient to propel them and there is no need to invoke special motor organs like flagella. There is no evidence to show that the flagella-like appendages of bacteria act as motile organs—in fact all the evidence when critically examined points the other way.

Analysis of the structure of bacteria excludes the possibility that tails, "flagella" or the thin wavy threads are live organs, or that they are in direct communication with the living parts of the cell. There is no evidence from either electron pictures or stained preparations that it is otherwise.

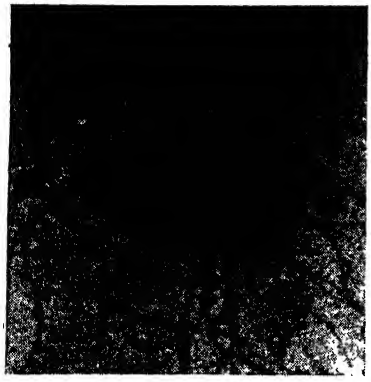


FIG. 36.—*Azotobacter vinelandii*, electron micrograph ×27,000. Note the large number of flagella arranged around the cell (peritrichous). (*After Hofer.*)

Not only does the visible gyratory undulating movement of motile bacteria satisfy all requirements for locomotion, but it is possible for bacteria grown under special conditions to swim in this fashion without showing tails or other supposed motor organs.

The staining of flagella is a difficult technique, especially in the hands of the beginner. For this reason, many methods have been proposed. Regardless of which method is employed, the film must be first treated with a mordant to make the flagella take the stain. Mordants consist usually of a mixture of tannic acid and some metallic salt. In some methods, the mordant and stain are applied separately; in others they are combined in one solution.

For an excellent method for staining flagella, see the report of Fisher and Conn (1942).

**Motion of Colonies.**—Muto (1904) described an organism, *Bacillus helixoides*, that exhibited motility of colonies, when grown on a solid medium. Smith and Clark (1938) and Shinn (1938) made similar observations on an organism named *B. alvei*.

Shinn prepared lapse-time motion pictures of the individual colonies on agar plates and was able to measure their velocities. He reported that the linear motions of colonies measuring 0.2 to 0.5 mm. in diameter averaged about 14 mm. per hour. Comparing this figure with the speed of individual cells of other species of motile bacteria, he obtained the following results:

| | |
|---|---|
| *Salmonella typhosa* | 65 mm. per hr. |
| *Bacillus megatherium* | 27 mm. per hr. |
| *B. alvei* (colonies) | 14 mm. per hr. |

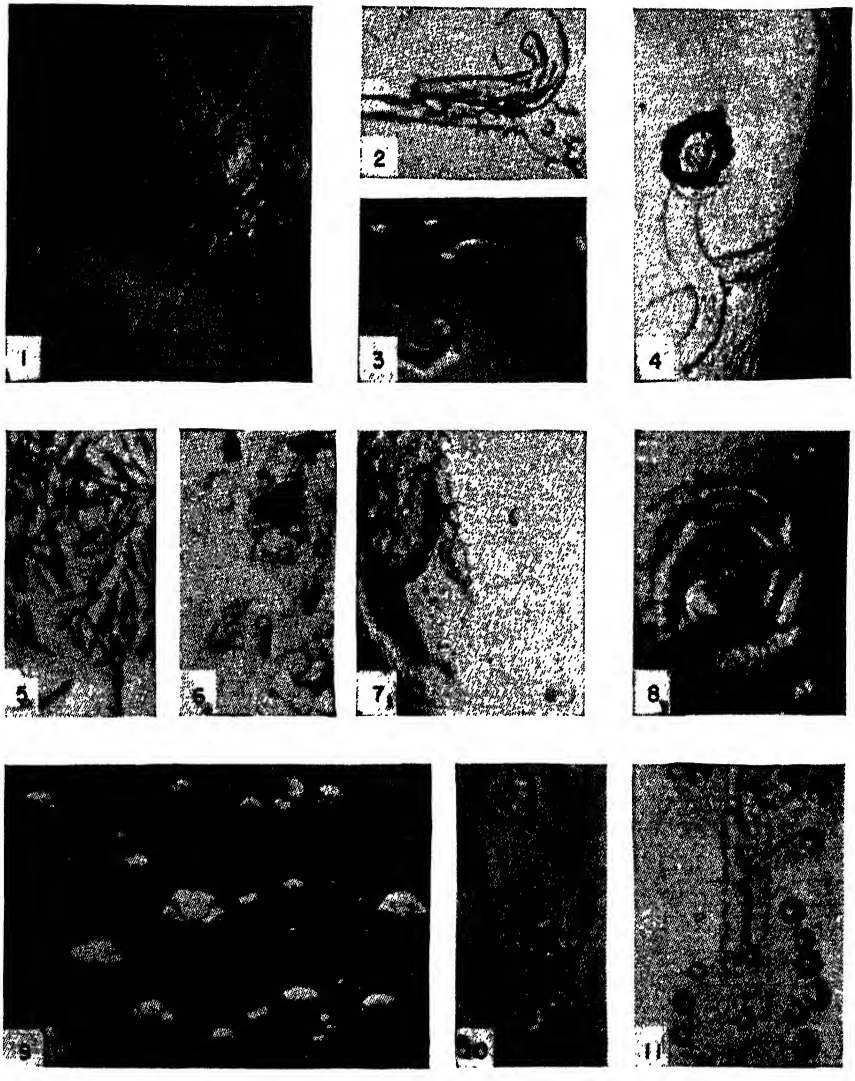



FIG. 37.—Rotating and migrating colonies of an aerobic, sporulating bacillus. 1, Smear, 24-hr. agar culture, ×1360; 2, same, showing flagella; 3, 24-hr. agar culture showing intersecting tracks somewhat grown up and colonies that have developed after ceasing to migrate, ×10; 4, 48-hr. agar culture showing two intersecting tracks, ×3; 5, 24-hr. agar culture showing track at end of which is a developed colony wider than the track, ×10; 6, 24-hr. agar culture showing effect produced when a large colony migrates along a circular or spiral path of small radius, ×10; 7, 72-hr. anaerobic culture showing migration of rotating colonies along short straight paths, ×10; 8, 48-hr. culture showing track of a wandering colony that has apparently rested temporarily after a slightly curved path, later began a spiral path, eventually resting and increasing in size, ×10; 9, 24-hr. culture showing a migrating colony, ×10; 10, 48-hr. culture showing a migrating colony, ×10; 11, 24-hr. culture showing a migrating colony moving through the swarm film, ×10. (*After Turner and Eales.*)

The colonies exhibited not only linear motion but also a slow rotary movement. The direction of rotation of 200 to 300 colonies observed was counterclockwise, with the exception of two colonies that moved clockwise.

FIG. 38.—Sketch of convoluted track of a wandering colony, showing two series of clockwise spirals followed by a final counterclockwise spiral. The colony had increased considerably in size after coming to rest and showed curved radial markings indicating rotation. The total length of the track was about 2 cm. (*After Turner and Eales.*)

Turner and Eales (1941*a*, *b*) described the rotational and migrational motilities of a number of aerobic and anaerobic species. Rotation of an aerobe was found to occur very early during growth (Fig. 37). The cells segregated in small groups and aligned themselves concentrically around a common center to form disk-like plaques one or a few cells thick. The rate of rotation was greater in smaller groups. As multiplication continued, successive layers were gradually built up in terrace fashion and the colony grew in height.

The colonies then began to migrate. When a colony migrated, it

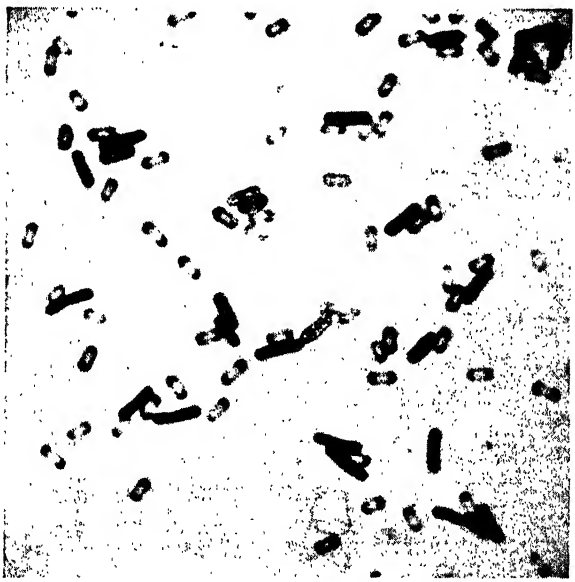


FIG. 39.—Spores and bacilli of *Bacillus subtilis*. Culture 24 hr. old.

left a peculiar "track" on the surface of the agar. A small number of cells were left behind, mostly at the edges of the track which formed two parallel lines separated by the width of the moving colony.

Typical migrating colonies, especially the small active type, pursued

curved or spiral paths which were often very elaborate and of relatively great length, even 2 or 3 cm. (Fig. 38). The direction of rotation was either clockwise or counterclockwise. After wandering for a variable distance, a colony approached the center of its spiral path with rapidly shortening radius, ceased to migrate, began to rotate around its center, lost its elongated shape, and increased in size to several times the width of the track at the end of which it was formed.

It is a difficult matter to explain the motility of entire colonies of bacteria. It is true that the individual cells are motile, but that does not explain the unified action of the cells so that the colony moves as a whole rather than spreading in all directions, as is the case with other highly motile species of bacteria.

**Spores of Bacteria.**—Spores are resistant bodies produced within the cells of a considerable number of bacterial species. They are more resistant to unfavorable environmental conditions, such as heat, cold, desiccation, osmosis, and chemicals, than the vegetative cells producing them. Spores are generally believed to be produced when the environment becomes too unfavorable for the existence of the vegetative forms. As far as is known, all spores produced by the true bacteria are nonmotile.

Apparently the first sign preceding the appearance of a spore is the cessation of reproduction. The cytoplasm of the cell becomes vacuolated and filled with granules. The granules gradually enlarge and increase in refractility. As the spore develops, refractility increases. As refractility increases, permeability of the spore membrane to dyes decreases. The membrane of a fully developed spore is only faintly stained by the usual methods of staining (Fig. 39).

Klieneberger-Nobel (1945) studied the changes in the nuclear structure of bacteria during spore formation by means of the Feulgen technique. The nuclear changes in the spore-bearing organisms, *Bacillus mycoides*, *Clostridium perfringens*, *C. septicum*, and *C. oedematiens* var. *gigas*, are drawn in Fig. 40. The dumbbell bodies which are dispersed in young cells (1) become aligned in the long axis of the cell (2), where they eventually fuse into an axial nuclear cylinder (3, 4). These cells break up into fusion cells of approximately the same length (5). The development of the chromosome stage (1) into the fusion cell (5) is the first step in the process of sporulation. During its further development, the fusion cell or spore mother cell divides twice (6, 7), with the result that it is segregated into four structures which often assume a dumbbell shape. Therefore, the chromatin cylinder of the individual spore mother cell seems to be equivalent to four nuclear elements, one of which functions as the spore chromosome or nucleus, whereas the other three disintegrate (8, 9).

As a general rule, each species of spore-bearing bacillus has its own characteristic size, shape, and position for the spore, but this is subject

to considerable variation under different environmental conditions. Bayne-Jones and Petrilli (1933) and others have given figures to indicate the degree of variation of spore size in *Bacillus megatherium*. It has already been shown that the vegetative cells producing the spores may also show considerable variation in size. The shape of spores of *B. megatherium* is usually described as being ovoid with squared ends, but it has been shown that spherical and bean-shaped forms may also be present in the same culture. The position of the spore in the cell may be central, subterminal, or terminal, but here again variation may be shown to exist in a culture of the same organism. Each cell usually produces only one spore; occasionally two spores may be present. It may be concluded that although the size, shape, position, and number of spores in a cell are usually quite constant, variations do exist under changing environmental conditions.

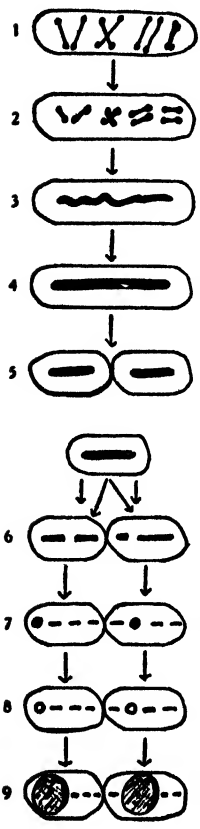



FIG. 40.—An outline of the changes in the nuclear structure of *Bacillus mycoides*, *Clostridium perfringens*, *C. septicum*, and *C. oedematiens* var. *gigas*, during spore formation. See text for legend. (*After Klieneberger-Nobel.*)

A fully grown spore may possess a diameter greater than that of the vegetative cell. The resulting forms are known as clostridium, if central, and plectridium, if terminal. Such spores are common among the anaerobic bacteria. As the spore becomes fully ripened, the sporangium gradually degenerates and finally disappears, leaving the spore in a naked state.

Very little is known concerning the composition of spores. It is generally stated that the great resistance exhibited by them is due to their low water content. It is known that the temperature required to coagulate protoplasm increases as the water content decreases. Henry and Friedman (1937) and Friedman and Henry (1938) showed that spores have about the same water content as the vegetative cells producing them. However, the spores were found to have a far greater water-binding capacity than the vegetative cells. They advanced the theory that the greater heat resistance of spores was due, in part at least, to the relatively high percentage of water in the bound state. Spores contain a high lipoid content, which is probably largely responsible for their increased impermeability to dyes and to other substances.

Roberts and Baldwin (1942) reported that *Bacillus subtilis* sporulated more profusely in peptone broth when a small quantity of activated charcoal was added to the medium. The percentage of cells in the spore state

was independent of the concentration of the treated peptone solution. They suggested that spore formation in peptone water was inversely proportional either to some particular food factor or factors, or to some factor directly inhibitory to the spore-forming process. The percentage of sporulating cells could be increased by adsorption of the medium with kaolin, ferric hydroxide, or aluminum hydroxide. The most effective adsorption by charcoal occurred in the pH range of 3 to 5.

Knaysi (1945*a*, *b*) made a study of a number of environmental factors that control spore formation in a strain of *B. mycoides* and reported as follows:

1. The accumulation of the acid by-products of glucose metabolism tended to inhibit sporulation. Oxygen might be necessary for the formation of spores, but its principal effect was to increase the rate of metabolism and the degree to which nutrients were utilized. Since the end products were more highly oxidized, there was a decreased accumulation of by-products accompanied by an increase in sporulation.

2. Although spores were formed within a relatively wide range of pH, the optimum was found to be between 6.6 to 6.8.

3. Vitamin $B_1$ stimulated sporulation on glucose agar slants but had no effect on nutrient agar without added sugar. The effect of vitamin $B_1$ was to increase the rate of decarboxylation of the acid by-products.

4. Gradual drying did not affect sporulation unless it affected growth and availability of nutrients. In general, drying decreased sporulation.

5. Suspending vegetative cells in distilled water increased sporulation of the uninjured cells.

6. Knaysi concluded that spores of this strain of *B. mycoides* were formed most readily by healthy cells faced with starvation in the presence of oxygen.

When a spore is removed from an unfavorable environment and placed in a suitable medium, germination occurs. Spores germinate in a variety of ways. There is a considerable degree of constancy in the method of spore germination for each species. Lamanna (1940) classified the modes of germination as follows:

I. Spore germination by shedding of spore coat. Characteristics of this method are
   A. Spore does not expand greatly in volume previous to the germ cell breaking through the spore coat. The limit of volume increase of the spore may be considered to be twice its original volume.
   B. Spore coat does not lose all of its refractive property previous to germination.
   C. After the second division of the germ cell, giving a chain of three organisms, the original spore coat, remaining attached to the cells, is visible for a long time after germination.

1. Equatorial germination (Figs. 41 and 42).
2. Polar germination (Fig. 43).
3. Comma-shaped expansion (Fig. 44).

II. Spore germination by absorption of the spore coat. Characteristics of this method are

A. The spore expands greatly during germination. A tripling or greater increase of the original volume occurs (Fig. 45).

B. The spore loses its characteristic refractiveness during germination, so that it is

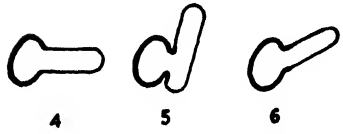



FIG. 41.—Equatorial germination without splitting along transverse axis. (*After Lamanna.*)

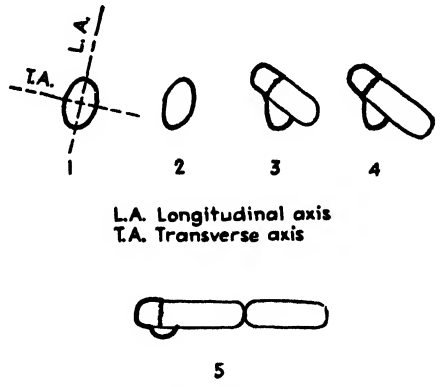



FIG. 42.—Equatorial germination with splitting along transverse axis. (*After Lamanna.*)

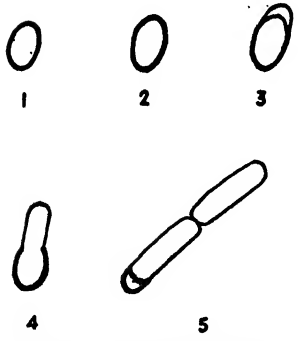



FIG. 43.—Polar germination. (*After Lamanna.*)

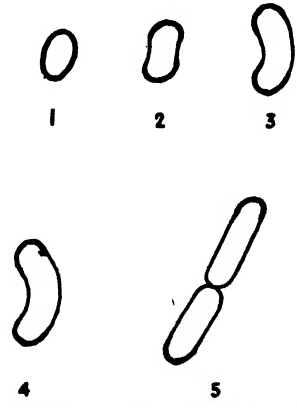



FIG. 44.—Spore germination by comma-shaped expansion. (*After Lamanna.*)

difficult to say when the spore has disappeared and the germ cell appeared.

C. After the second division of the germ cell, even if a thin capsule originally remains, all traces of the spore coat are gone.

Some strains germinating by absorption regularly show a thin capsule remaining about one end of the growing cell. This would appear as a polar germination

(Fig. 45, 7). In other cases, equatorial capsules are seen (Fig. 45, 8). Yet, in all instances, the spore is considered to germinate by absorption inasmuch as the three characteristics of the method are still adhered to.

Spore production is limited almost entirely to the rod-shaped organisms. None of the common spherical bacteria sporulate. Some spore-bearing species can be made to lose their ability to produce spores (asporogenous). When the ability of a species to produce spores is once lost, it is seldom regained.

Sporulation is not a process to increase bacterial numbers, because a cell seldom produces more than one spore. Since spores are very resistant to adverse environmental conditions, they are a means of keeping a species alive, even for many years.

For more information, see Breed, Murray, and Hitchens (1944), Cohen (1942), Dubos (1945), Gulick (1941), Knaysi (1944, 1946*c*), Knaysi, Baker and Hillier (1947), Lewis (1941), Mudd, Heinmets, and Anderson (1943*a*, *b*).

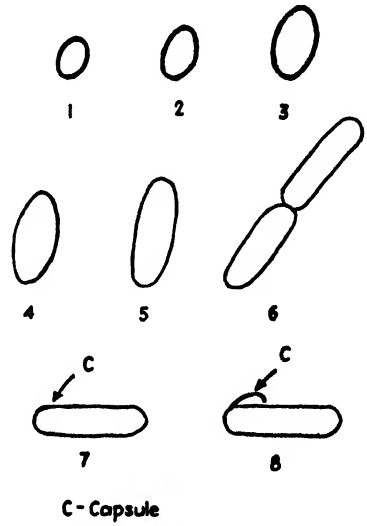



FIG. 45.—Spore germination by absorption. (*After Lamanna.*)

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# CHAPTER V

## YEASTS

The yeasts are spherical, ovoid, or rod-shaped ascomycetous fungi, in which the usual and dominant growth form is unicellular (Fig. 46). They appear as a surface froth or as a thick sediment in fruit juices, malt worts, and other saccharine liquids.

Under certain conditions, the yeasts may reproduce by elongation of their cells, forming structures having the appearance of a mycelium, but

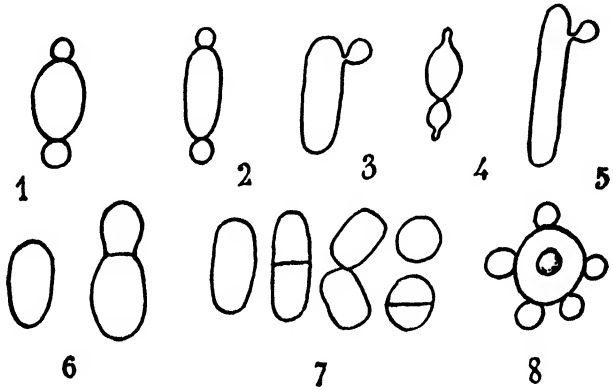



FIG. 46.—Various types of yeast cells. 1, *Saccharomyces cerevisiae;* 2, *S. ellipsoideus;* 3, *S. pastorianus;* 4, *S. apicule;* 5, *Mycoderma;* 6, *Saccharomycodes;* 7, *Schizosaccharomyces;* 8, *Torula.* (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

these never show the picture of a true fungus (Fig. 60*B*). In this respect, yeasts differ from molds which develop mycelia. On the other hand, many molds may grow temporarily as single cells and take on the appearance of yeasts. Many of the mucors grow as single cells, capable of budding under reduced oxygen tensions. The organisms causing sporotrichosis, coccidioidal granuloma, and blastomycosis appear as single cells in the tissues of the host, whereas mycelia appear on artificial culture media. Members of the genus *Monilia* grow as single cells on artificial culture media, but mycelia develop under reduced oxygen tensions (partial anaerobic conditions).

The fact that molds display dimorphism has led some investigators to believe that the yeasts were at one time mold-like but have permanently lost the ability to produce a mycelium. Fuchs (1926) grew the mold

*Aspergillus oryzae* under a reduced oxygen tension and noted that yeast-like cells developed from the conidia that were submerged in the medium. A slight fermentation occurred, which resulted in the formation of ethyl alcohol. The cells failed to revert to the mycelial stage but grew permanently as yeasts, even under aerobic conditions.

## CYTOLOGY OF YEASTS

Examination of yeasts under the microscope reveals the presence of a cell wall, a relatively large nucleus with which is associated one or more vacuoles, and various granules.

**Cell Wall.**—The individual cell is bounded by a delicate membrane, which is relatively thin in young cells and becomes thicker in more mature ones. The cell wall is composed of a carbohydrate known as yeast cellulose, although different from the cellulose found in the cell walls of higher plants.

Special staining methods reveal the presence of a cytoplasmic membrane, which is surrounded by the cell wall. This membrane corresponds in function to that found in bacterial cells (see page 64). It is believed to function as a selectively permeable membrane in determining which substances may enter and leave the cell.

**Nucleus.**—Yeast cells differ from the true bacteria in being usually much larger and possessing well-defined nucleuses. The nucleus is usually round, sometimes kidney-shaped owing to compression by a neighboring vacuole, and is situated near the center of the cell. It is difficult to see in hanging-drop preparations but may be seen in stained smears. Some believe that the nucleus possesses the power of ameboid movement through the cytoplasm. This has been observed in connection with the process of budding. After the bud has started to form, the nucleus wanders to that side of the cell and begins to elongate in the form of a dumbbell with one end passing into the bud. Finally, the portion in the bud pinches off from the parent nucleus to form two nucleuses, one for the mother cell and one for the bud. There is evidence to support the statement that in budding, as well as in spore formation, the nucleus divides by mitosis as in higher organisms.

It is well established that the nucleus of yeasts and of higher organisms consists largely of nucleoprotein. A nucleoprotein is a high-molecular-weight compound consisting of nucleic acid in combination with a protein. Since nucleic acid is acid in character, it is combined with a basic protein, probably a histone or a protamine. The protamines are proteins that are formed from a few amino acids only, and these are mainly the basic amino acids arginine, lysine, and histidine. The histones are similar to the protamines but contain a lower concentration of the basic amino acids. On hydrolysis, the nucleic acid portion of the molecule yields four molecules

of phosphoric acid, four molecules of a sugar, two molecules of purine bases, and two molecules of pyrimidine bases (see page 586). It is fairly well established that the nucleus of both plant and animal cells contains the same type of nucleic acid, namely, desoxyribonucleic acid as distinguished from another type present in metachromatic granules known as ribonucleic acid.

**Vacuoles.**—Vacuoles are cavities in the protoplasm, which are especially characteristic of plant cells and protozoa but occur also in the cells of higher animals. In plant cells, the vacuoles contain a fluid known as the cell sap, which is commonly an aqueous solution of various organic acids, and their salts. In protozoa, they may contain secretions of the protoplasm or substances about to be excreted, or food in various stages of digestion and assimilation.

**Granules.**—The cell contents of yeasts are more clearly differentiated than those of bacteria. Young cells have a very thin cell wall and a relatively homogeneous cytoplasm. As the cells become older, granules and vacuoles appear. The granules present in yeast cells consist of metachromatic granules or volutin, glycogen, and fat.

*Metachromatic Granules.*—Metachromatic granules or volutin constitute the most important elements found in yeasts. These granules are located almost exclusively in vacuoles present in yeast cells. They are also present in the cytoplasm that surrounds the vacuoles. The granules probably originate in the cytoplasm and localize in the vacuoles later. In old cells, the metachromatic granules may appear in relatively large masses. The granules appear as refractive bodies in unstained preparations and show a great affinity for methylene blue and other basic dyes, indicating that they are acid in character.

Metachromatic granules are composed of nucleic acid, either free or combined with basic proteins or organic bases. It is fairly well established that the granules contain ribonucleic acid as distinguished from desoxyribonucleic acid of the nucleus (see page 586). Metachromatic granules are believed to function as reserve food material for the cell. These granules are present in greatest amount in old cells. In young cultures, the cells are so active that very little volutin is able to accumulate. As the cells become older and less active, the granules appear. When old cells containing a large number of volutin granules are transferred to a new medium, the granules disappear, only to reappear again as the cells become old.

*Glycogen.*—Glycogen is a white, amorphous carbohydrate, related to starch and dextrin. It is hydrolyzed to glucose on boiling with dilute mineral acids, and also by the action of amylolytic enzymes. Glycogen is easily recognized by the brown color produced on the addition of a dilute solution of iodine. The color disappears when the solution is heated to 60°C. and reappears when it cools.

Glycogen has been shown to be abundant in well-nourished yeast cells and disappears during starvation. The concentration increases with age and reaches a maximum after 48 hr. It diminishes gradually and finally disappears completely toward the end of fermentation. Glycogen is usually localized in the vacuoles distinct from those which contain the metachromatic granules. It accumulates in the asci during sporulation and is absorbed by the spores during their maturity.

*Fat.*—Fat globules of variable size are distributed throughout the cytoplasm of yeast cells. They are stained brown with osmic acid. Fat globules are prominant in yeast cells, especially during sporulation, and serve as food for the ascospores. They are considered to be reserve food materials.

For more information on carbohydrate and fat reserves in yeasts, see Lindegren (1945*a*).

## MULTIPLICATION IN YEASTS

Yeasts multiply by budding, by fission, by asexual spore formation, and by sexual reproduction. However, the usual type of multiplication observed is by budding. Sometimes, chlamydospores are produced but, since only one spore is produced in a cell, they are not a means of increasing the numbers of yeast cells but a method of perpetuating the species.

**Budding.**—In almost all the yeasts, division of the cell occurs by budding. Budding occurs after the organism acquires a certain definite size. The bud appears as a small prominence separated from the wall of the mother cell by a very narrow collar (Fig. 46), and gradually enlarges until it finally separates from the mother cell. As a rule, it never reaches the same size as the mother cell before separation. During periods of rapid division, buds may be formed at different points on the surface of the cell. The daughter cells likewise bud at different points before separation from the mother cell occurs. This results in the formation of a small colony or of a chain of yeast cells.

For information on the mechanism of budding in yeasts, see Lindegren (1945*b*).

**Transverse Fission.**—There are a few species in which multiplication occurs by transverse fission. These yeasts resemble the bacteria in their mode of division.

In *Schizosaccharomyces octosporus*, the spherical or ovoid-shaped cells elongate to a certain size and then form cross walls in the middle. The two cells pull apart, and the ends become rounded. When the two new cells reach maturity, they elongate and repeat the cycle. During periods of rapid multiplication, cells divide without separating. Transverse partitions also form in the new cells. In this manner, a chain of cells is produced, resembling a mycelium, which eventually breaks apart (Fig. 47).

In the genus *Saccharomyces*, a form of division intermediate between budding and fission occurs. Buds are generally produced at the extremities of the cells. The cells first elongate, then a tube puffs out at one end. This enlarges and is slowly transformed into a bud, which remains at-

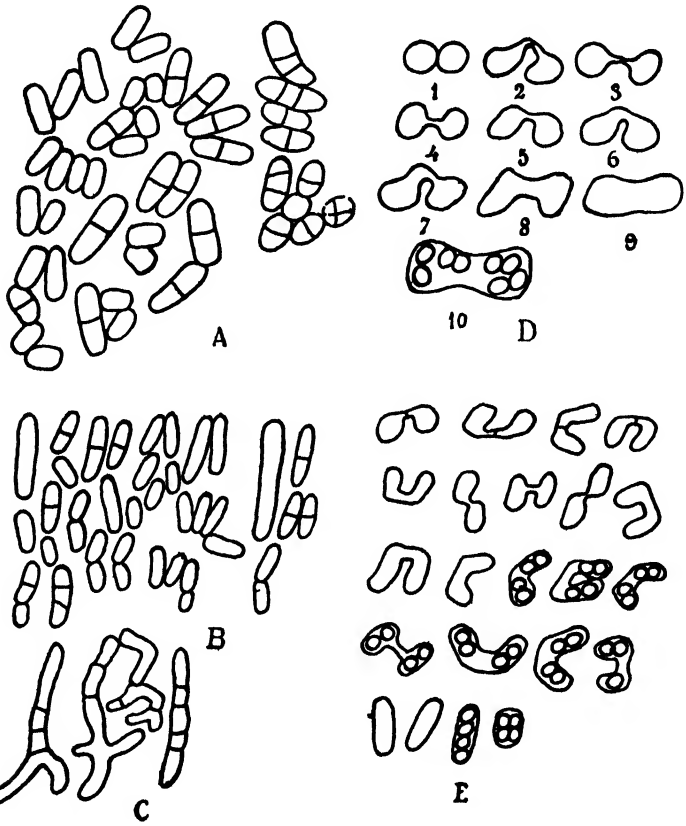



FIG. 47.—*Schizosaccharomyces*. *A*, *B*, multiplication by transverse fission; *C*, mycelial growth; *D*, *E*, formation of asci by isogamic copulation. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

tached to the cell by a collar. Finally, a wall is formed, which separates the cell from the bud.

**Asexual Spore Formation.**—Although budding is the usual process of multiplication in yeasts, such a method does not perpetuate the species. The usual process for perpetuating the species is by spore formation, which is a form of resistance that permits yeast cells to remain viable after budding has stopped. Sporulation may be observed in old cultures where the environmental conditions have become unfavorable for the growth of vegetative cells. Spores are easily produced by inoculating

yeasts into a variety of special culture media. When the environment again becomes favorable, the spores germinate into vegetative cells (Fig. 48).

A definite number of spores is usually produced in the cells of each species. The cells bearing spores are called "asci" (singular, ascus) and the spores are known as "ascospores." In the majority of species, the spores are formed by a division of the nucleus twice, giving rise to four-

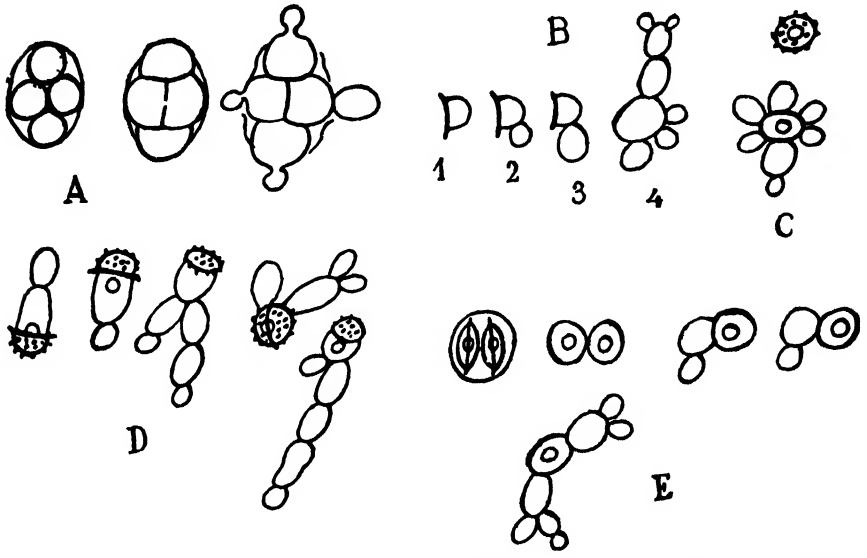



FIG. 48.—Germination of ascospores. *A, Saccharomyces cerevisiae; B, Hansenula anomala; C, Debaryomyces; D, Schwanniomyces; E, Hansenula saturnus. (From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.)*

spore nucleuses. The nucleuses become surrounded by some cytoplasm around which are formed the spore walls. In other species the number of spores in a cell may be 1, 2, more rarely 8 and 16.

The spores assume various shapes, and some species may be easily recognized by this character. Yeast spores are usually spherical or ovoid (Fig. 49). Such spores are found in *Saccharomyces cerevisiae*, the common bread or beer yeast, and in other less known species. The spores in *Hansenula anomala* and in the genus *Hanseniaspora* are hemispherical, and their adjacent surfaces are provided with a projecting border giving them the appearance of a hat (Fig. 49*B*). In the species *Pichia membranaefaciens*, the spores are irregularly shaped into ovoid, elongated, triangular, kidney-shaped, or hemispherical forms (Fig. 49*C*). Cells of *Hansenula saturnus* produce spores that are lemon-shaped and are surrounded by a projecting ring (Fig. 49*D*). In the species *Schwanniomyces occidentalis*, the spores are surrounded by a projecting ring and the membrane is covered

with stiff, erect protuberances (Fig. 49 *E*). The spores of *Debaryomyces* are globular and are also covered with protuberances (Fig. 49 *F*). Other shapes are illustrated in Fig. 49 *G*, *H*, *I*.

**Sexual Reproduction or Copulation.**—Sexual multiplication occurs by (1) isogamic copulation, (2) heterogamic copulation, (3) a form of copulation intermediate between isogamy and heterogamy, and (4) copulation of ascospores.


FIG. 49.—Various types of ascospores. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

*Isogamic Copulation.*—Isogamic copulation may be defined as the fusion of two similar gametes. Guilliermond (1920) showed that the asci of *Schizosaccharomyces octosporus* result from the fusion of two cells.

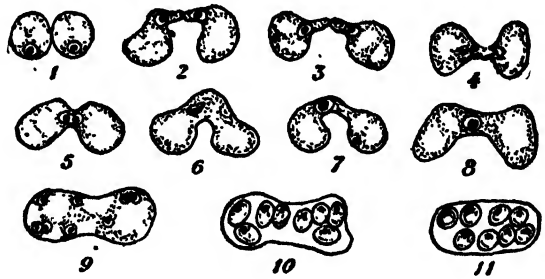



FIG. 50.—*Schizosaccharomyces octosporus.* Isogamic copulation of two cells to form an ascus containing eight ascospores. (*Reprinted from Guilliermond-Tanner, The Yeasts, John Wiley & Sons, Inc.*)

When the cells cease to multiply, copulation occurs. Two identical cells lying adjacent in a colony are joined by a copulation canal (Fig. 50). The two cells are now known as gametes. The wall that separates the two cells quickly disappears, and the nucleus of each passes through the copulation canal. This results in the formation of a single cell or zygospore. Since the gametes are similar, the zygospore is formed by isogamic copulation. The zygospore increases in size, followed by a division of the nucleus into four or eight separate nucleuses. The nucleuses become surrounded

with cytoplasm around which are formed the spore walls. The zygospore now becomes an ascus.

In *S. pombe*, copulation occurs as in *S. octosporus*, except that fusion

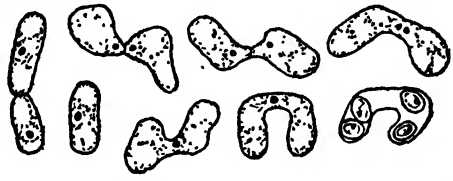


FIG. 51.—*Schizosaccharomyces pombe.* Copulation and incomplete fusion of two cells to form an ascus containing four ascospores. (*Reprinted from Guilliermond-Tanner, The Yeasts, John Wiley & Sons, Inc.*)

remains usually incomplete. Copulation takes place between two adjacent cells in the same colony. The gametes are joined by means of a canal through which nuclear and protoplasmic fusion occurs. The nucleus result-

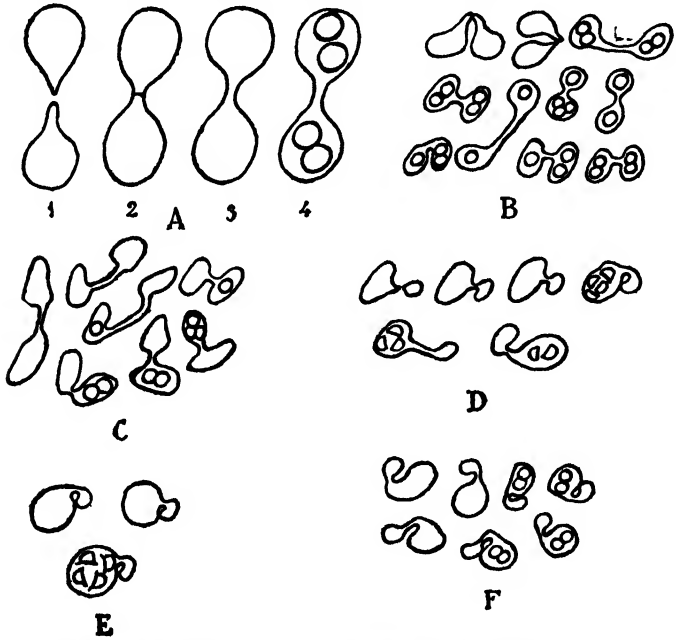



FIG. 52.—*Zygosaccharomyces. A, B,* ascus produced by isogamic copulation; *D, E, F,* ascus produced by heterogamic copulation; *C,* ascus produced by copulation intermediate between isogamic *and* heterogamic. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

ing from the fusion quickly divides, and the two nucleuses migrate to both enlargements of the zygospore. The nucleuses undergo a second division resulting in the formation of four spores. The zygospore becomes an ascus, and the spores are known as ascospores (Fig. 51).

*Heterogamic Copulation.*—Heterogamic copulation may be defined as the fusion of two unlike gametes. Sometimes it is possible to see a fusion between a cell and an undeveloped bud on the latter. This may be observed in the species *Zygosaccharomyces priorianus*. The ascus that develops is composed of two unequal enlargements, the larger representing the mother cell and the other the bud. Because of lack of space in the bud, the spores develop in the mother cell. This is known as heterogamic copulation (Fig. 52 *D*, *E*, *F*).

In the genus *Nadsonia*, copulation occurs by heterogamy between an adult cell and one of its buds. After the two cells fuse, the contents of the

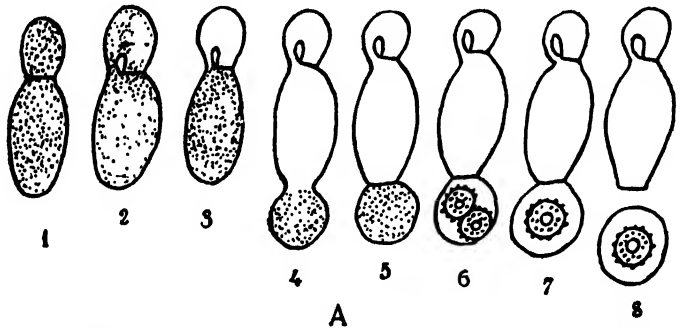



FIG. 53.—*Nadsonia.* Formation of an ascus by heterogamic copulation. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

male gamete or bud pass into the female gamete or mother cell. A new cell then forms by budding, into which pass the contents of the mother cell. This new cell now becomes the ascus, and it usually contains only a single ascospore (Fig. 53).

*Intermediate Form of Copulation.*—A rarer form of copulation, intermediate between isogamy and heterogamy, has been observed. In these yeasts, the two cells or gametes are of the same dimensions and do not show any sexual differentiation. After fusion takes place, the contents of one cell pass into the other. The former may be regarded as the male cell and the latter as the female cell. The ascospores originate from the female cell and are usually two in number (Fig. 52 *C*).

*Copulation of Ascospores.*—In some species such as in *Saccharomycodes ludwigii*, *S. johannisbergensis*, and *Hansenula saturnus* an isogamic copulation occurs between the ascospores originating from an ascus. This is shown in Fig. 54. In the species *S. ludwigii*, an ascus contains usually four spores. The ascospores copulate, two by two, by means of a copulation canal. This is a true copulation and is accompanied by a fusion of the nuclear material. The fusion remains incomplete, and a zygospore is formed from each pair of ascospores united by a copulation canal. The

fusion of the two spores results in the formation of a zygospore. It elongates into a germination tube from which develop numerous vegetative cells.

Copulation occurs normally between two spores in the same ascus. It has also been observed between ascospores from different asci and even those more distantly related. This may be observed in old asci in which many of the spores are dead. Some of the spores can germinate alone; others must fuse in pairs before this occurs. This means that some of

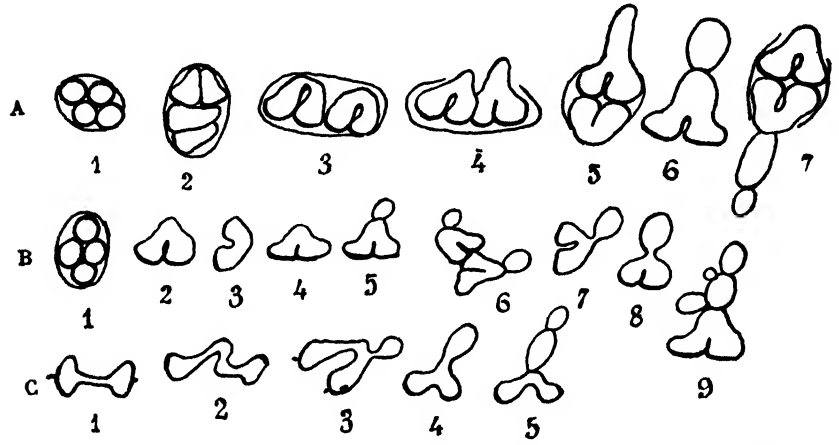



FIG. 54.—Conjugation of ascospores. *A, Saccharomycodes ludwigii; B, Saccharomyces johannisbergensis; C, Hansenula saturnus. (From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.)*

those in the latter group are forced to fuse with spores from different asci. The fusion of ascospores is not regarded as a true copulation but as a new process that takes the place of normal sexual fertilization.

**Haploid and Diploid Yeasts.**—Haploid yeasts have a single set of genes, whereas the diploid yeasts possess two complete sets of genes. The genes are factors concerned with the transmission and development or determination of hereditary characters. A gene makes up a small part of a chromosome.

Haploid yeasts are capable of fusing in pairs to produce diploid yeasts. According to Lindegren (1944)

> There are two mating types or "sexes" of haploid yeasts and one haploid cell of one "sex" can fuse with another haploid cell of the opposite "sex" to produce a diploid cell of the type known as a legitimate diploid or "heterozygous" cell. In heterozygous diploids the paired genes in one or more pairs are different from each other, as contrasted to "homozygous" diploids, in which both members of every pair of genes are identical. An "illegitimately" diploid, homozygous cell is formed when fusion occurs between two haploid cells of the same "sex." Most

bakers', brewers', and distillers' yeasts which we have examined are illegitimately diploid strains. They were probably produced from wild yeasts by the chance selection of single ascospore cultures homozygous for some desirable modifier of a gene affecting some chemical stage of the fermentation process. Their high stability impressed the manufacturer and led to their selection.

The sexual mechanism of yeasts suggests the possibility of producing many new varieties. For example, no natural yeast is capable of fermenting both lactose and maltose. Some can ferment one and some the other, but none can ferment both. It is conceivable that, if a haploid lactose-fermenter were mated with a haploid maltose-fermenter, the hybrid could ferment both.

*Saccharomyces cerevisiae* exists in both haploid and diploid forms. The ordinary vegetative cells are diploid but, under certain conditions, diploid cells become converted into asci containing four haploid ascospores. The ascospores germinate to produce four different haploid cultures which are generally easily distinguishable from the diploid cultures.

Lindegren and Lindegren (1943) showed that cultures of the haploid cells, when paired, will copulate to produce diploid cells, provided the haploid cells are of the opposite sexes. The haploid ascospores from the four-spored ascus were designated A, B, C, and D, and the haplophase cultures from them were paired in all possible combinations. They found that A and D belonged to the same mating type; B and C to the complementary type


A & B, A & C, D & B, D & C

Copulation tubes and zygotes were produced when A & B, A & C, D & B, and D & C were paired. When placed in a sporing medium, the diploid cells produced four-spored asci, whereas the A & D and B & C combinations failed to produce spores. Thousands of experiments have confirmed the fact that there are two mating types in *Saccharomyces cerevisiae*. Lindegren (1944) designated these different mating types as "$a$" and "$\alpha$." An interpretation of the mating phenomena is schematically represented in Fig. 55. The large diploid zygotes are readily recognized as *S. cerevisiae*, whereas different stabilized haploid cultures are indistinguishable from *Torula* or *Zygosaccharomyces*, depending upon their characteristic morphology.

To quote from Lindegren (1944),

> The legitimate diploids are heterozygous for the $a/\alpha$ genes, while the illegitimate diploids are homozygous, being either $aa$ or $\alpha\alpha$. Legitimately diploid vegetative cells sporulate to produce four haploid ascospores. These ascospores can be separated from each other and each can multiply to produce a culture of haploid cells. Haploid cells are capable of acting as gametes or sex cells. When two of

these gametes of opposite sex fuse, a legitimately diploid heterozygous vegetative cell is produced. These cells are generally quite vigorous. When conditions are favorable for sporulation, they produce four spores in each cell. When fusion occurs between two gametes of the same sex, derived from the same ascospore, an illegitimately diploid homozygous cell is formed. This type of cell is often as capable of growth and fermentation as the legitimate diploid, and some illegitimate diploids have some very desirable industrial characteristics. However, illegitimate

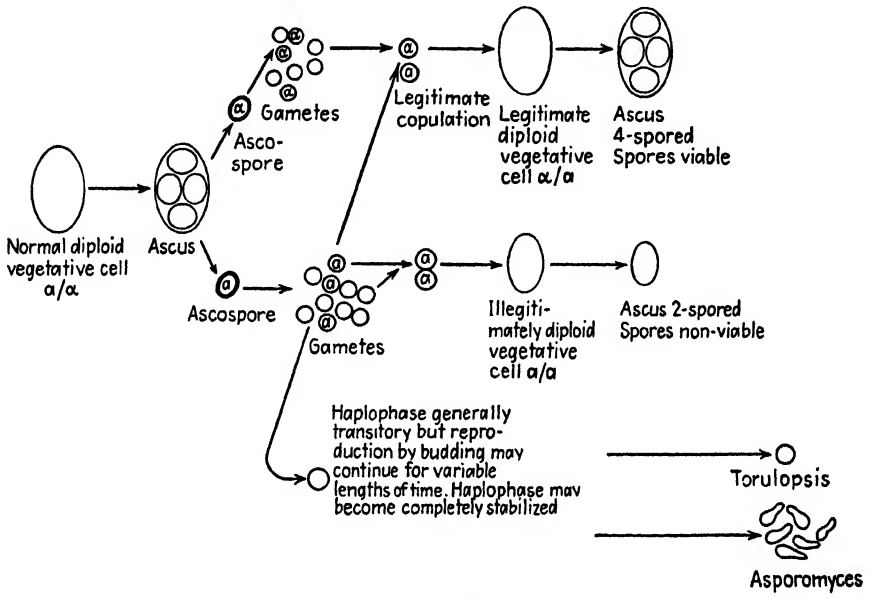



FIG. 55.—Life cycle of *Saccharomyces cerevisiae* showing the relationship with *Torula* and *Zygosaccharomyces*. The ascospores belong to two mating types, *a* and α, and the heterozygote is the legitimate diploid. The homozygotes are called illegitimate diploids. (*After Lindegren.*)

diploids are often smaller and usually do not produce viable ascospores, and the asci are generally two-spored.

In Fig. 55, the mating types of the haplophase cells are designated as "*a*" and "α," and cells of each specific mating type are indicated by letter. Diplophase cultures from nature are generally heterozygous for the *a*/α mating type genes and are marked "*a*/α" in the diagram. Diploid cultures, homozygous for *a* or α ("*aa* or αα") tend to be less vigorous in regard to growth rate, dry weight, yield, and ascospore viability than the *a*/α heterozygotes.

The superiority of the heterozygote and its normal occurrence in nature has led us to call it a legitimate diploid, while the homozygotes, with their poorly viable spores, have been called illegitimate diploids.

**Parthenogenesis.**—In the majority of yeasts, and especially in those of industrial importance, sexuality has not been observed. These yeasts

represent parthenogenetic forms derived from primitive sex cells. If the development of the ascus is not the result of copulation, it represents a gamete that has developed by parthenogenesis (Fig. 56). Parthenogenesis

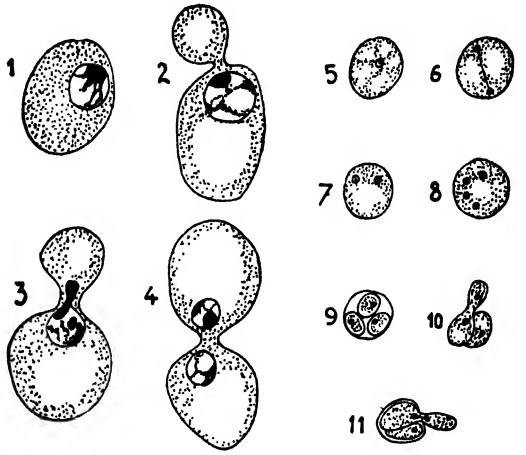



FIG. 56.—*Saccharomyces cerevisiae.* 1–4, formation of a bud with division of the nucleus. 5–11, formation of an ascus from the bud and germination of an ascospore. (*From Gäumann and Dodge, Comparative Morphology of Fungi.*)

may be defined as the development of an organism from an unfertilized cell.

In the *Schwanniomyces*, the cells forming the asci produce projections

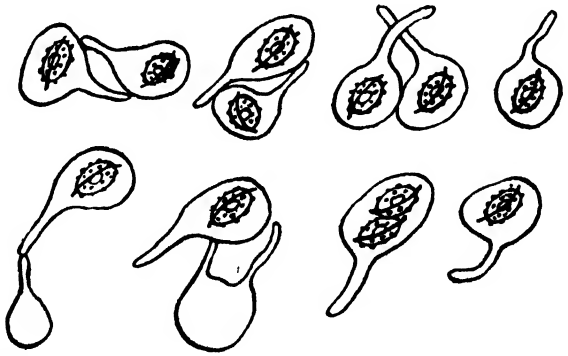


FIG. 57.—*Schwanniomyces.* Asci with ascospores. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

of different lengths, which attempt to fuse together as in true copulation. However, fusion fails to occur, and it appears that the cells have retained only a portion of their sexual characteristics. This same phenomenon has been observed in the *Torulaspora* and in other yeasts (Figs. 57 and 58).

**Chlamydospores.**—Under unfavorable conditions, yeast cells cease to

multiply. When this occurs, some organisms become filled with reserve food products, such as fat and glycogen granules, and enclose themselves in a thick wall. These cells are known as durable cells or chlamydospores. When conditions become favorable again, the chlamydospores germinate into vegetative cells, which in turn multiply by the usual method of bud-

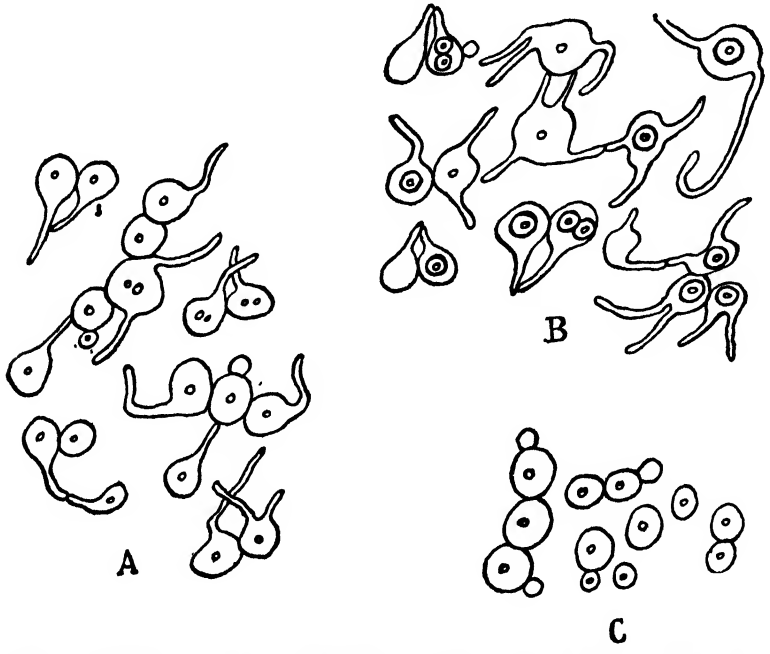



FIG. 58.—*Torulaspora.* (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

ding. Since only one chlamydospore is formed in a cell, it is not considered a method of reproduction but rather a method for perpetuating the species.

## CLASSIFICATION OF YEASTS

Yeasts have been classified with difficulty, and considerable confusion still exists. The classification given here is taken from the works of Stelling-Dekker (1931), Guilliermond (1928, 1937), and Henrici (1941).

### Ascosporogenous Yeasts

Family. *Endomycetaceae.* Growth forms mycelium, pseudomycelium, oidia, or yeast cells, together or singly. Multiplication by transverse fission or budding. Asci produced by isogamic or heterogamic copulation or by parthenogenesis. Ascospores spherical, hemispherical, angular, sickle- or spindle-shaped, smooth, warty, or with a projecting border.

Subfamily I. *Eremascoideae.* Growth form entirely mycelial. Multiplication by transverse fission. Spores hat-shaped and formed by isogamic copulation.

Subfamily II. *Endomycoideae.* Growth form either mycelium with oidia or only oidia. Multiplication by transverse fission.

Genus I. *Endomyces.* Growth form true mycelium with oidia. Asci contain four spherical or hat-shaped ascospores.

Genus II. *Schizosaccharomyces.* No mycelium but with oidia. Asci contain four or eight spherical ascospores.

Subfamily III. *Saccharomycoideae.* Growth form either mycelium with conidial buds and occasionally oidia, or only budding yeast cells. Multiplication by transverse fission or budding. Spores spherical, hemispherical, angular or sickle-shaped, or with a projecting border. Asci formed by isogamic or heterogamic copulation, or by parthenogenesis. Subfamily is divided into three tribes.

Tribe I. *Endomycopseae.* Growth form mycelium with conidial buds, occasionally

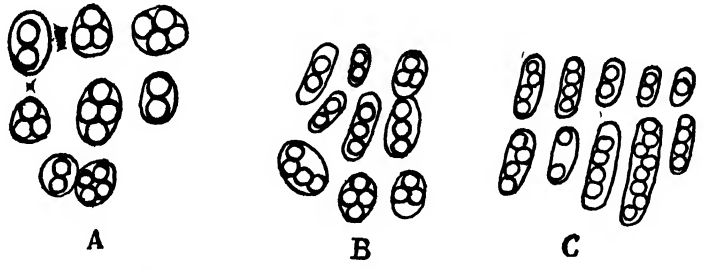



FIG. 59.—*Saccharomyces.* Asci and ascospores. *A*, *S. cerevisiae; B*, *S. ellipsoideus; C*, *S. pastorianus.* (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

oidia. Multiplication by transverse fission and by multipolar budding. Ascospores hat-shaped, sickle-shaped, or with a projecting border, and produced by isogamic copulation or by parthenogenesis.

Genus III. *Endomycopsis.* Characteristics same as for tribe.

Tribe II. *Saccharomyceteae.* Growth form yeast cells, no mycelium. Multiplication by multipolar budding. Ascospores produced by isogamic or heterogamic copulation, or by parthenogenesis. Spores variously shaped.

Genus IV. *Saccharomyces.* Cells spherical, ovoid, or elongated. Asci formed by parthenogenesis and contain one to four spherical, smooth ascospores. Spores germinate by budding (Figs. 56, 59).

Genus V. *Zygosaccharomyces.* Cells spherical, ovoid, or elongated. Asci formed by isogamic or heterogamic copulation or by a process intermediate between the two. Asci contain from one to four ascospores (Fig. 52).

Genus VI. *Pichia.* Cells ovoid or elongated. Asci formed by isogamic or heterogamic copulation, or by parthenogenesis. Asci contain from one to four spherical, hemispherical, or triangular spores (Fig. 60).

Genus VII. *Torulaspora.* Cells spherical, with a large fat globule in the center. Asci show a trace of copulation by development of projections, but union does not occur. Asci produced by parthenogenesis, and contain one or two spherical, smooth spores (Fig. 58).

Genus VIII. *Debaryomyces.* Cells spherical or ovoid. Asci formed by isogamic or heterogamic copulation and contain one or two, sometimes four, spores (Fig. 61).

Genus IX. *Hansenula.* Cells ovoid or elongated, occasionally spherical. Asci

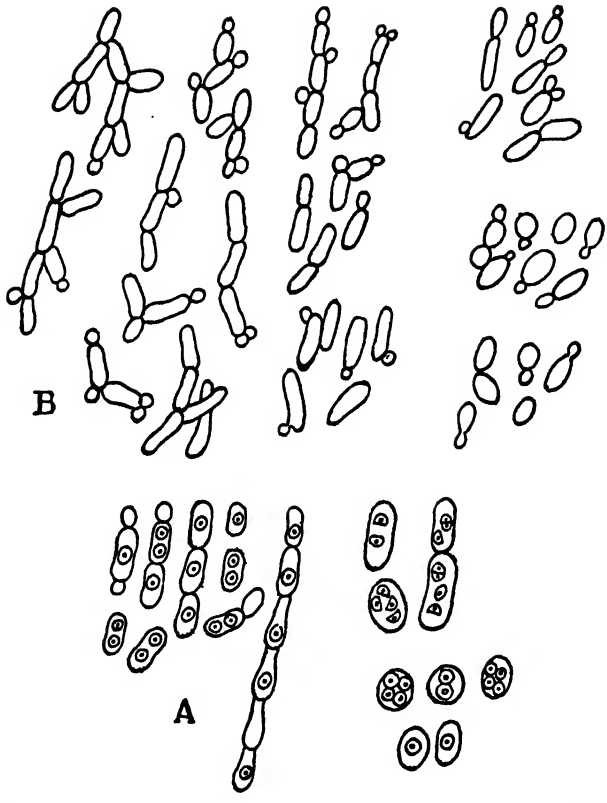



FIG. 60.—*Pichia*. *A*, asci and ascospores. *B*, budding cells. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

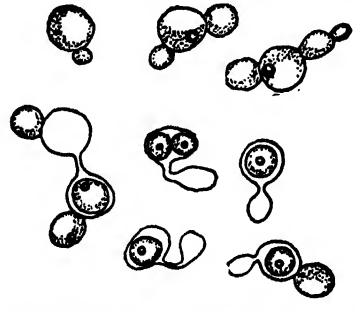


FIG. 61.—*Debaryomyces*. (*Reprinted from Guilliermond-Tanner, The Yeasts, John Wiley & Sons, Inc.*)

formed by parthenogenesis and contain from one to four lemon- or hat-shaped spores (Fig. 62).

Genus X. *Schwanniomyces*. Cells spherical or ovoid, occasionally show rudimentary mycelia. Asci formed by parthenogenesis and contain one or two

spores, provided with a projecting collar and with a verrucose wall (Fig. 57).

Tribe III. *Nadsonieae.* Growth form yeast cells, no mycelium. Multiplication

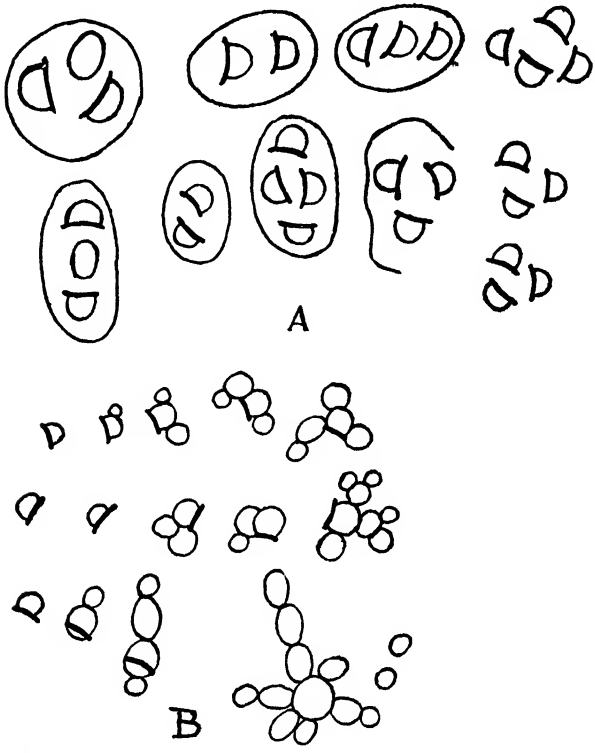



FIG. 62.—*Hansenula.* *A*, asci and ascospores; *B*, germination of ascospores by budding. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

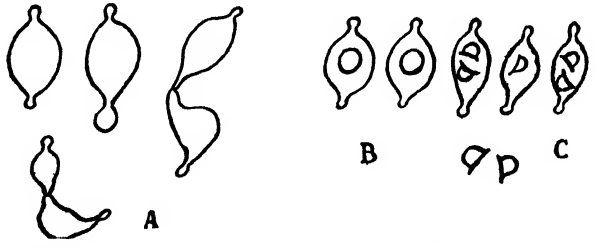



FIG. 63.—*Hanseniaspora.* *A*, cells; *B*, *C*, asci and ascospores. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

by bipolar budding. Ascospores produced by heterogamic copulation or by parthenogenesis.

Genus XI. *Saccharomycodes.* Cells large and show bipolar budding. Asci

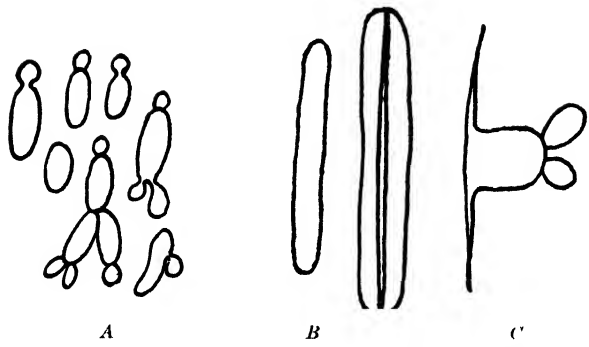



FIG. 64.—*Monosporella.* *A*, budding cells; *B*, ascus with one ascospore; *C*, germination of ascospore by budding. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

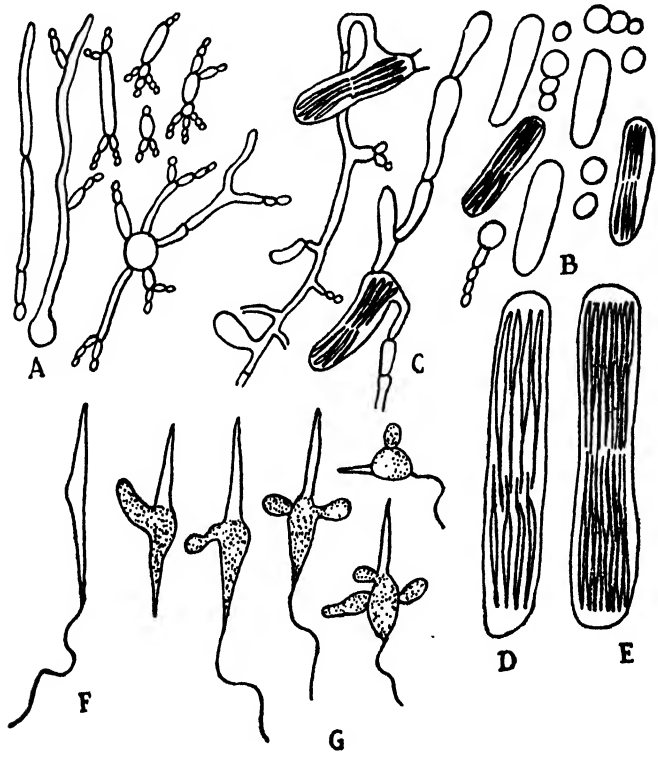



FIG. 65.—*Nematospora.* *A*, mycelial growth; *B*, *C*, formation of asci; *D*, *E*, asci filled with 8 or 16 ascospores. *F*, *G*, germination of ascospores. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

formed by heterogamic copulation and contain four spherical, smooth spores. Spores conjugate on germination (Fig. 54*A*).

Genus XII. *Hanseniaspora.* Cells lemon-shaped. Multiplication by budding. Asci produced by parthenogenesis and contain from one to four smooth, hat-shaped spores (Fig. 63).

Genus XIII. *Nadsonia.* Cells spherical, ovoid, ellipsoidal, or lemon-shaped. Asci produced by heterogamic copulation between a bud and the mother cell. One or two, sometimes up to four spherical, verrucose spores produced in each ascus (Fig. 53).

Subfamily IV. *Nematosporoideae.* Growth form mycelium and budding yeast cells. Multiplication by multipolar budding.

Genus XIV. *Monosporella.* Cells ovoid-shaped. Multiplication by budding.

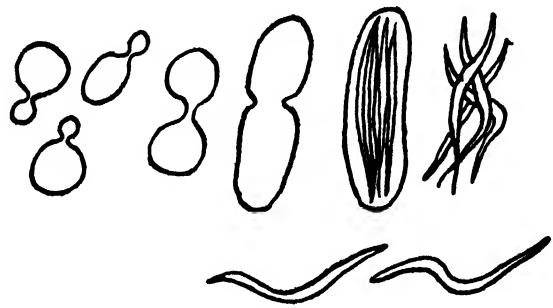


FIG. 66.—*Coccidiascus.* Ascus produced by isogamic copulation. Four ascospores in each ascus. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

Asci produced by parthenogenesis and contain one needle-shaped spore (Fig. 64).

Genus XV. *Nematospora.* Cells of variable form and multiply by budding. Asci produced by parthenogenesis and contain 8 or 16 flagellated, spindle-shaped spores (Fig. 65).

Genus XVI. *Coccidiascus.* Cells ovoid-shaped and multiply by budding. Asci produced by isogamic copulation and contain four spindle-shaped, non-flagellated spores (Fig. 66).

**Saccharomyces.**—Since this genus includes almost all the species of industrial importance, it is by far the most important group of yeasts. The cells are spherical, ovoid, or elongated. Asci are formed by parthenogenesis and contain from one to four spherical, smooth spores. At least 24 species are recognized.

The most important species is *S. cerevisiae*, the common yeast used by bakers, brewers, and distillers. It is the same species that is extensively employed therapeutically as a natural source of vitamins of the B complex.

### Asporogenous Yeasts

The term *Torulae* (singular, *Torula*) is generally applied to the species of yeasts that are not capable of producing spores. They are sometimes

called "false" or "wild" yeasts. Will (1916) placed all of the nonsporing yeasts in the family *Torulaceae*, and Lodder (1934) recognized two families and a provisional third family containing a single doubtful genus.

Family I. *Rhodotorulaceae.* Produce carotenoid pigments, colonies being yellow, orange, pink, or red. They are commonly found in air and are the frequent cause of contaminations in bacteriological work. All species are nonfermentative and of no practical importance. The family includes only one genus, *Rhodotorula.*

Family II. *Torulopsidaceae.* Do not produce carotinoid pigments. This family includes the remainder of the nonsporing yeasts. It is further divided into the two subfamilies, *Torulopsidoideae* and *Mycotoruloideae.*

Subfamily I. *Torulopsidoideae.* Do not produce pseudomycelium. The subfamily includes a majority of the wild yeasts common as contaminants in bacteriological procedures. It embraces seven genera, *Kloeckera*, *Trigonopsis*, *Pityrosporum*, *Asporomyces*, *Torulopsis*, *Mycoderma*, and *Schizoblastosporion.*

Subfamily II. *Mycotoruloideae.* Produce pseudomycelium, with buds at the nodes.

**Pityrosporum.**—A number of species of the genus *Pityrosporum* have been isolated from skin, but they all appear to be sufficiently alike to be considered identical with *P. ovale.* The organism is usually referred to as the bottle bacillus because of its resemblance to a bottle. Since the organism is present on skin, especially of the scalp, it is claimed by some to be the etiological agent of dandruff. However, there appears to be no evidence that it has anything to do with the formation of dandruff or any pathological condition of the skin or scalp.

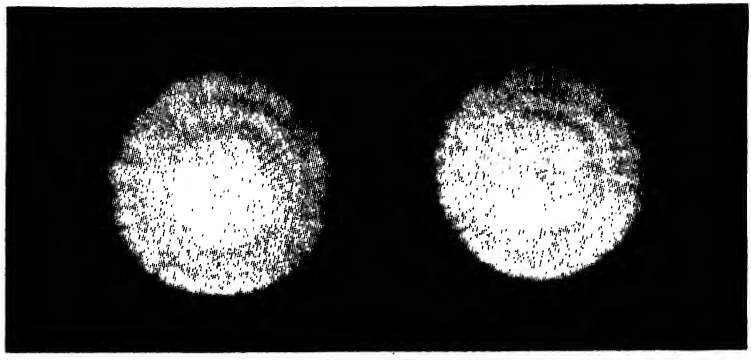
**Brettanomyces.**—This genus includes a number of species of industrial importance. They are concerned with the afterfermentation of certain English and Belgian beers and ales. The afterfermentation is a secondary fermentation taking place in bottles in which the residual oxygen is utilized and replaced by carbon dioxide. Fermentation under anaerobic conditions is very slow, requiring several months for the reaction to go to completion. Under aerobic conditions, the organisms oxidize alcohol to acetic acid.

The cells are ovoid, or globular, often elongated and pointed at both ends. Budding may occur on all parts of the cell, forming irregular clusters. They show a tendency to form a poorly developed pseudomycelium. Ascospores are not produced. The organisms grow very slowly on culture media.

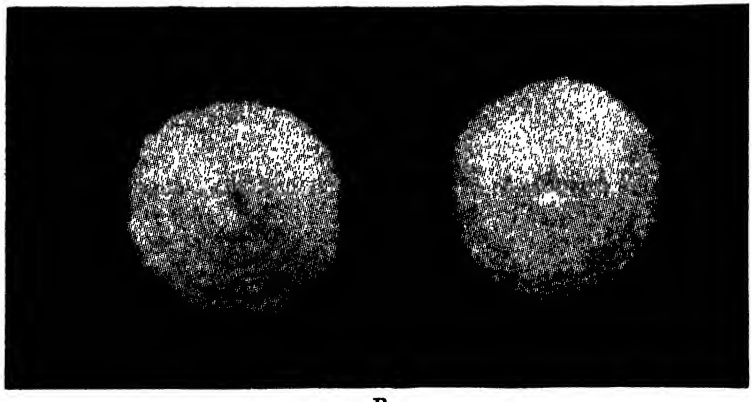
## HYBRIDIZATION

A hybrid may be defined as the development of a new organism from two cells of different species or different genetic make-up.

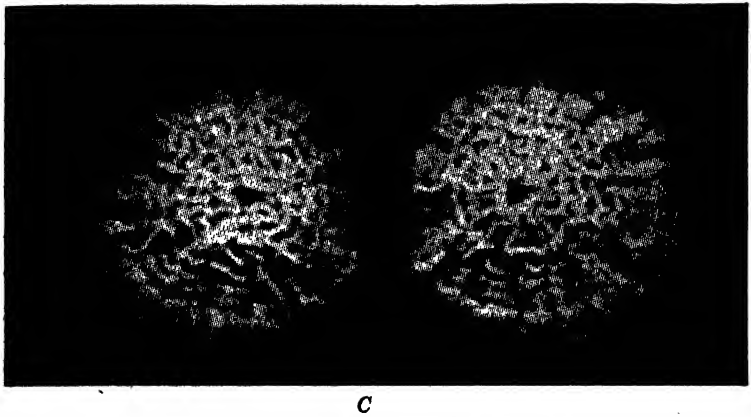
Winge and Laustsen (1938, 1939) showed that it is possible to breed new varieties of yeasts by hybridization (Fig. 67). They placed two spores from different species in a drop of culture solution, so as to enable them to copulate to form a zygospore from which the hybrid yeast germinated. The copulation was observed under the microscope. These



*A*



*B*



*C*

FIG. 67.—Hybridization of yeasts. *A*, *Saccharomyces cerevisiae*, Danish baking-yeast strain; *B*, *Saccharomyces cerevisiae*, strain II; *C*, hybrid produced by crossing the two strains, 30 days old. (*After Winge and Laustsen.*)

workers succeeded in producing 14 new yeast types from 8 different species and strains of *Saccharomyces* and one of *Zygosaccharomyces*.

They observed that the ability of a yeast to produce a certain enzyme was always dominant in the hybrid. This means that, if one of the species or strains hybridized elaborates a sucrase and the other does not, the hybrid will always possess the ability to produce a sucrase.

Most strains of *S. cerevisiae* do not synthesize biotin, vary from good to poor in the ability to synthesize pantothenic acid, but do manufacture pyridoxin in large quantities. Lindegren and Lindegren (1945) showed that *S. carlsbergensis* is capable of synthesizing both pantothenic acid and biotin in large quantities, but is unable to synthesize pyridoxin. However, mating of the two species produced a hybrid that synthesized all three vitamins in large quantities.

Improved yeast types may be produced by hybridization and selection. In spite of the limited number of experiments performed by the above workers, one of the hybrids has already been employed commercially with excellent results. Hybridization of yeasts appears to have far-reaching possibilities.

## LABORATORY TECHNIQUE

The methods used for the isolation and study of yeasts in pure culture are, in general, the same as those employed for bacteria. Most species of yeast grow best at a temperature of about 25°C.

**Induced Sporulation.**—Most yeasts do not sporulate freely on the commonly used laboratory media. The environment must be made unfavorable for active sporulation to occur. The conditions generally considered necessary for active sporulation to occur include the following:

1. The yeast cells must be young and vigorous.
2. An abundance of moisture and oxygen must be present.
3. The medium must be deficient in nutrients.
4. The organisms must be incubated at a suitable temperature, usually 25°C.
5. The medium must be adjusted to a suitable hydrogen-ion concentration.
6. Inhibitory substances must be absent from the medium.

One of the earliest methods used to induce sporulation in yeasts is the classic method of Engel (1872). He employed plaster of Paris blocks. A block is placed in a glass container or a Petri dish and water added until the lower portion is immersed. The glass container and contents are sterilized in an autoclave. The upper surface of the block is heavily inoculated from a young broth culture and the container incubated at 25°C. for about one week. This method usually produces satisfactory sporulation.

Potato, carrot, cucumber, and beet slices, either raw or cooked, and an agar medium containing a weak infusion of carrots with a small amount of calcium sulfate, have been substituted for the plaster of Paris blocks with good success.

Gorodkowa (1908) developed a medium containing 1 per cent peptone and 0.25 per cent glucose solidified with agar. Sporulation occurred after 3 to 4 days. A similar medium containing 5 per cent glucose failed to induce sporulation, indicating that the conditions essential to sporulation involved primarily the sugars in the media rather than the other components.

A starvation medium apparently does not explain all the facts. Welten (1914) found that yeasts sporulated readily on prune extract agar. More spores were produced in concentrated than in dilute prune extract. He showed that a high acidity was necessary for sporulation to occur. No spores were encountered in an alkaline medium.

Mrak, Phaff, and Douglas (1942) found that agar slants made from a water extract of carrots, beets, cucumbers, and potatoes induced sporulation and, at the same time, served as an excellent stock culture medium. The medium was prepared by grinding equal weights of the vegetables and then mixing with a quantity of water equal to the weight of the ground mass. The mixture was autoclaved at 10 lb. pressure for 10 min., after which the extract was separated from the pulp by passage through a cloth filter. Two per cent agar was dissolved in the extract. The medium was tubed, autoclaved at 15 lb. pressure for 15 min., and slanted.

Nickerson and Thimann (1941) found that copulation and sporulation in a *Zygosaccharomyces* occurred more abundantly when many dead cells were present, and postulated that some stimulating substance was derived from the dead cells. They also found that an extract from *Aspergillus niger* increased copulation and sporulation. Later Nickerson and Thimann (1943) showed that riboflavin and sodium glutarate were probably the substances in the extract responsible for the stimulation.

Lindegren and Lindegren (1944*a*) recommended the following presporulation medium:

| | |
|---|---|
| Beet (leaves) extract | 10 cc. |
| Beet (roots) extract | 20 cc. |
| Apricot juice | 35 cc. |
| Grape juice | 16.5 cc. |
| Yeast (dried) | 2 gm. |
| Glycerin | 2.5 cc. |
| Agar | 3 gm. |
| Calcium carbonate | 1 gm. |

Water was added to give a final volume of 100 cc. The mixture was steamed for 10 min. and tubed. Tubes were sterilized at 15 lb. pressure for 20 min. and slanted. Most strains of yeasts will produce spores directly

on the slants if allowed to grow for a few weeks. However, if spores are needed sooner, transfer to plaster of Paris blocks is necessary.

It may be concluded that a specific nutrient is essential for abundant sporulation to occur. If this nutrient is satisfactory and the sugar content of the medium is not too high, sporulation may occur, even on agar slants. If plaster of Paris blocks are used, the water should be acid in reaction and should contain by-products of yeast growth and possibly some substances produced on the death and disintegration of the yeast cells.

**Isolation of Pure Cultures.**—Many methods have been recommended for the isolation of yeasts in pure culture. Most of the methods are concerned with the isolation of single cells and their propagation in a suitable culture medium. Such methods require considerable skill and patience. Yeasts may also be isolated in pure culture by the same methods employed for the separation of bacterial species (see page 134).

For more information on yeasts, see Henrici (1947), Leonian and Lilly (1943), Lindegren (1944, 1945, 1945*c*), Lindegren and Hamilton (1944), Lindegren and Lindegren (1944*a*, *b*, 1945), Lindegren, Spiegelman, and Lindegren (1944, 1945), Spiegelman (1945), Spiegelman and Lindegren (1944, 1945), Spiegelman, Lindegren, and Hedgecock (1944), and Spiegelman, Lindegren, and Lindegren (1945).

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# CHAPTER VI

## MOLDS

Molds are minute saprophytic or parasitic filamentous fungi, which reproduce by means of sexual and asexual spores. They differ from the algae in not containing chlorophyll, the green pigment that enables plants to synthesize carbohydrates from carbon dioxide and water in the presence of sunlight. Typical plants are chemosynthetic. They utilize simple substances and build them up into compounds of greater complexity. They utilize carbon dioxide to form carbohydrates and fats and eliminate oxygen. Generally speaking, animals are chemoanalytic. They break down complex organic matter into simple compounds, and differ from plants in that they take in oxygen and eliminate carbon dioxide. Molds resemble plants in structure and in being nonmotile but appear to be more related to animals in that they require oxygen in their metabolism and eliminate carbon dioxide.

Most species of molds possess a thallus, which is a colony composed of a mass of threads. The threads are known as hyphae (singular, hypha). A mass of threads taken collectively is spoken of as a "mycelium." The filaments or hyphae are usually colorless. Hyphae that are concerned in the production of spores are the fertile hyphae; those which serve to secure nutrients are the vegetative hyphae.

**Structure of Hyphae.**—Hyphae may be single-celled (nonseptate) or composed of many cells (septate). The transverse walls in the septate molds are known as septa (singular, septum). Longitudinal or oblique septa are very rare. Hyphae are more or less branched, continuous tubes. In most molds increase in size of hyphae occurs by apical growth. Cells lying back from the tips of hyphae may start to grow and develop into branches. In a few species, all cells may continue to grow and divide. The nonseptate hyphae form one large cell containing many nucleuses. The septate hyphae may contain one, two, or many nucleuses in each cell. Septa are rare in the *Phycomycetes* except in the fruiting bodies, though they may be occasionally present in old cultures (Fig. 68).

The nucleuses are usually easily differentiated from the remainder of the cell contents. Young cells show a relatively dense cytoplasm whereas old ones become vacuolated. The protoplasm (cytoplasm + nucleus) of mature cells usually makes up only a small portion of the total cell volume. Various reserve food materials such as fat globules, glycogen, and metachromatic granules or volutin, may also appear (see page 82).

**Cell Wall.**—The cell wall shows some variation in composition. In many *Archimycetes* and *Oömycetes* and in the yeasts (*Ascomycetes*) the cell wall is composed largely of cellulose, but in the *Zygomycetes* and higher fungi the cellulose is replaced by a polymerized acetylated glucosamine

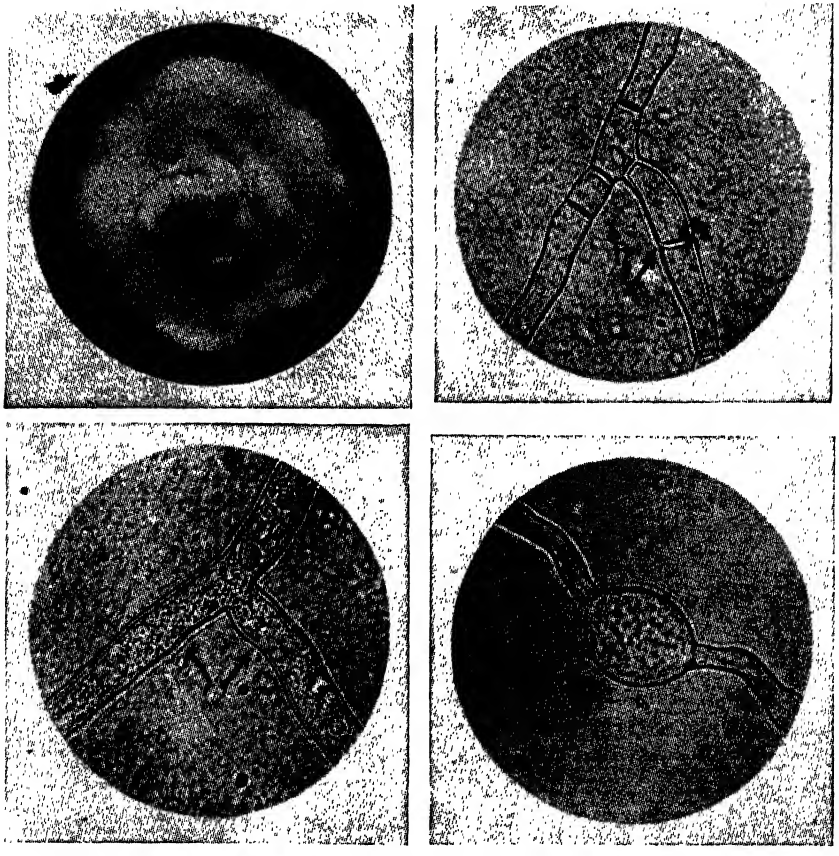


FIG. 68.—Illustrations of some important morphological terms used in mycology. Upper left, thallus—a colony; upper right, septate mycelium; lower left, nonseptate mycelium; lower right, chlamydospore. (*After Kurung.*)

known as chitin, and by other substances of an obscure nature. Chitin is also present in the shells of crabs and lobsters and in the shards of beetles.

## MULTIPLICATION IN MOLDS

Most of the common molds may be cultivated by transferring any part of the plant to fresh medium, but the normal process of development begins with the germination of a spore. Spores are produced in great numbers. They are very resistant bodies, being capable of withstanding adverse

conditions over long periods of time. Spores are of different shapes and sizes and may be composed of one cell or more than one cell.

A spore consists of an outer wall, the epispore, and an inner wall, the endospore. The epispore may be smooth, pitted, or roughened by small projections; the endospore encloses the protoplasm in which may be seen droplets of oily or fatty material and one or more nucleuses. Under favorable conditions, the spore first swells, then throws out one or more germ tubes (Fig. 76). Each germ tube elongates and becomes branched, forming a network of hyphae or a mycelium. Later spore-bearing bodies develop on the fertile hyphae, or some of the hyphae show the presence of special fruiting bodies in which spores are formed.

Two types of spores are produced: sexual and asexual. An asexual spore is not the result of the fusion of two gametes or sex cells. A few of the molds produce several kinds of spores, corresponding to different stages in their development. Practically all the molds commonly encountered produce asexual spores. Some of these produce both asexual and sexual spores.

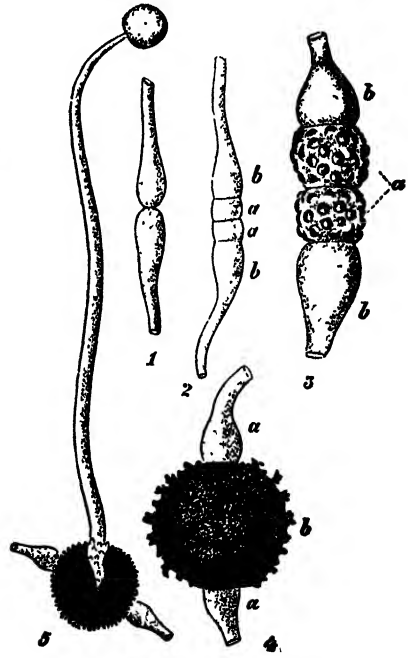



FIG. 69.—*Mucor mucedo.* Formation of zygospore. 1, two hyphae in terminal contact; 2, articulation into gamete *a* and suspensor *b*; 3, fusion of gametes *a*; 4; ripe zygospore *b* supported by the suspensors *aa*; 5, germination of zygospore. (*After Brefeld; from Lafar's Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

**Sexual Multiplication.**—The nonseptate molds produce sexual spores known as zygospores. In zygospore formation, two filaments lying near each other send out branches which finally touch. The apical cells of the two branches fuse together. Cell walls are then formed, which separate the fused mass of protoplasm from the remainder of the hyphae. The resulting cell greatly enlarges in size and develops a very thick and irregular cell wall. This cell is known as a zygospore. The zygospore germinates into a new fungus when conditions become favorable (Fig. 69).

The septate molds produce sexual spores known as ascospores. Two cells (gametes) on the same or adjoining hyphae coil together, the tips fuse, and the contents of one cell pass into the other, resulting in the development of one cell (Fig. 80). The fertilized cell grows into a mass of branching threads or hyphae, certain cells of which give rise to spore sacs or asci.

Each ascus contains from two to many spores, the usual number being eight. Frequently, the hyphae lying near the sex cells branch many times to form a covering for the mass of asci. This results in the formation of a structure known as a perithecium (plural, perithecia). The principal molds producing perithecia are those of the genus *Aspergillus* (Fig. 80).

**Asexual Multiplication.**—Asexual multiplication in molds occurs in both nonseptate and septate molds. The spores produced by nonseptate molds may be either free or enclosed in spore cases known as sporangia (singular, sporangium). The septate molds never produce sporangia.

A sporangium is produced at the upper end of a fertile hypha. The

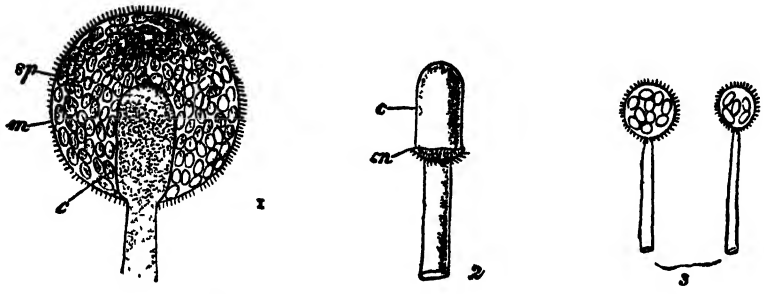



FIG. 70.—*Mucor mucedo.* 1, sporangium; 2, remains of an emptied sporangium; 3, stunted dwarf sporangia, with only a few spores and devoid of columella. (*After Brefeld; from Lafar's Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

hypha becomes enlarged at the end, increases in length, and finally consists of a long filament with a swollen tip. The enlarged portion is filled with cytoplasm in which are present many nucleuses. The nucleuses surround themselves with some cytoplasm and develop cell walls to produce spores. The portion of the hypha reaching into the interior of the sporangium is called a "columella." The filament bearing the sporangium is known as a sporangiophore. When the sporangium ripens the wall ruptures, scattering the spores. Each spore is capable of repeating the cycle (Fig. 70.)

Molds that do not produce sporangia develop definite fertile threads known as conidiophores. The conidiophores produce asexual spores known as conidia (singular, conidium). Conidia are borne on the tips of branches in the form of chains. In some species, the tip of the conidiophore constricts and pinches off spores. The spore nearest the mother cell is the youngest (Fig. 71). In other species, spores are formed by budding from the terminal cell of the conidiophore. The spore then increases in size after which another spore pushes out from the tip of the new spore. This continues until a chain of spores is formed. In these molds, the terminal spore of the chain is the youngest (Fig. 72).

Sometimes cells in a filament surround themselves with very thick

walls to form spores. These are asexual spores and are known as chlamydospores (Fig. 68). They occur either singly or in chains in ordinary vegetative hyphae or in special branches. Chlamydospores are produced by transverse division of the hyphae at irregular intervals.

In some species, the vegetative hyphae break up into short segments or spores. These segments break apart and are capable of giving rise to new molds. The segments are called oidia (singular, oidium). This is shown in (Fig. 73).

## RESISTANCE OF MOLD SPORES

Molds have been responsible for enormous losses in the home and in the industries. These losses are due largely to the fact that molds produce spores in great masses. Mold spores are very resistant to unfavorable conditions, such as heat, cold, desiccation, ultraviolet light, high osmotic pressures, and deficient food supply. Generally speaking, mold spores are more resistant to heat than mycelium and less resistant than bacterial spores.

Spores are easily disseminated by wind and air currents. They are commonly present in the air of laboratories and are the frequent cause of contaminations of cultures and culture media. Therefore, laboratory windows should be kept closed to prevent the wind from stirring up the dust and spores in the laboratory air.

Molds occur particularly in damp places. Spores will not germinate in a dry environment. Many industrial products, such as paper, leather, textiles, and foods readily absorb moisture from a moist atmosphere and are susceptible to attack by molds. In order to decrease mold contaminations, laboratories and rooms should be kept as free as possible from any excessive amount of moisture.

## CLASSIFICATION OF MOLDS

The general principles followed for the classification of molds are similar to those employed for the classification of bacteria (page 397). A condensed classification of the commonly encountered genera and species of molds is as follows:

Class I. *Phycomycetes.* Vegetative mycelium typically nonseptate. Sporangia with motile or nonmotile sporangiospores, or conidia produced. Resting sporangia, oöspores, or zygospores formed, sometimes after fertilization, sometimes without any preliminary fusion of gametes or gametangia. Some are parasitic; others are saprophytic.

Order. *Mucorales.* Accessory multiplication by sporangiospores.

Sporangia globose to ovoid, usually contain numerous spores, sometimes one or a few. Zygospores formed from the whole of the two gametangia.

Columella present. Zygospore naked or invested by outgrowths from its own wall or from those of the suspensors.

Principal sporangia contain numerous spores.

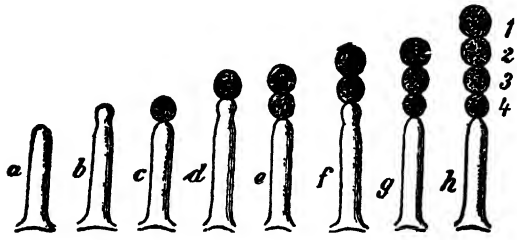



FIG 71.—Formation of conidia. The topmost (1) is the oldest. (*After Zopf, from Lafar's Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

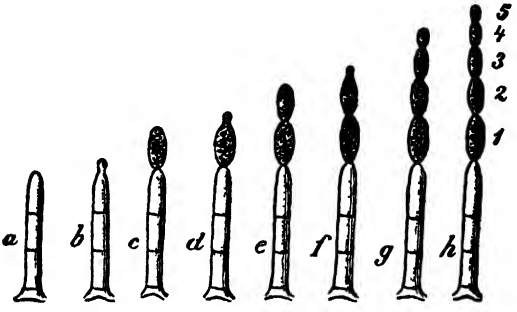



FIG 72.—Formation of conidia. The topmost (5) is the youngest. (*After Zopf, from Lafar's Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

FIG 73.—Oospora. (*After Kurung.*)

Family. *Mucoraceae.* Sporangiola, if any, developed on lateral branches of principal sporangiophores. Sporangia of one kind. Sporangiophore simple or branched but not repeatedly dichotomous. Suspensors without appendages at maturity.

Genus 1. *Mucor.* Stolons absent. Sporangia single and terminal.

Sporangiophores rarely or never branched.

Species 1. *hiemalis.* Columella spherical. Sporangium olive to grayish-brown when ripe.

Species 2. *piriformis.* Columella pear-shaped. Sporangium very large.

Species 3. *mucedo.* Columella pear-shaped to cylindrical. Sporangium grayish.

Species 4. *ramannianus.* Columella spherical. Sporangium reddish.

Sporangiophores usually branched.

Species 5. *spinosus* (*plumbeus*). Columella usually spiny near tip.

Species 6. *rouxii.* Rapidly converts starch to sugar. Used in manufacture of alcohol.

Sporangiophores with main stem and secondary lateral branches, racemose.

Species 7. *racemosus.* Columella ovoid.

Species 8. *erectus.* Columella spherical. Sporangium gray yellow.

Species 9. *fragilis.* Columella spherical. Sporangium black.

Branches of sporangiophore nearly equal, cymose.

Species 10. *ambiguus.* Sporangia borne irregularly.

Species 11. *circinelloides.* Sporangia in two rows, alternating. Spores spherical to ellipsoidal.

Species 12. *alternans.* Sporangia in two rows, alternating. Spores longer, ellipsoidal.

Genus 2. *Rhizopus.* Stolons present.

Species 1. *nigricans.* The common black bread mold.

Species 2. *oryzae.* Employed in the hydrolysis of starch to sugar.

Species 3. *japonicus.* Also employed in the hydrolysis of starch to sugar.

Class II. *Ascomycetes.* With the exception of the yeasts, all possess a well-developed mycelium of branched and septate hyphae. Cells of mycelium may be uninucleate or may contain several nucleuses. Multiplication takes place by conidia and by chlamydospores but the characteristic method is by means of ascospores. An ascus contains usually 8 spores, more rarely a smaller or a larger number. Some of the *Aspergillus, Penicillium,* and *Alternaria* produce ascospores and should be included here (see Fig. 74). However, asci have not been identified in the great majority of the species and for that reason all of them are grouped under the *Fungi Imperfecti* for convenience.

Class III. *Basidiomycetes.* All possess well-developed mycelium. Basidiospores produced which are borne externally on the mother cell or basidium. The young basidium contains two nucleuses that fuse, then divides to provide the nucleuses of the spores. The spore is formed on a sterigma through which the nucleus passes from the basidium to enter the developing spore. The basidiospores are unicellular, round or oval, asymmetrically attached to their sterigmata, usually with a smooth, rather thin wall. Echinulate spores occur in a few species.

Class IV. *Fungi Imperfecti.* Characteristic method of reproduction by means of conidia. In some species oidia and chlamydospores may be present. Sporangiospores, ascospores, and basidiospores are not produced.

Order. *Moniliales.* Conidiophores free, arising irregularly from the mycelium Conidiophores detached, not compacted.

Family 1. *Moniliaceae.* Hyphae colorless or in pale or bright colors.

Genus 1. *Trichothecium.* Spores two-celled, in small clusters on ends of erect conidiophores.

Species. *roseum.* A commonly occurring pink mold found on decaying fruit. At times causes contamination of laboratory media.

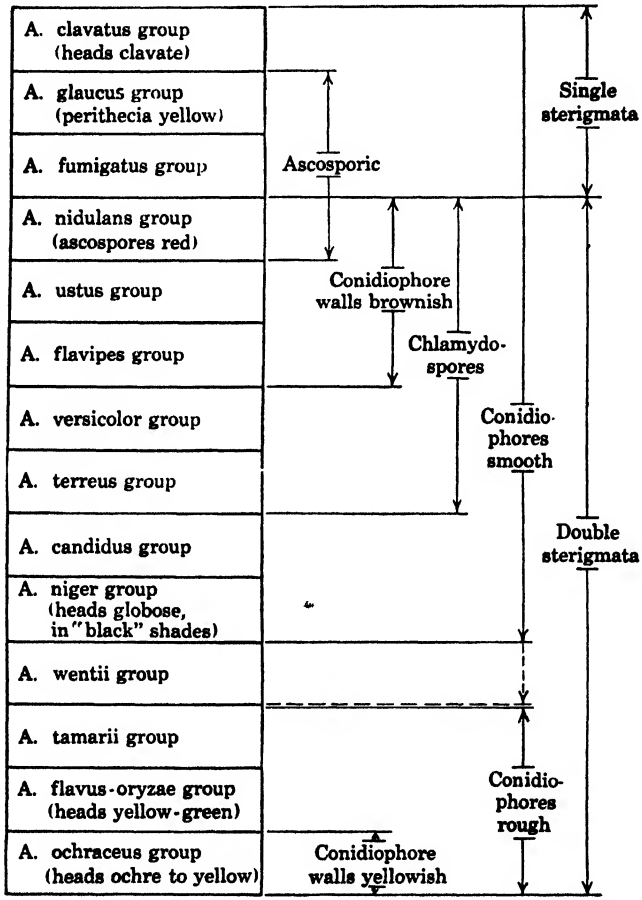



FIG. 74.—Natural relationships among the groups of the *Aspergillus* (*Adapted from Thom and Raper.*)

Genus 2. *Oöspora* (*Oidium*). Reproduction occurs by fragmentation of mycelium. Conidia exogenous, globose, or suboblong.

Species. *lactis.* Commonly found in milk and cheese. Imparts flavor and aroma to many types of cheeses.

Genus 3. *Monilia.* Spores distinct from mycelium. Reproduction by ovate spores, increasing by budding and forming branched chains; also by fragmentation of mycelium in old cultures.

Species. *sitophila.* Commonly found in air. Grows rapidly and is frequently the cause of laboratory contaminations.

Genus 4. *Aspergillus*. Vegetative mycelium consisting of septate hyphae, branching, colorless or colored. Conidiophores arising from specialized foot cells, usually nonseptate, terminating in a swelling which bears the sterigmata. Conidia borne in chains formed by abscission from sterigmata. Conidia vary greatly in color, size, shape, and markings. Perithecia found in some groups (unknown in most species) producing asci and ascospores within a few weeks.

*A. clavatus* group. Conidial heads clavate pale blue-green. Conidiophores generally coarse, smooth-walled, uncolored. Conidia elliptical, smooth, thick-walled.

*A. glaucus* group. Perithecia yellow, thin-walled, suspended in networks of red or yellow hyphae. Asci contain eight lenticular, smooth, or rough-walled ascospores. Conidiophores smooth-walled, terminating in dome-like vesicles. Conidia elliptical to subglobose, uniformly roughened.

*A. fumigatus* group. Conidial heads columnar, green to dark green. Vesicles flask-shaped. Conidiophores smooth-walled, usually green. Conidia globose, echinulate, green.

*A. nidulans* group. Conidial heads short columnar, usually dark green. Conidiophores smooth-walled, terminating in dome-like or hemispherical vesicles. Perithecia usually present. Ascospores purple-red. Conidia globose.

*A. ustus* group. Conidiophores yellow-brown, smooth. Conidial heads irregular. Vesicles hemispherical. Conidia roughened, ranging in color from pale blue-green to deep brown.

*A. flavipes* group. Conidiophores smooth, yellow, with color often confined to outer layer. Heads barrel-shaped to columnar. Vesicles subglobose to elliptical. Conidia colorless, smooth, thin-walled.

*A. versicolor* group. Conidial heads hemispherical to almost globose, usually green or blue-green. Conidiophores smooth, colorless, more or less sinuous. Vesicles globose to elliptical. Conidia globose or subglobose, echinulate.

*A. terreus* group. Heads columnar, pale buff or light flesh shades. Conidiophores smooth, colorless. Vesicles hemispherical with upper portion covered by sterigmata. Conidia small, smooth, globose to slightly elliptical.

*A. candidus* group. Conidial heads white or becoming cream-colored with age, globose, but approaching columnar in small heads. Conidiophores smooth, colorless, or slightly yellow. Conidia smooth, globose or subglobose.

*A. niger* group. Conidial heads black, brownish-black, or purple-brown. Heads large and globose. Conidiophores smooth, colorless, or slightly yellow-brown. Vesicles globose in large heads, dome-like apices in small heads. Conidia rough, showing mostly bars or bands of brown-black coloring matter.

*A. wentii* group. Conidial heads large, globose, varying greatly in color. Conidiophores smooth-walled or nearly so, often appearing finely roughened. Vesicles globose. Conidia commonly elliptical, smooth, or somewhat roughened depending upon species.

*A. tamarii* group. Conidial heads radiate, hemispherical to globose, yellow-brown to olive-brown in color. Conidiophores colorless, roughened throughout a part or all of their length. Vesicles globose to subglobose. Conidia heavy-walled, rough, elliptical, pyriform, or subglobose.

*A. flavus-oryzae* group. Conidiophores colorless, rough, or pitted. Heads hemispherical to columnar to subglobose. Vesicles hemispherical to dome-shaped in small heads, globose in large heads. Conidia roughened, varying in color.

*A. ochraceus* group. Conidial heads yellow to ochraceus. Heads globose or radiate with conidial chains commonly adhering into divergent columns. Conidiophores yellowish. Conidia in some series thin-walled and smooth, in others double-walled and echinulate.

Genus 5. *Penicillium.* Conidiophores little or not inflated, unequally verticillate at tip. Conidia globoid.

Species 1. *camemberti.* Found in Camembert or Brie cheese. Colonies floccose, white or grayish green in color.

Species 2. *brevicaule* var. *glabrum.* Found on Camembert or Brie cheese. Powdery colonies, yellowish white in color.

Species 3. *brevicaule.* Found on Camembert or Brie cheese. Yellow-brown areas formed. Spores rough.

Species 4. *roqueforti.* Found on Roquefort cheese. Forms green streaks inside of cheese.

Species 5. *italicum.* Found on citrus fruits. Colonies blue-green

Species 6. *digitatum.* Found on citrus fruits. Colonies olive-green.

Species 7. *expansum.* Found on apples and pears. Colonies blue-green.

Family 2. *Dematiaceae.* Mycelium, spores, or both, dark brown to black. Conidiophores detached, not compacted.

Genus 1. *Cladosporium.* Spores increase by budding, forming branched chains. Spores one-celled but become two-celled in old cultures.

Species. *herbarum.* Found on decaying paper, straw, and similar materials.

Genus 2. *Alternaria.* Spores many-celled, club-shaped, and in chains.

Species. *tenuis.* Found on moldy grains and in soil. Frequently found in laboratory air.

## MORPHOLOGY OF THE COMMON MOLDS

Several hundred genera and thousands of species of molds have been described. Only a few genera are of common occurrence, and these may be easily recognized. The commonly occurring genera include: *Mucor, Rhizopus, Trichothecium, Oidium* and *Oöspora, Monilia, Aspergillus, Penicillium, Cladosporium,* and *Alternaria.* Their characteristics are as follows:

**Mucor.**—This is the largest genus of the order *Mucorales.* The species belonging to this genus are found on decaying vegetables and on bread. The vegetative mycelium penetrates the food material and sends out long slender threads known as aerial hyphae. The mycelium is white in color. A septum forms near the apex of each hypha. The tip of the hypha then swells into a globular sporangium within which develop numerous oval spores. The sporangia are almost black in color. The wall of the ripe sporangium easily breaks, discharging the enclosed spores. Each spore is capable of repeating the cycle. (Figs. 75 and 76). Under certain conditions, conjugation of two cells from different hyphae precedes spore formation, resulting in the development of a zygospore. This is sexual

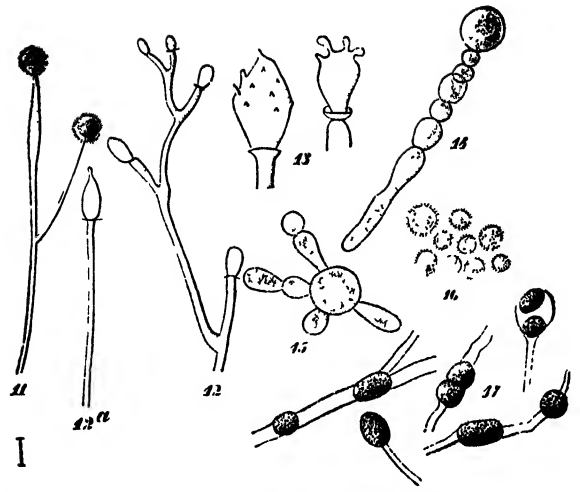



FIG. 75.—*Mucor plumbeus.* 11, sporangiophores and sporangia; 12-12*a*, sporangiophores and columella; 13, columella; 14, spherical cells; 15, budding spherical cells; 16, spores; 17, chlamydospores. (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

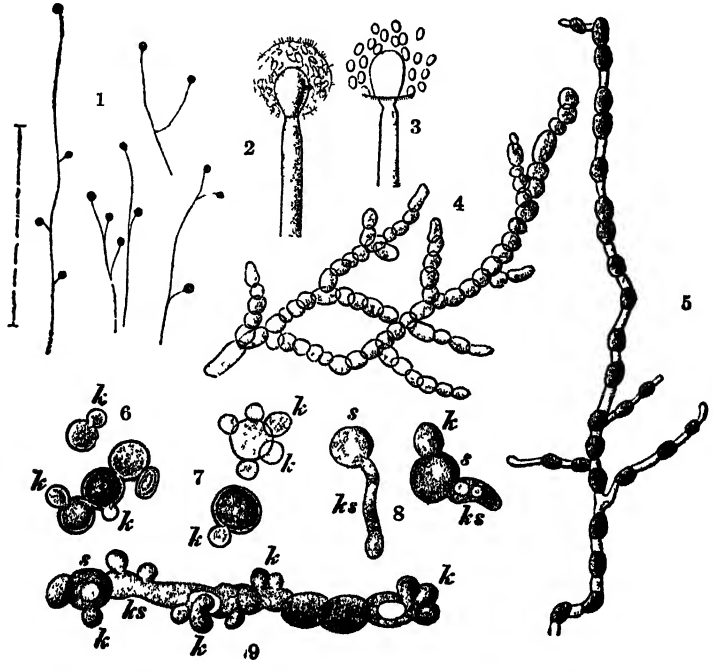



FIG. 76.—*Mucor racemosus.* 1, aerial hyphae with sporangia; 2, sporangium; 3, columella; 4, spherical cells; 5, chlamydospores; 6–7, germination of spherical cells by budding (*k*); 8, germination of a spore; 9, degenerate hypha. (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

reproduction. A germ tube arises from the matured zygospore, which develops a sporangium at the apex (Fig. 69).

Separation of the various species is based on the length and diameter of the sporangiophores, the type of branching, if any, the size and color of the sporangia, the character of the sporangial wall, the characteristics of zygospores and chlamydospores, if any, the size and shape of the columellae, the size and shape of spores, and general colony characteristics, such as color and height of aerial growth.

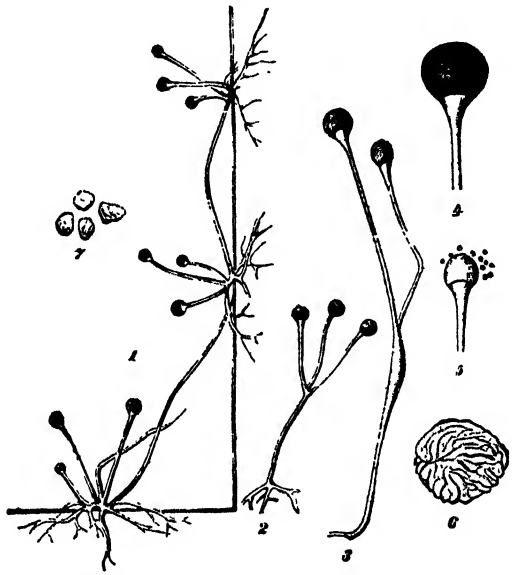



FIG. 77.—*Rhizopus nigricans.* 1, 2, 3, growth showing aerial hyphae and sporangia; 4, sporangium; 5, columella; 6, spore showing wrinkled surface; 7, spores under low power. (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

**Rhizopus.**—Members of this genus are of common occurrence and frequently cause laboratory contaminations. Growth on the usual laboratory media is very rapid. The molds spread widely by means of stolons or runners. Culture tubes and Petri dishes soon become filled with a dense cottony mycelium. Species in this genus are easily distinguished from the mucors by the presence of stolons. Stolons often reach a length of several centimeters and bear tufts of root-like hyphae known as rhizoids, which emerge from the points where the stolons come in contact with the medium or the surface of the glass. Spores may be ovoid, polygonal, or striated. The members are usually grayer in color and produce a more luxurious growth than the mucors (Figs. 77 and 78).

**Trichothecium.**—This genus contains several species, but only one, *T. roseum*, is of common occurrence. The colonies are thin, spreading,

floccose, at first white in color, then becoming slowly pale pink. The conidiophores bear clusters of spores attached to the tip. The spores are ovoid with a nipple-like projection at the point of attachment and are composed of two cells.

**Oidium and Oöspora.**—There appears to be some doubt as to the correct use of these two terms. Some authorities use the generic name *Oidium*

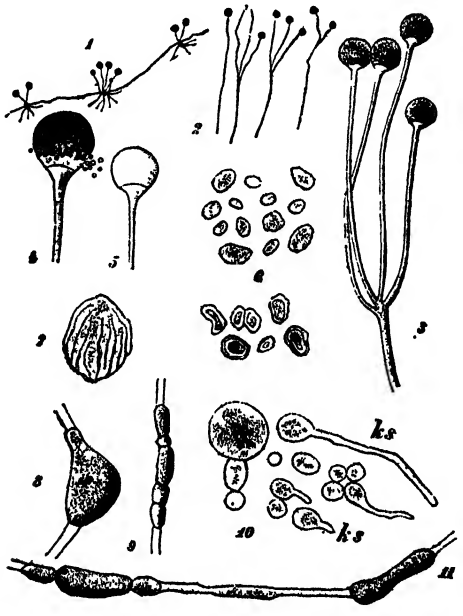



FIG. 78.—*Rhizopus oryzae.* 1, 2, 3, aerial hyphae and sporangia; 4, sporangium; 5, columella; 6, spores under low power; 7, spore under high power; 8, 9, 11, chlamydospores; 10, germination of spherical cells. (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

for the parasitic members and the term *Oöspora* for the saprophytic species. Others classify under *Oidium* those species having rectangular mycelial fragments and as *Oöspora* those species having rounded cells.

A well-known saprophytic species is *Oöspora lactis.* It is found in various milk products. It grows readily on milk or wort agar and produces a thin, spreading, slimy growth. The colonies are creamy white in color. Young cultures show long hyphal threads, whereas old cultures are composed entirely of short rectangular fragments (oidia). Each fragment is capable of producing a new culture (Fig. 73).

Another species, *Oöspora crustacea,* is found on cheese rind and produces an orange-colored pigment. It produces a powdery type of growth. The optimum temperature is about 18°C. It grows rapidly on culture media and produces a bright orange to scarlet pigment. The cells com-

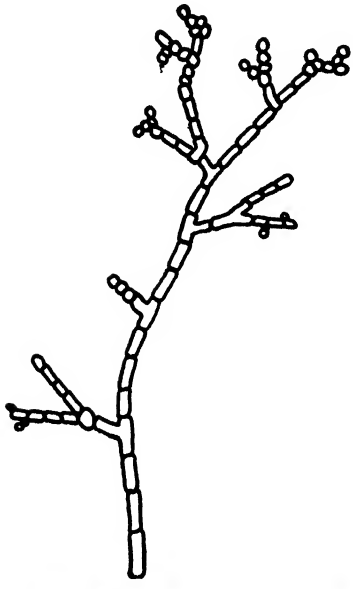


FIG. 79.—*Monilia sitophila.* Aerial hyphae (conidiophores) with budding yeast conidia. (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

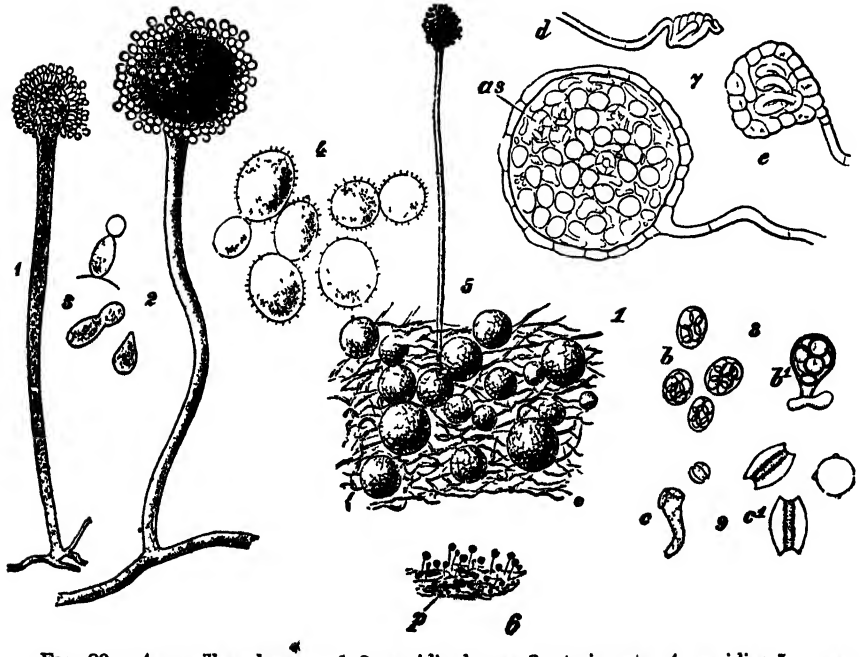



FIG. 80.—*Aspergillus glaucus.* 1–2, conidiophores; 3, sterigmata; 4, conidia; 5, a portion of mycelium with perithecia and conidiophore; 6, same, natural size; 7, sections of perithecia; 8, isolated asci; 9, spores. (*After De Bary and Wehmer; from Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

posing a young mycelium round up before breaking apart and resemble a chain of conidia.

**Monilia.**—This genus contains only one species of common occurrence. *M. sitophila* is found in laboratory air and causes contaminations of cultures and media. It grows rapidly on media in loosely floccose masses and produces a pale pink to salmon pink color. The aerial hyphae contain oval-shaped conidia at their tips. The conidia increase by budding, eventually producing large, irregular masses. As the hyphae age, the

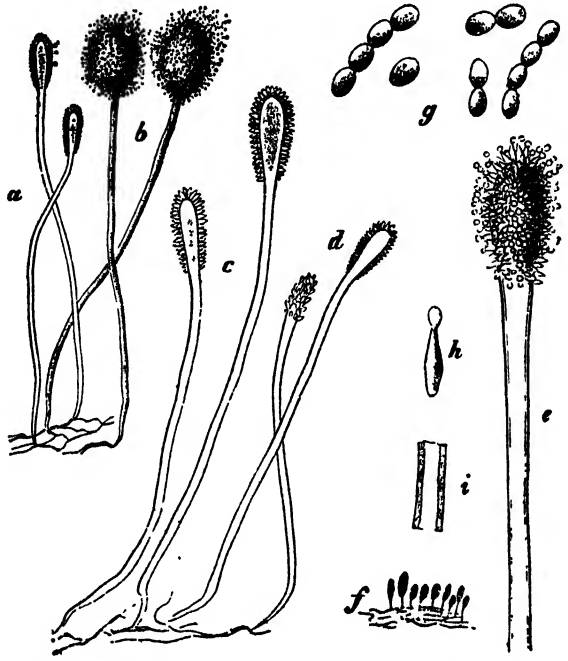



FIG. 81.—*Aspergillus clavatus*. *a, b, c, d, e*, conidiophores in various stages of development; *f*, conidial herbage; *g*, conidia; *h*, sterigma; *i*, section of stem. (*After Wehmer; from Lafar's Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

individual cells break apart. Each cell is capable of producing a new plant (Fig. 79).

**Aspergillus.**—The species of this genus are relatively common in air. They are found almost everywhere on nearly all types of substrates (Figs. 80 to 84). The organisms are found on decaying fruits, vegetables, grains, bread, and other articles of food. Aspergilli are commonly found in incompletely sterilized culture media. The color may vary considerably. It may appear green, yellow, orange, black, or brown. The molds have a powdery appearance. In marked contrast to *Mucor*, the hyphae are branched and septate. The hyphae enlarge at the apices to form conidio-

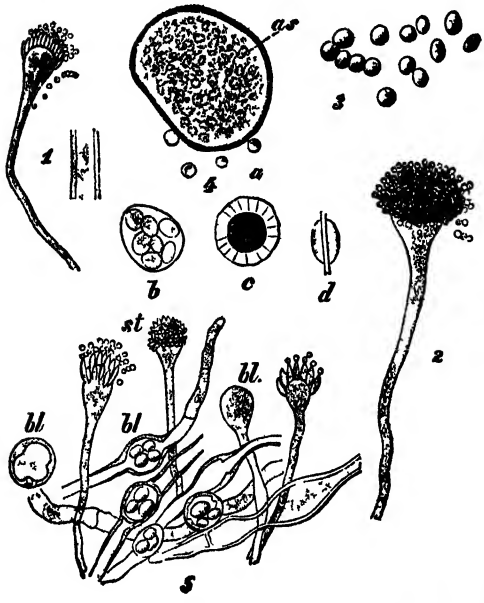



FIG. 82.—*Aspergillus fumigatus* 1–2, club-shaped conidiophores, 3, conidia, 4, ascus and spores, 5, hyphae with globular swellings (*After Gruns and Wehmer, from Lafar's Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena*)

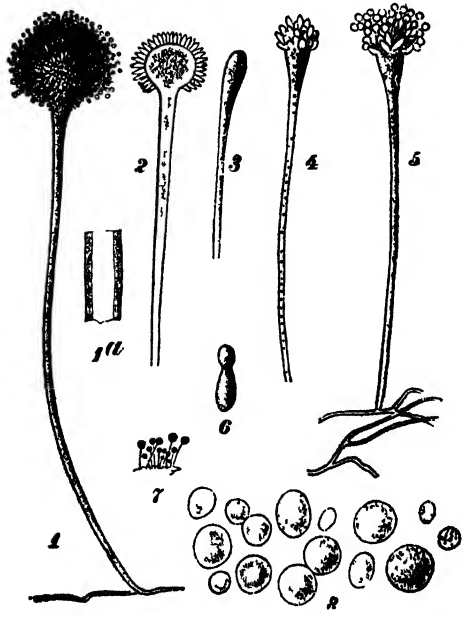



FIG. 83.—*Aspergillus oryzae* 1, conidiophore, 2, longitudinal section, 3–5, development of conidiophore, 1*a*, longitudinal section of stem, 6, sterigma, 7, conidial herbage, 8, conidia (*After Wehmer, from Lafar's Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena*)

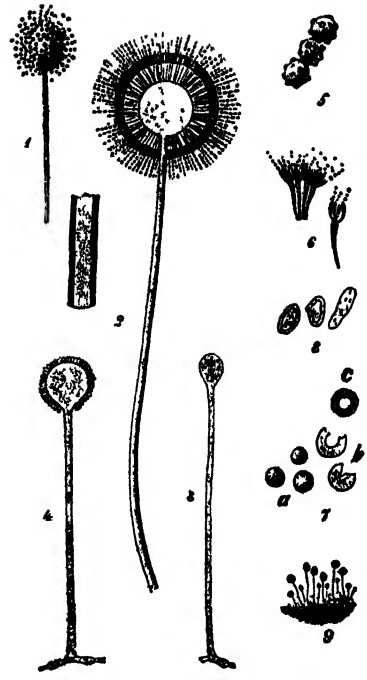


FIG 84 —*Aspergillus niger* 1, 2, conidiophores, 3, young conidiophore before formation of sterigmata, 4, young conidiophore during formation of sterigmata, 5, globular, warty conidia, 6, sterigmata, 7, sclerotia, 8, tough skinned spotted cells from interior of sclerotia, 9, conidial herbage (*After Wehmer, from Lafar's Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena*)

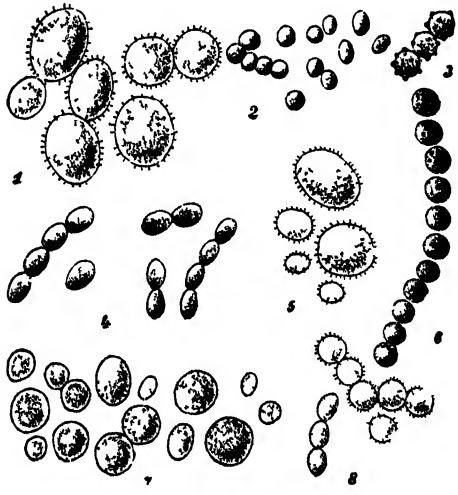


FIG. 85.—Conidia of various species of *Aspergillus*. 1, *A. glaucus;* 2, *A. fumigatus;* 3 *A. niger;* 4, *A. clavatus;* 5, *A. tokelau;* 6, *A. varians;* 7, *A. oryzae;* 8, *A. wenti.* (*From Lafar Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

phores. The conidiophores are not branched. Numerous short stalks called "sterigmata" develop from the apical or swollen ends of the condiophores. Chains of spores are produced from the tips of the sterigmata, sometimes developing to a considerable length (Fig. 85). A few species produce perithecia. These are spherical, cylindrical, or flask-shaped, hollow structures, which contain the asci, and usually open by a terminal pore. The asci contain the ascospores.

For excellent discussions of the aspergilli, see the monographs by Smith (1942) and Thom and Raper (1945).

**Penicillium.**—The members of this genus are closely related to the aspergilli and are also widely distributed in nature. The genus includes the characteristic blue-green colored mold so often observed on citrus and

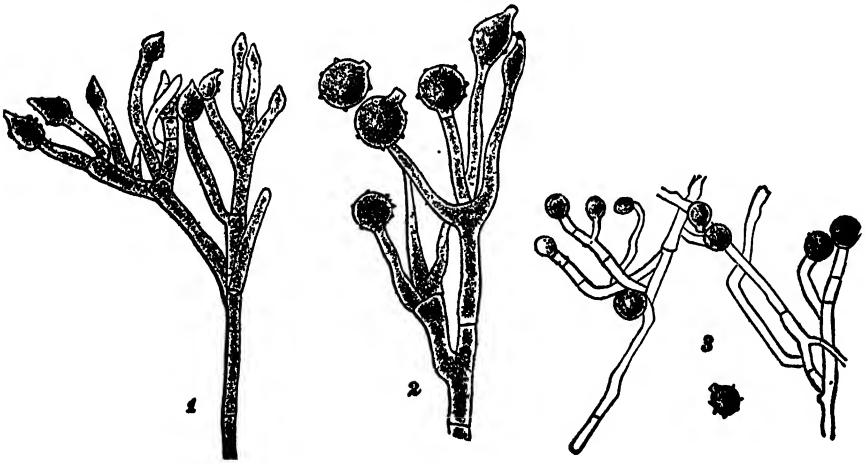



FIG. 86.—*Penicillium brevicaule*. 1, 2, formation of conidia on special conidiophores; 3, formation of conidia on the mycelium. (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

other fruits, vegetables, grains, hay, organic infusions, cheeses, and other food materials (Figs. 86 to 88). The vegetative mycelium penetrates the food substances, after which aerial hyphae or conidiophores appear. The conidiophores branch one or more times from the same joint, giving rise to a terminal cluster of parallel hyphae. The small terminal branches are known as sterigmata. A chain of conidia develop from each sterigma (Fig. 89).

Some species are destructive whereas others are beneficial. Probably the most important species are *P. roqueforti* and *P. camemberti*, which are responsible for the desirable changes occurring in Roquefort, Camembert, Gorgonzola, and other cheeses. The penicillia are employed in the manufacture of a considerable number of substances of commercial importance. These are discussed under Biochemistry of the Molds (page 126).

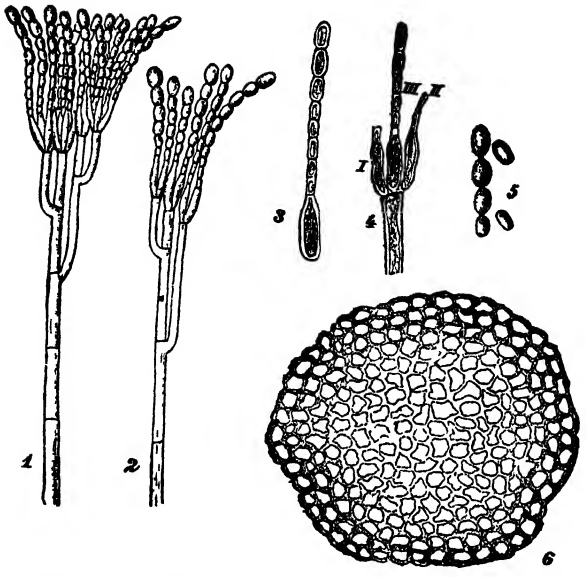



FIG. 87.—*Penicillium italicum.* 1, 2, conidiophores; 3, 4, sterigmata; 5, conidia; 6, section through an old sclerotium. (*After Wehmer; from Lafar's Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

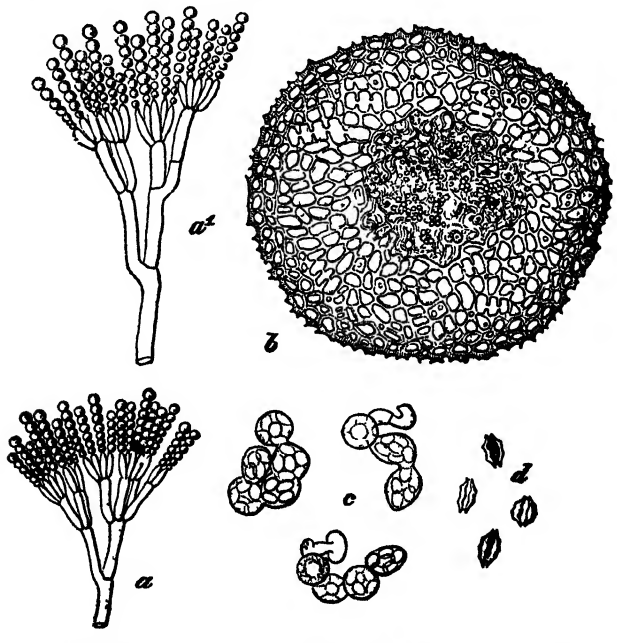



FIG. 88.—*Penicillium glaucum.* *a*, *a'*, conidiophores showing branching; *b*, perithecium with ripening asci; *c*, ascus showing germination; *d*, spores, viewed laterally. (*After Brefeld; from Lafar's Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

The species of *Penicillium* are more difficult to classify than are the members of the genus *Aspergillus*. The conidia show less variation in color. Most species show some shade of green during the period of active growth. The colors vary in shade under different environmental conditions and with the age of the cultures. Also, there are considerably more species of *Penicillium* than of *Aspergillus*.

For excellent discussions of the penicillia, see the monographs by Smith (1942) and Thom (1930).

**Cladosporium.**—The most common species in this genus is *C. herbarum*. It is widely distributed, being found on rubber, leather, textiles, foodstuffs, and decaying vegetable matter (Fig. 90). On culture media, it produces a thick, velvety growth and the color varies from deep green to dark gray-green. When examined microscopically in the dry state, the spores occur in large, almost tree-like clusters. In mounted specimens, the structure breaks up, the spores detach themselves, and the hyphae separate into rod-like cells. The oval-shaped spores increase by budding in a manner similar to the yeasts. Young spores are usually single-celled; old spores frequently show two cells.

**Alternaria.**—The species are commonly found on organic compounds. Parasitic forms have been isolated from cultivated plants. On culture media, the organisms grow rapidly and produce dense, floccose, greenish-colored mycelium. The mycelium is septate and may form chains of short, swollen cells similar to oidiospores. The conidia vary from oval-shaped to roughly club-shaped forms, with a pronounced beak at the tip. The spores are produced in short chains, sometimes branched, and are greenish brown to dark brown in color. They show both transverse and longitudinal septa and the degree of division increases with age.

### Laboratory Technique

In order that accurate studies be made on molds it is necessary to grow them in pure cultures. The methods employed for isolating and studying the growth of molds in pure cultures are, in general, similar to those used for bacteria (page 134). Most molds grow best at a temperature of about 25°C.

**Culture Media.**—Many types of solid and liquid media are employed for the cultivation of molds but only a few of these are for general use. Media employed for the cultivation of molds are usually slightly acid in reaction. Many species are able to tolerate relatively high acidities. Vegetables and vegetable extracts are commonly employed as culture media. These are suitable with or without the addition of sugar. The solid media may be composed of solid substances, such as potatoes, carrots, and beans, or liquid media made solid by the addition of agar or gelatin.

Some prefer to use pure synthetic media, composed of inorganic salts. These are of constant composition and strictly comparable when prepared in different laboratories. One of the simplest synthetic media employed for the cultivation of molds is Czapek's solution. This is easy to prepare and is probably as good as any other for general use. A modification of the original formula is given on page 126.

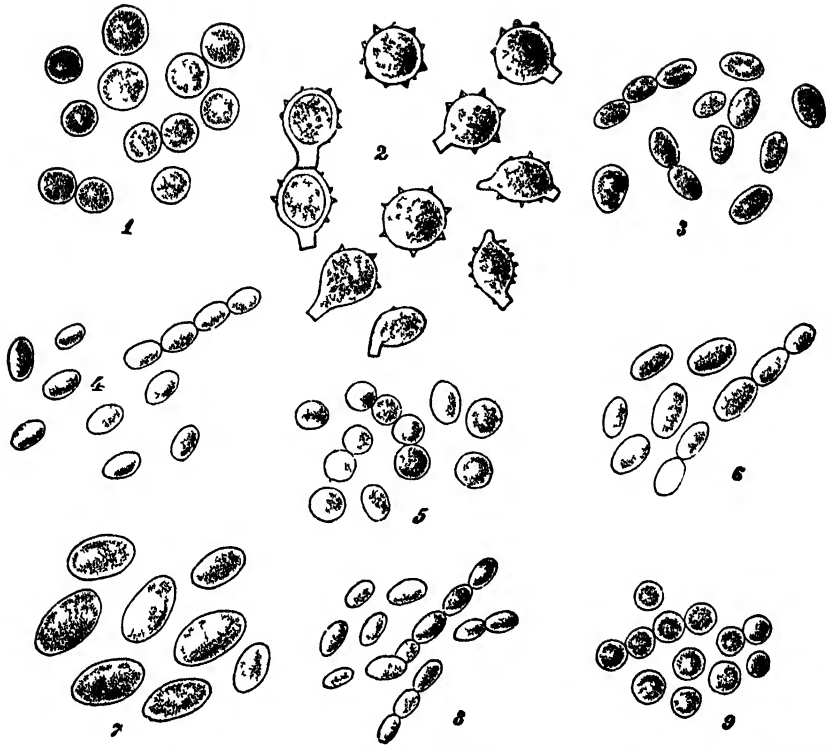



FIG 89 —Conidia of various species of *Penicillium* 1, *P camemberti*, 2, *P brevicaule*, 3, *P purpurogenum*, 4, *P claviforme*, 5, *P rubrum*, 6, *P italicum*, 7, *P olivaceum*, 9, *P luteum*, *P glaucum* (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena*)

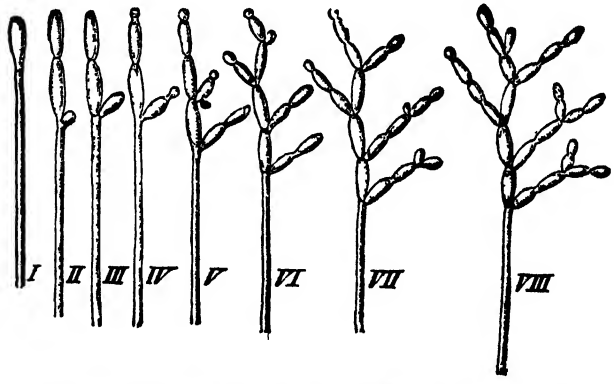



FIG 90 —*Cladosporium herbarum* Successive steps in the development of conidia (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

| | | |
|---|---|---|
| Sodium nitrate | 2.0 | gm. |
| Potassium chloride | 0.5 | gm. |
| Magnesium sulfate | 0.5 | gm. |
| Ferrous sulfate | 0.01 | gm. |
| Potassium acid phosphate | 1.0 | gm. |
| Distilled water, to make | 1000 | cc. |

Various carbohydrates, such as glucose and sucrose, may be incorporated. Usually 50 gm. of glucose or 30 gm. of sucrose are added. The medium is acid (pH4.2) which is more favorable to mold growth than a neutral reaction. The medium may be solidified by the addition of 15 to 20 gm. of agar per liter. This is probably the most useful solid medium employed for the cultivation of molds.

**Isolation and Purification of Molds.**—Molds may be easily and satisfactorily purified by the streak-plate and pour-plate procedures as used for the separation and propagation of bacterial species. These are discussed in detail on page 134.

*Microscopical Methods.*—Considerable information may be obtained by examining first dry, living cultures under the low-power objective. Petri dish cultures are placed on the stage of the microscope, with the lids removed, and examined by transmitted or reflected light. Aerial mycelium, conidiophores, fruiting heads, chains of spores, and other structures may be easily examined by this method. This gives a preliminary idea of what to look for when slide preparations are examined, since mold structures are easily broken when disturbed.

For high-power examination, slide preparations are necessary. Mold specimens are very difficult to remove from culture media without being greatly broken. Therefore, great care must be exercised in preparing satisfactory mounts. Water should not be used for the mounting fluid since it rapidly evaporates, produces a shrinkage of the hyphae by osmosis, and causes the various parts to adhere together as a tangled mass. Obviously, such preparations are unsatisfactory for accurate observations. Probably the most useful mounting fluid is that known as lactophenol. It has the following composition:

| | |
|---|---|
| Phenol, c.p. crystals | 20 gm. |
| Lactic acid, c.p. | 20 gm. |
| Glycerol, c.p. | 10 gm. |
| Distilled water | 20 gm. |

The solution is prepared by first dissolving the phenol in the water, then adding the lactic acid and glycerol.

This fluid does not cause shrinkage of the cells and does not evaporate, thus permitting permanent preparations to be prepared. A dye may be added to the fluid to stain the various mold structures. This is especially desirable for mounting molds that are to be photographed.

Molds are mounted by first placing a drop of lactophenol in the center of a clean glass slide. A small portion of the mold growth is removed from the culture and placed in the drop of fluid. It is gently teased out with a pair of needles until the various parts are well separated and wetted by the fluid. It is then carefully covered with a cover slip to avoid as far as possible air bubbles being entrapped.

For an excellent discussion of mycological methods, see the monograph by Smith (1942).

## BIOCHEMISTRY OF THE MOLDS

The biochemical activities of molds are of great importance in the industrial world. They are probably not so important in this respect as

are the yeasts and bacteria, but they do, nevertheless, produce certain changes not carried on by the other two members of the fungi group. A few of the more important biochemical changes induced by molds are the following:

**Alcoholic Fermentations.**—Alcohol is produced industrially by the fermentation of various sugars by yeasts. The raw materials consist of

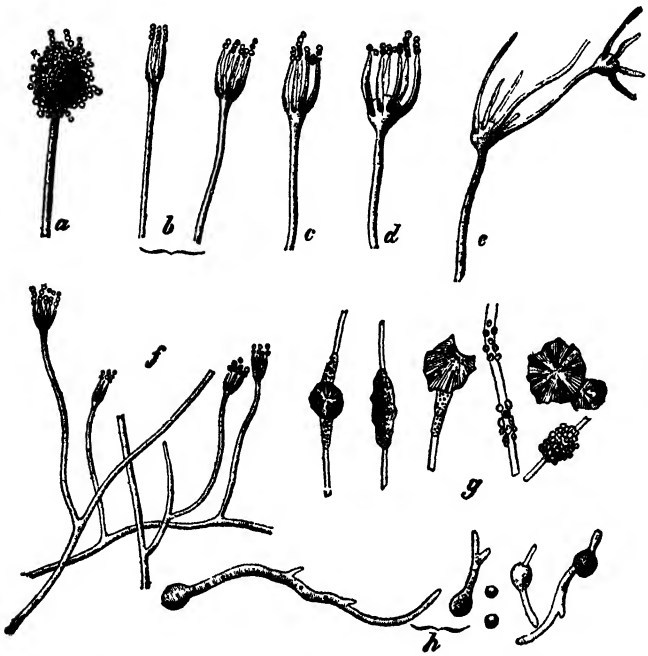



FIG. 91.—*Citromyces* (*Penicillium*) *pfefferianus*. *a*, conidiophore; *b-e*, conidiophores after removal of the conidia; *f*, conidiophores slightly magnified; *g*, hyphae, showing spherical, granular, or compact deposits of calcium citrate; *h*, germinating conidia. (*After Wehmer, from Lafar's Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

sugars, such as cane sugar, molasses, and glucose, and the polysaccharides, such as potato starch, cornstarch, barley starch, and cellulose. As a preliminary to their utilization by yeasts, starches and other polysaccharides must be first hydrolyzed to soluble sugars. A number of molds elaborate the necessary enzyme or enzymes that effect the saccharification of the various starches, after which certain yeasts are capable of fermenting the sugars to alcohol. Yeasts do not produce an amylase and are unable to ferment starches directly to alcohol.

Fitz (1873) was probably the first to show that the mold *Mucor mucedo* (now recognized as *M. racemosus*) was capable of elaborating the starch-hydrolyzing enzyme amylase. Since that time, many other molds have been shown to be capable of elaborating an amylase. The commercial

product, Takadiastase, which was first prepared by Takamine (1914) from some species of *Aspergillus*, is extensively used for the saccharification of starches (see Underkofler and Fulmer, 1943).

**Citric Acid.**—Wehmer (1893) showed that two species of *Citromyces* (now *Penicillium*) were capable of fermenting sugar with the production of citric acid (Fig. 91). Subsequently, it was shown that many species of *Penicillium* are also able to produce citric acid in varying amounts, but in no instance is the yield sufficiently large to enable the method to compete with the extraction of citric acid from lemons and other citrus fruits. Currie (1917) found that the yield could be greatly increased by employing the mold *Aspergillus niger*. The mold is employed commercially for this purpose, and in some localities it is as cheap to prepare citric acid by this method as it is from natural sources.

The carbohydrates that have been found suitable for citric acid fermentation include starch, sucrose, glucose, fructose, lactose, invert sugar, maltose, molasses, and the alcohol, glycerol. The yield varies considerably, depending upon the carbohydrate employed. The highest yield of citric acid is obtained when the medium contains about 15 per cent sucrose.

Currie found that the most favorable medium for citric acid production contained the following:

| | | |
|---|---|---|
| Sucrose | 150 | gm. |
| Ammonium carbonate | 2.5 | gm. |
| Potassium acid phosphate | 1.0 | gm. |
| Magnesium sulfate | 0.25 | gm. |
| Distilled water, to make | 1000 | cc. |

HCl to give a pH of about 3.5.

The fermentation yields oxalic acid and carbon dioxide besides citric acid. The oxalic acid is separated from the fermented medium by evaporation and crystallization leaving the citric acid in solution.

The reaction for the formation of citric acid from glucose is as follows:

$$C_6H_{12}O_6 + 3O \longrightarrow \begin{matrix} CH_2\cdot COOH \\ | \\ COH\cdot COOH \\ | \\ CH_2\cdot COOH \end{matrix} + 2H_2O$$

Glucose — Citric Acid

The intermediate steps are not clearly understood.

**Gluconic Acid.**—Molliard (1922) was one of the first to detect the presence of gluconic acid in cultures of molds. He used the organism *Sterigmatocystis nigra* and a culture medium containing sucrose. Later, Butkewitsch (1923) identified the same acid in cultures of *Aspergillus niger*. The acid is now known to be produced by a number of aspergilli and penicillia.

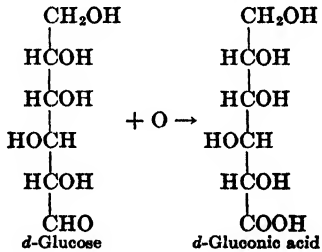
Gluconic acid can be more cheaply prepared by means of molds than by chemical methods. Herrick and May (1928) employed the mold *Penicillium purpurogenum* var. *rubrisclerotium* and found that a high yield of gluconic acid could be produced to the exclusion of other acids. They used the following culture medium:

| | |
|---|---|
| Glucose | 200.00 gm. |
| Magnesium sulfate | 0.25 gm. |
| Disodium phosphate | 0.10 gm. |
| Potassium chloride | 0.05 gm. |
| Sodium nitrate | 1.00 gm. |
| Distilled water, to make | 1000 cc. |

The medium produced a good yield in about 10 days at 25 to 30°C.

Moyer, May, and Herrick (1936) found that the mold *Penicillium chrysogenum* showed the greatest capacity for producing gluconic acid. However, the mold does not produce large quantities of spores, required for inoculating media. Wells, Moyer, Stubbs, Herrick, and May (1937) selected a strain of *Aspergillus niger* for gluconic acid production because it possessed certain desirable characteristics. It readily sporulated and produced uniform fermentations.

The reaction for the oxidation of glucose to gluconic acid is as follows:




**Cheeses.**—A number of cheeses are ripened by means of molds. These may be placed in two groups: (1) the soft cheeses of the Camembert and Brie types and (2) the green-streaked cheeses of the Roquefort, Gorgonzola, and Stilton types.

The cheeses in the first group are ripened by the mold *Penicillium camemberti*. The prepared curd is shaped into cakes, salted on the surface, and inoculated with the spores of the mold. The cakes are placed in a damp room where the mold rapidly multiplies on the surface, then gradually softens the entire mass of curd. The process requires about four weeks.

The cheeses in the second group are prepared by first inoculating the curd with a pure culture of *P. roqueforti*. The curd is then pressed so as to leave irregular cracks in the cake. The cake is aerated from time to time during the ripening process by piercing it with wires. The mold

produces a dense growth along the cracks, giving the finished product a streaked appearance.

**Antibiotic Substances.**—A large number of molds elaborate compounds which are actively bactericidal or bacteriostatic against certain bacteria. The best known representative of this group is penicillin, produced by the mold *P. notatum*. This important subject is discussed under Antibiosis (page 446).

**Miscellaneous Compounds.**—Molds produce a large number of compounds of minor industrial importance. They are nonnitrogenous, metabolic products and are probably the result of the action of the organisms on carbohydrates or carbohydrate-like compounds. The outstanding contributions on this subject have been made by Raistrick and his coworkers.

It should be noted that, on the whole, molds produce compounds of greater complexity than do bacteria. Most of the compounds that have been isolated and characterized are the following:

*Acids.*—Acetic, aconitic, allantoic, byssochlamic, carlic, carlosic, carolic, carolinic, citric, dimethylpyruvic, formic, fulvic, fumaric, fusarinic, gallic, gentisic, glaucic, glauconic, *d*-gluconic, glycuronic, glycolic, glyoxylic, helvolic, 2-hydroxymethylfurane-5-carboxylic, isovaleric, itaconic, kojic, $\gamma$-ketopentadecoic, lactic, luteic, malic, *d*-mannonic, 1- $\gamma$-methyltetronic, methyl salicylic, minioluteic, mycophenolic, oxalic, penicillic, puberulic, pyruvic, spiculisporic, stipitatic, succinic, and terrestric.

*Alcohols.*—Erythritol, ethyl alcohol, glycerol, and mannitol.

*Pigments.*—Aspergillin, aurantin, aurofusarin, auroglaucin, boletol, $\beta$-carotene, catenarin, chrysogenin, citrinin, citromycetin, cynodontin, erythroglaucin, flavoglaucin, fulvic acid, helminthosporin, monascoflavin, monascorubrin, ochracin, oösporin, phoenicin, physcion, ravenelin, rubrofusarin, and tritisporin.

*Polysaccharides.*—Capreolinose, galactocarolose, glycogen, luteic acid, mannocarolose, mycodextrin, rugulose, starch (mold), trehalose, and varianose.

*Sterols.*—Cholesterol, ergosterol, fungisterol, and phytosterol.

*Miscellaneous.*—Acetaldehyde, alboleersin, dimethylselenide, ergot, ergosteryl palmitate, ethyl acetate, gums, hydroxylamine, lipins, luteoleersin, palitantin, phenylethylamine, terrein, erdin, geodin, and griseofulvin.

For further reading, consult Birkinshaw (1937), Clutterbuck (1936), Fitzpatrick (1930), Henrici (1947), Johansen (1940), Lockwood and Moyer (1938), Prescott and Dunn (1940), Raistrick (1932, 1938, 1940), and Tatum (1944).

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# CHAPTER VII

## TECHNIQUE OF PURE CULTURES

### GENERAL CONSIDERATIONS

A culture may be defined as the active growth of microorganisms in or on nutrient media.

A mixed culture consists of two or more species of organisms growing together.

A pure culture consists of only one species of microorganism growing in or on nutrient media. Pure cultures are required for studying the morphology and physiology of organisms. All laboratory studies, with few exceptions, are based on the use of pure cultures. In a few instances two species are grown together in making studies of the various types of bacterial associations.

A plate culture consists of an organism growing on a solid medium contained in a Petri dish.

A slant culture consists of an organism growing on the inclined surface of a solid medium, such as nutrient agar. This is referred to specifically as a nutrient agar slant culture. Other types of solid media include coagulated blood serum, potato wedges, and coagulated egg. Cultures prepared in this manner are sometimes referred to as streak cultures. A nutrient agar slant culture may be called a "nutrient agar streak culture"; a coagulated blood serum slant culture may be called a "coagulated blood serum streak culture"; etc. Solid media prepared in the slanted position greatly increase the surface area exposed to air and result in a much greater growth of organisms.

A stab culture is one prepared by stabbing a solid medium, such as nutrient gelatin or nutrient agar, to a considerable depth with a previously inoculated straight wire needle. Gelatin medium is used for studying the character of liquefaction produced by certain organisms. If an agar medium containing a fermentable carbohydrate is used, the production of gas may be detected by the appearance of gas bubbles in the agar. In some cases, the agar may be split into disks with a layer of gas separating each disk.

A liquid culture consists of an organism growing in a liquid medium, such as nutrient broth, milk, or Dunham's peptone solution.

A shake culture is one prepared by inoculating a liquefied agar medium and rotating or shaking the tube to obtain a uniform suspension of organisms and agar before solidification occurs. A shake culture is valuable for indicating the oxygen requirements of an organism. Obligate anaerobic

organisms grow in the deeper portions of the medium; obligate aerobic organisms grow near or at the surface in the presence of oxygen.

### METHODS EMPLOYED FOR INOCULATION OF CULTURE MEDIA

The following procedures are recommended for the inoculation of various types of culture media:

**Agar Deep Cultures.**—Sterilize a wire needle in a flame and allow it to cool for about 5 sec. Remove the cotton stopper from an agar slant culture by grasping it with the small finger of the right hand, and flame the neck of the tube. Hold the tube slanted, not upright, to minimize aerial contamination. Remove a small amount of growth with the sterilized wire needle. Again flame the neck of the agar slant culture, replace the cotton stopper, and set the tube in the test-tube block. Remove the cotton stopper from the tube to be inoculated, by grasping it with the small finger of the right hand. Flame the neck of the tube. Stab the straight wire, containing the inoculum, to the bottom of the tube. Withdraw the needle carefully. Again flame the neck of the tube and replace the cotton stopper. Flame the wire needle before setting it down on the table. Mark the tube with a china marking pencil and incubate at the proper temperature.

If a transfer is to be made from a liquid culture, use a wire loop instead of a needle. Remove a loopful of the medium and force the wire loop to the bottom of the tube. Withdraw the loop carefully. The procedure in every other detail is the same as above.

**Agar Slant Cultures.**—Sterilize a wire needle or wire loop in the flame, depending upon whether a solid or a liquid culture is to be used. Allow the wire to cool for about 5 sec. Remove the cotton stopper from the culture, by grasping it with the small finger of the right hand, and flame the neck of the tube. Remove a small amount of the growth with the sterilized wire needle, or a loopful of the liquid culture with the wire loop. Again flame the neck of the culture, replace the cotton stopper, and set the tube in the test-tube block. Remove the cotton stopper from the agar slant to be inoculated, by grasping it with the small finger of the right hand. Flame the neck of the tube. Spread the inoculum over the surface of the agar slant by making streaks back and forth a few millimeters apart. Start at the butt of the slant and work up to the top. Withdraw the needle or loop from the tube. Again flame the neck of the tube and replace the cotton stopper. Flame the wire needle or loop before setting it down on the table. Mark the tube with a china marking pencil and incubate at the proper temperature.

**Broth Cultures.**—Follow the same procedure as used for the preparation of agar slant cultures except that the inoculating needle or loop is plunged into broth and shaken to dislodge the inoculum from the wire.

## ISOLATION OF SPECIES IN PURE CULTURE

Bacteria are rarely found in nature in pure culture. Mixed species are the rule. An organism must first be isolated and grown in pure culture before it can be studied accurately. Two different species growing together may produce reactions quite different from those given by each organism when studied separately.

A number of methods have been employed for the propagation of cultures from single cells. Most of these are too difficult and time-consuming to be of practical value except in certain special instances.

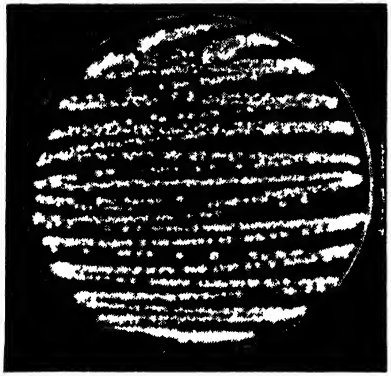
Plate cultures offer a means for isolating pure species of organisms in a comparatively simple manner. Two methods are generally followed: (1) the streak-plate method and (2) the pour-plate method.

**Streak-plate Method.**—Melt two tubes of nutrient agar in boiling water or in an Arnold sterilizer. Allow the agar to cool to about 50°C. Remove the cotton stopper from one of the tubes and flame the neck of the tube. Lift the lid of a sterile Petri dish just high enough to insert the opening of the test tube and pour the melted agar into the plate. In like manner, pour the second tube of melted agar into another sterile Petri dish. Great care must be observed in pouring agar from a test tube into a Petri dish to avoid external contamination. Always flame the neck of the test tube. Also, never raise the lid of the Petri dish any more than is necessary. Set both plates aside until the agar has become firm.

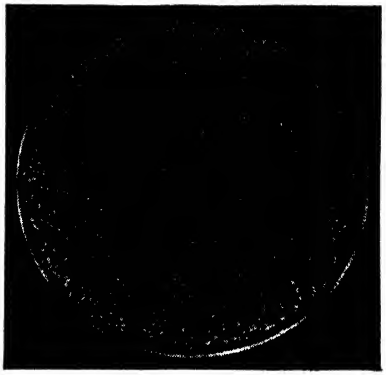
Sterilize the wire loop in a flame and allow it to cool for about 5 sec. Remove the cotton stopper from the broth culture, by grasping it with the small finger of the right hand, and flame the neck of the tube. Remove a loopful of the culture with the wire loop. Again flame the neck of the culture tube, replace the cotton stopper, and set the tube in the test-tube block. Raise the lid of the Petri dish high enough to insert the wire loop. Spread the loopful of culture at the upper end of the dish to thin it out; then make streaks back and forth with a free arm movement from the elbow, over the surface of the agar, about ¼ in. apart. The first streak will contain more of the culture than the second, the second streak more than the third, etc. The last streaks should thin out the culture sufficiently to give isolated colonies. It is usually advisable to streak a second plate, without reinoculating the wire loop. This gives greater certainty in securing well-isolated colonies. Each colony usually represents the growth from a single organism. Mark the plates with a china marking pencil and incubate at the proper temperature.

The colonies appear only on the surface of the agar. A pure culture may be obtained from a well-isolated colony by transferring a portion with the wire needle to an appropriate culture medium (Fig. 92).

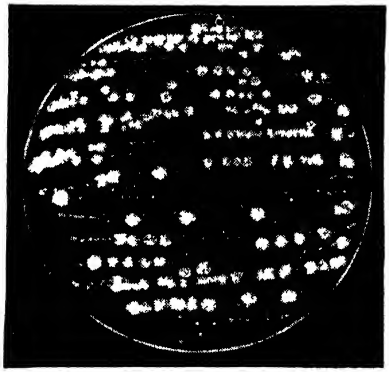
**Pour-plate Method.**—Melt three tubes of nutrient agar in boiling water or in an Arnold sterilizer. Allow the agar to cool to about 50°C.



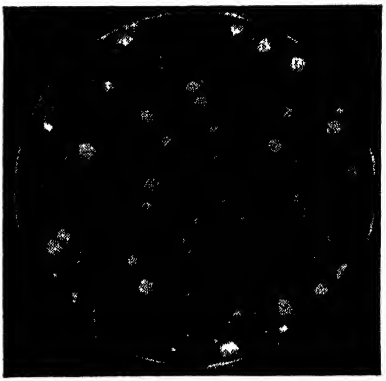
A



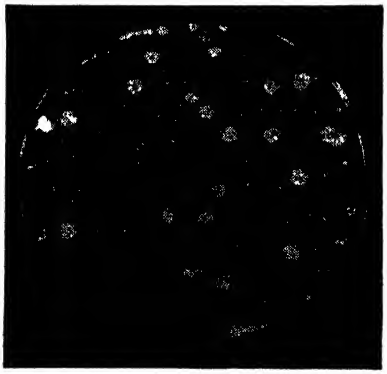
D



B



E



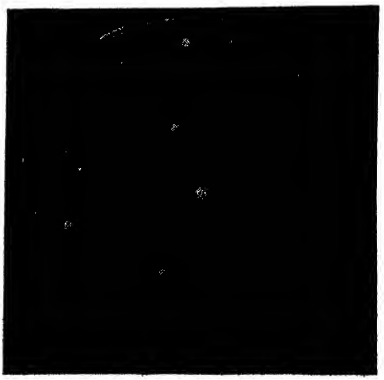


FIG. 92.—Isolation of species from a mixed culture of *Micrococcus pyogenes* var. *aureus* and *Bacillus subtilis*. Streak-plate method: *A*, one loopful of culture streaked over the surface of agar; *B*, without recharging loop, a second plate was streaked; *C*, without recharging loop, a third plate was streaked. Pour-plate method: *D*, tube of agar mixed with 1 loopful of culture and poured into a Petri dish; *E*, agar mixed with 1 loopful from tube *A* and poured into a Petri dish; *F*, agar mixed with 1 loopful from tube *B* and poured into a Petri dish.

Sterilize the wire loop in a flame and allow it to cool for about 5 sec. Remove the cotton stopper from the broth culture, by grasping it with the small finger of the right hand, and flame the neck of the tube. Remove a loopful of the culture with the wire loop. Again flame the neck of the

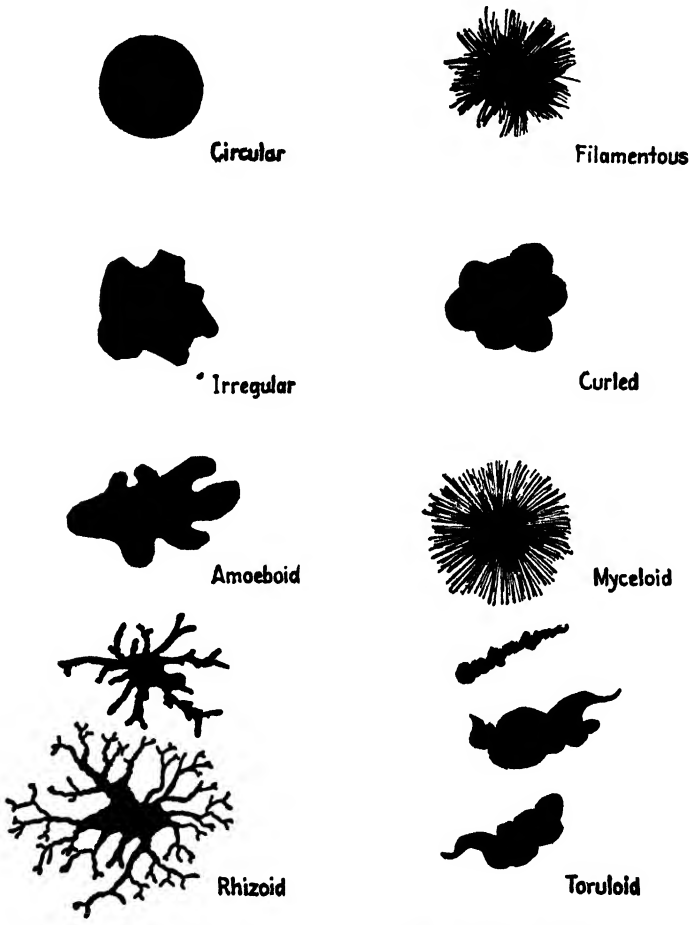



FIG. 93.—Shape or form of colonies. (*After Thomas.*)

culture tube, replace the cotton stopper, and set the tube in the test-tube block. Remove the cotton stopper from one of the tubes of melted and cooled agar, by grasping it with the small finger of the right hand, and flame the neck of the tube. Plunge the inoculating loop into the agar and shake to remove the inoculum from the wire before withdrawing from the tube. Again flame the neck of the tube and replace the cotton stopper. Flame the wire loop before setting it down on the table. Mix thoroughly by rotating the tube between the palms of the hands to obtain a uniform

suspension of organisms. Remove a loopful from tube 1 and transfer to tube 2. Again rotate the tube between the palms of the hands to mix thoroughly. Remove a loopful from tube 2 and transfer to tube 3. Mix thoroughly as before. Pour the inoculated agar into three sterile Petri

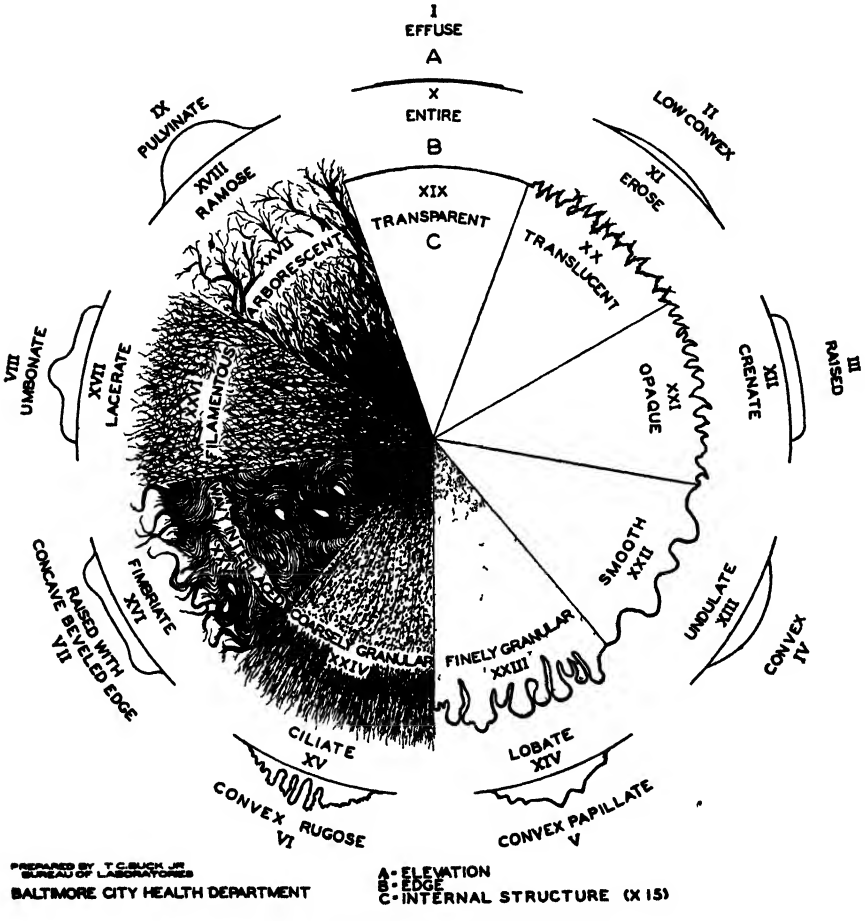



FIG. 94.—Bacterial colony formations.

dishes. When firm, incubate the plates in the inverted position at the proper temperature (Fig. 92).

In this procedure, most of the colonies are embedded in the agar, only a few appearing on the surface. The first agar plate usually contains too many organisms with the result that it is a difficult matter to find a well-isolated colony. The colonies are so crowded that they are not able to develop to their normal size. The second or third plate should show well-separated colonies of normal size. A pure culture may be obtained by

removing a portion of a well-separated colony with the wire needle and transferring it to an appropriate medium.

Well-isolated surface colonies are usually round and quite characteristic

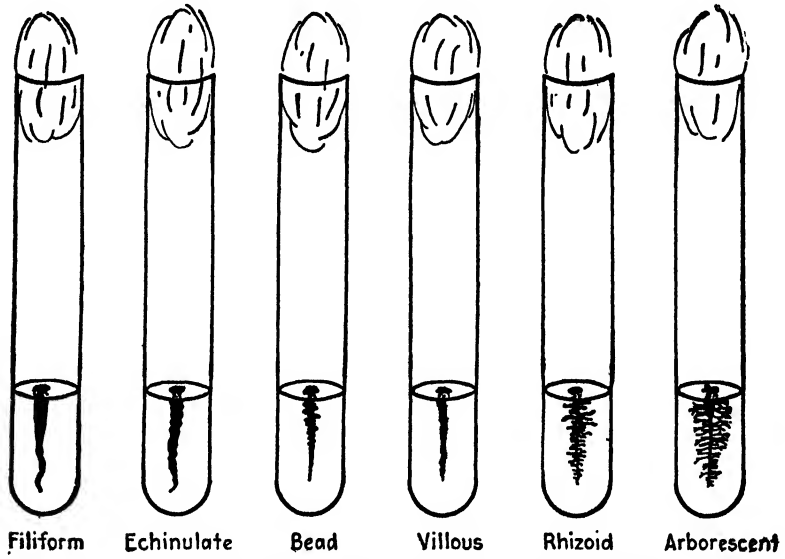



FIG. 95.—Growth in agar stab cultures. (*After Thomas.*)

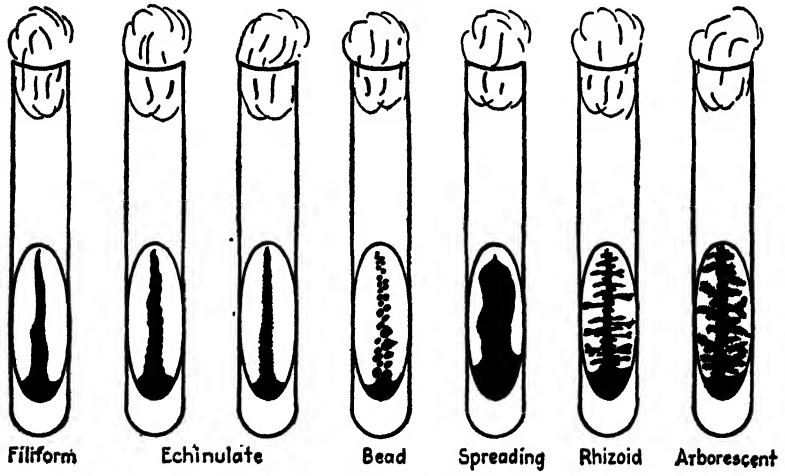



FIG. 96.—Growth on agar streak cultures. (*After Thomas.*)

for each species. On the other hand, colonies embedded in the agar are smaller in size and usually lenticular in shape. As a rule, it is not possible to distinguish between different species of subsurface colonies by their colonial appearance.

It is usually easier to obtain pure cultures by the pour-plate method because the organisms separate better when mixed with melted agar. Bacteria that produce mucoid colonies are very difficult to separate from nonmucoid organisms by the streak-plate method.

## IDENTIFICATION OF BACTERIAL SPECIES

A descriptive chart, prepared by the Committee on Bacteriological Technic of the Society of American Bacteriologists, will be found on pages 140 to 143. The chart is used for the identification and classification of bacteria.

**Colony Formation.**—Generally speaking, each bacterial species when

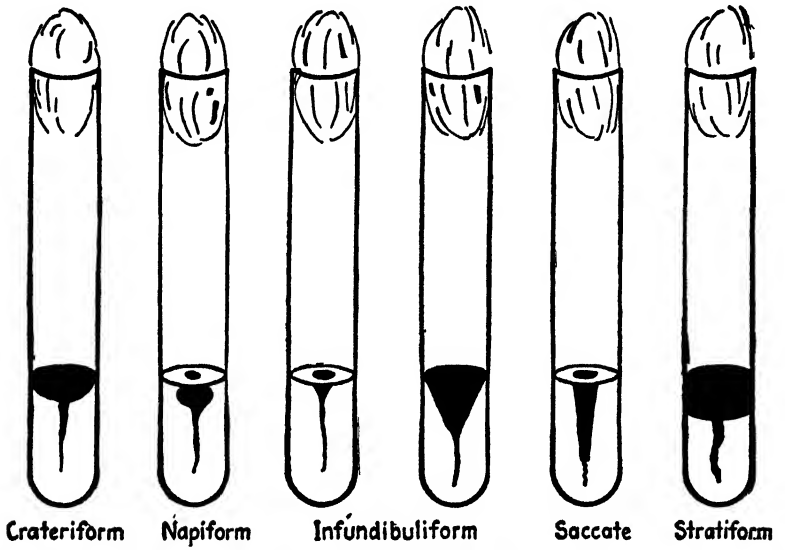



FIG. 97.—Growth in gelatin stab cultures. (*After Thomas.*)

grown on a standard solid medium, forms a characteristic type of colony. Colonies differ in size, shape, edge, elevation, and internal structure. The various characteristics are illustrated in Figs. 93 and 94.

**Stab and Streak Cultures.**—The type of growth on the surface and in the depth of agar media is characteristic for many bacterial species (Figs. 95 and 96). This applies also to the character of the liquefaction of gelatin stab cultures (Fig. 97). These various characteristics are made use of in the differentiation and classification of bacteria.

## PITFALLS IN THE USE OF THE CHARTS

In making routine tests for the identification of bacterial species the beginner is likely to run into a number of pitfalls, the most important of which are (1) the danger of contaminated cultures, (2) the dissociation of species into two or more phases, and (3) differences in methods of study.

Name of organism _____ Source _____

Date of isolation _____ Habitat _____

Is phase variation observed? _____ Phase on this Chart: S, R, M, G (smooth, rough, mucoid, gonidial) _____

Underscore required terms.

**Sketches**

**Vegetative Cells:** Medium used _____
Reaction (pH) _____ Temp. _____ Age _____ d.
Size of Majority _____
Ends, *rounded, truncate, concave, tapering*
**Motility:** In broth _____ On agar _____
**Sporangia and Endospores:** *present, absent.*
Medium used _____ pH _____ Temp. _____ Age _____ d.
Endospore Form: *spherical, ellipsoid, cylindrical*
**Irregular Forms:**
Present on _____ in _____ days at _____ °C.

**Agar Colonies:** Temperature _____ °C. Age _____ d.
Form, *punctiform (i.e. under 1 m.m. diam.), circular (i.e. over 1 m.m. diam.), filamentous, irregular, rhizoid.*
Surface, *smooth, rough, concentrically ringed, radiately ridged.*
Edge, *entire, undulate, lobate, erose, filamentous, curled.*
Elevation of growth, *effuse, flat, raised, convex.*
Optical Characters, *opaque, translucent, opalescent, iridescent.*

| Surface Colonies | Deep Colonies |
|---|---|
| | |

**Gelatin Colonies:** Temperature _____ °C. Age _____ d.
Form, *punctiform, circular, irregular, filamentous.*
Elevation, *flat, raised, convex, pulvinate, crateriform (liquefying).*
Edge, *entire, undulate, lobate, erose, filamentous, curled.*
Liquefaction, *cup, saucer, spreading.*
Surface, *smooth, contoured, rugose.*
Optical Characters, *opaque, translucent, opalescent, iridescent.*

| Surface Colonies | Deep Colonies |
|---|---|
| | |

**Agar Streak:** Temperature _____ °C. Age _____ d.
Growth, *scanty, moderate, abundant, none.*
Form of growth, *filiform, echinulate, beaded, spreading, arborescent, rhizoid.*
Lustre, *glistening, dull.*
Chromogenesis _____ *photogenic, fluorescent.*
Odor, *absent, decided, resembling* _____
Consistency, *butyrous, viscid, membranous, brittle.*
Medium, *grayed, browned, reddened, blued, greened, unchanged.*

Medium: Temperature _____ °C. Age _____ d.

**Nutrient Broth:** Temperature _____ °C. Age _____ d.
Surface growth, *ring, pellicle, flocculent, membranous, none.*
Clouding, *slight, moderate, strong, transient, persistent, none, fluid turbid, granular growth.*
Odor, *absent, decided, resembling* _____
Sediment, *compact, flocculent, granular, flaky, viscid.*
Amount of sediment, *abundant, scanty, none.*

Medium: Temperature _____ °C. Age _____ d.

**Gelatin Stab:** Temperature _____ °C. Age _____ d.
Growth, *uniform, best at top, best at bottom.*
Line of puncture, *filiform, beaded, papillate, villous, arborescent.*
Liquefaction, *none, crateriform, infundibuliform, napiform, saccate, stratiform:* begins in _____ d. complete in _____ d.
Degree of liquefaction in _____ days
Method used _____
Medium, *fluorescent, browned, unchanged.*

Medium: Temperature _____ °C. Age _____ d.

**Fermentation** Temperature _____ °C

| Medium _____ containing _____ _____ and: | Monosaccharides | | | | | | | Disaccharides | | | | | | | Polysaccharides | | | | | | Alcohols | | | | | | | Glucosides | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Arabinose | Rhamnose | Xylose | Glucose | Fructose | Galactose | Mannose | Lactose | Sucrose | Maltose | Trehalose | Melibiose | Cellobiose | | Raffinose | Melezitose | Starch | Inulin | Dextrin | Glycogen | Glycerol | Erythritol | Arabitol | Adonitol | Mannitol | Sorbitol | Dulcitol | Salicin | Aesculin | Coniferin | a-Methyl Gluc. | |
| *Gas in _____ fermentation tube* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Amt. $CO_2$ in Eldredge tube* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Reaction (pH) after _____ d.* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Titrable acidity in ml. of N/ _____ Na OH* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

Studied by _____ Culture No. _____
Optimum conditions: Media _____ Temp. _____ °C.
Phases recorded on other charts: _____

Brief Characterisation

As each of the following characteristics is determined, indicate in proper marginal square by means of figure, as designated below. In case any of these characteristics are doubtful or have not been determined, indicate with the letters U, V, and X according to the following code:

U, undetermined; V, variable; X, doubtful.

| | | | |
|---|---|---|---|
| Morphological | Vegetative Cells | Form & arrangement: 1, streptococci; 2, diplococci; 3, micrococci; 4, sarcinae; 5, rods; 6, commas; 7, spirals; 8, branched rods; 9, filamentous | |
| | | Diameter: 1, under 0.5$\mu$; 2, between 0.5$\mu$ and 1$\mu$; 3, over 1$\mu$ | |
| | | Gram stain: 0, negative; 1, positive | |
| | | Flagella: 0, absent; 1, peritrichic; 2, polar; 3, present but undetermined | |
| | | Capsules: 0, absent; 1, present | |
| | | Chains (4 or more cells): 0, absent; 1, present | |
| | Sporangia: 0, absent; 1, elliptical; 2, short rods: 3, spindled; 4, clavate; 5, drumsticks | | |
| | Endospores: 0, absent; 1, central to excentric; 2, subterminal; 3, terminal | | |
| Cultural | Agar Stroke | Growth: 0, absent; 1, abundant; 2, moderate; 3, scanty | |
| | | Lustre: 1, glistening; 2, dull | |
| | Agar Colonies | Form: 1, punctiform; 2, circular (over 1 mm. diameter); 3, rhizoid; 4, filamentous; 5, curled; 6, irregular | |
| | | Surface: 1, smooth; 2, contoured; 3, rugose | |
| | Gelatin Colonies | Form: 1, punctiform; 2, circular (over 1 mm.); 3, irregular; 4, filamentous | |
| | | Surface: 1, smooth; 2, contoured; 3, rugose | |
| Physiological | Biologic relationships: 1, pathogenic for man; 2, for animals but not for man; 3, for plants; 4, parasitic but not pathogenic; 5, saprophytic; 6, autotrophic | | |
| | Relation to free oxygen: 1, strict aerobe; 2, facultative anaerobe; 3, strict anaerobe; 4, microaerophile | | |
| | In nitrate media: 0, neither nitrite nor gas; 1, both nitrite and gas; 2, nitrite but no gas; 3, gas but no nitrite | | |
| | Chromogenesis: 0, none; 1, pink; 2, violet; 3, blue; 4, green; 5, yellow; 6, orange; 7, red; 8, brown; 9, black | | |
| | Other photic characters: 0, none; 1, photogenic; 2, fluorescent; 3, iridescent | | |
| | Indole: 0, negative; 1, positive | | |
| | Hydrogen sulfide: 0, negative; 1, positive | | |
| | Hemolysis: 0, negative; 1, positive | | |
| | Methemoglobin: 0, negative; 1, positive | | |
| | Protein Liquefaction or Digestion | Gelatin: 0, negative; 1, positive | |
| | | Casein: 0, negative; 1, positive | |
| | | Egg albumin: 0, negative; 1, positive | |
| | | Blood serum: 0, negative; 1, positive | |
| | Indicator Reduction | Litmus: 0, negative; 1, positive | |
| | | Methylene blue: 0, negative; 1, positive | |
| | | Janus green: 0, negative; 1, positive | |
| | Rennet production: 0, negative; 1, positive | | |
| | | | |
| | | | |

SUPPLEMEN

### Temperature Relations

Medium _____ pH _____
Optimum temperature for growth _____ °C.
Maximum temperature for growth _____ °C.
Minimum temperature for growth _____ °C.

THERMAL DEATH POINT: Time 10 minutes: _____ °C.
Medium _____ pH _____

THERMAL DEATH TIME:
Medium _____ pH _____

| Temp. | Time | Temp. | Time |
|---|---|---|---|
| ____ °C. | ____ min. | ____ °C. | ____ min. |
| ____ °C. | ____ min. | ____ °C. | ____ min. |
| ____ °C. | ____ min. | ____ °C. | ____ min. |
| ____ °C. | ____ min. | ____ °C. | ____ min. |
| ____ °C. | ____ min. | ____ °C. | ____ min. |

### Chromogenesis

Gelatin _____
Agar _____
Potato _____

### Other Photic Characters

Photogenesis on _____
Iridescence on _____
Fluorescence in _____

### Relation to Reaction (pH) of Medium

Medium _____
Optimum for growth: *about pH* _____
Limits for growth: *from pH* _____ *to* _____

### Relation to Free Oxygen

Method _____
Medium _____ Temp. _____ °C.
Aerobic growth: *absent, present, better than anaerobic growth, micro-aerophilic*
Anaerobic growth: *absent, occurs in presence of glucose, of sucrose, of lactose, of nitrate; better than aerobic growth*
Additional data: _____

### Milk

Temperature _____ °C.

| | | | |
|---|---|---|---|
| Reaction: | ____ d. ____; | ____ d. ____; | ____ d. ____ |
| Acid curd: | ____ d. ____; | ____ d. ____; | ____ d. ____ |
| Rennet curd: | ____ d. ____; | ____ d. ____; | ____ d. ____ |
| Peptonisation: | ____ d. ____; | ____ d. ____; | ____ d. ____ |

### Litmus Milk

Temperature _____ °C

| | | | |
|---|---|---|---|
| Reaction: | ____ d. ____; | ____ d. ____; | ____ d. ____ |
| Acid curd: | ____ d. ____; | ____ d. ____; | ____ d. ____ |
| Rennet curd: | ____ d. ____; | ____ d. ____; | ____ d. ____ |
| Peptonisation: | ____ d. ____; | ____ d. ____; | ____ d. ____ |

Reduction of litmus *begins in* ____ *days, ends in* ____ *days*

## PATHOLOGY

### Animal Inoculation

Medium used _____ Age of culture _____ Amount _____ Incubation period _____

| | | Whole culture | | | Cells | | | Filtrate | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Animal | | | | | | | | | | |
| Type of Injection | Subcutaneous | * | | | | | | | | |
| | Intraperitoneal | | | | | | | | | |
| | Intravenous | | | | | | | | | |
| | Per os | | | | | | | | | |

* In each instance where pathogenicity is observed, indicate location of lesion, and type, *e.g.* edema, histolysis, gas, hemorrhage, ulcer, diphtheritic, etc.

### Antigenic Action

Animal _____ Medium used _____ Age of culture _____
Type injection _____ Number of injections _____
Culture causes production of *cytolysins, agglutinins, precipitins, antitoxin.*
Specificity: Antibodies produced effective against other antigens as follows _____

Immune sera from _____
_____ effective against this organism as antigen

This DESCRIPTIVE CHART presented at the annual meeting of the SOCIETY OF AMERICAN
Prepared by a sub-committee consisting

TARY DATA

**Action on Erythrocytes**

Cells: _____
Method: *plate, broth, filtrate*
Hemolysis: *negative, positive*
Methemoglobin: *negative, positive*

**Production of Indole**

Medium _____
Test used _____
Indole *absent, present in* _____ *days*

**Production of Hydrogen Sulfide**

Medium _____
Test used _____
$H_2S$ *absent, present in* _____ *days*

**Action on Nitrates**

Medium _____ Temp. _____ °C.
Nitrite: ___d.___; ___d.___; ___d.___; ___d.___
Gas ($N_2$): ___d.___; ___d.___; ___d.___; ___d.___
Medium _____ Temp. _____ °C.
Nitrite: ___d.___; ___d.___; ___d.___; ___d.___
Gas ($N_2$): ___d.___; ___d.___; ___d.___; ___d.___
Ammonia production (in amino-N-free nitrate medium): *negative, positive*
Complete disappearance of nitrate in _____ medium: *negative, positive*
Disappearance of 2 p.p.m. nitrite in _____ medium: *negative, positive*

**Reduction of Indicators**

Medium _____ pH _____ Temp. _____ °C.

| Indicator | Conc. | Reduction: | |
|---|---|---|---|
| _____ | _____ % | ___ hr. ___; | ___ hr. ___ |
| _____ | _____ % | ___ hr. ___; | ___ hr. ___ |
| _____ | _____ % | ___ hr. ___; | ___ hr. ___ |
| _____ | _____ % | ___ hr. ___; | ___ hr. ___ |

**Staining Reactions**

Gram: ___ d. ___; ___ d. ___; ___ d. ___; ___ d. ___
Method _____
Spores: Method _____
Capsules: Method _____
Medium _____
Flagella: Method _____
Special Stains _____

**Additional Tests**

Methyl red: *negative, positive*
Voges-Proskauer: *negative, positive*
Growth in sodium citrate: *absent, present*
Growth in uric acid: *absent, present*
Hydrolysis of starch: *complete (iodine colorless); partial (iodine reddish-brown); none (iodine blue)*
Nitrogen obtained from the following compounds: _____

SPECIAL TESTS

BACTERIOLOGISTS, Dec. 28, 1934, by the Committee on Bacteriological Technic.
of M. W. Jennison and H. J. Conn.

**Contaminated Cultures.**—Needless to say, the beginner cannot be too careful in handling and making transfers from cultures to prevent external contamination. Unless a culture is kept pure and viable, results of tests are certain to be misleading.

**Dissociation of Species.**—During the early years of bacteriology, most bacteriologists believed that a bacterial species could exist in more than one cell form. Some years later, this concept was altered in favor of the idea of a fixed cell form for each species. Forms that departed more or less widely from the normal types were usually dismissed as being either involution forms, degenerate cells, or different species present as contaminants. At the present time, sufficient evidence has accumulated to support the original hypothesis of variability in the morphological characters of an organism. As a result of this change in viewpoint, it is easy for a careless beginner to believe that he is observing two phases in a culture when in reality one of the "phases" observed is a contaminant. Conversely, it is also easy for the beginner to consider a culture composed of two different organisms when actually they are different phases of the same species.

**Differences in Methods of Study.**—In order that results of tests have significance, it is necessary to include the procedures employed. For example, it is not sufficient to state that an organism does or does not produce hydrogen sulfide without including the conditions under which it was investigated. It is necessary to mention the composition of the culture medium, the indicator incorporated in the medium to test for hydrogen sulfide, the temperature of incubation, and the length of the incubation period, otherwise disagreements in results are likely to occur.

### Glossary of Terms Used on the Descriptive Chart[1]

A number of scientific terms are used on the descriptive chart to describe the various characteristics of organisms growing on different media. These terms, together with a number of others, are defined as follows:

**Acid curd,** coagulation of milk due to acid production.

**Adherent,** applied to sporangium wall, indicates that remnants of sporangium remain attached to endospore for some time.

**Aerobic,** growing in the presence of free oxygen; strictly aerobic, growing only in the presence of free oxygen.

**Agglutinin,** an antibody having the power of clumping suspensions of bacteria.

**Anaerobic,** growing in the absence of free oxygen; strictly anaerobic, growing only in the absence of free oxygen; facultative anaerobic, growing both in presence and in absence of oxygen.

**Antibody,** a specific substance produced by an animal in response to the introduction of an antigen.

[1] Taken from Leaflet I of "Manual of Methods for Pure Culture Study of Bacteria," Society of American Bacteriologists, Biotech Publications, Geneva, N.Y., 1944.

**Antigen,** a substance which, when introduced into an animal body, stimulates the animal to produce specific bodies that react or unite with the substance introduced.
**Antigenic action,** behavior as an antigen.
**Antitoxin,** an antibody having the power of uniting with or destroying a toxic substance.
**Arborescent,** branched, tree-like in growth.
**Aseptically,** without permitting microbial contamination.
**Autotrophic,** able to grow in absence of organic matter.

**Bacteriocidal,** capable of killing bacteria.
**Bacteriostasis,** prevention of bacterial growth, but without killing the bacteria.
**Beaded,** (in stab or stroke culture) separate or semiconfluent colonies along the line of inoculation.
**Bipolar,** at both poles or ends of the bacterial cell.
**Bleb,** vesicle or blister-like swelling.
**Brittle,** growth dry, friable under the platinum needle.
**Butyrous,** showing growth of butter-like consistency.

**Capsule,** an envelope surrounding the cell membrane of some kinds of bacteria.
**Chains,** four or more bacterial cells attached end to end.
**Chromogenesis,** the production of color.
**Clavate,** club-shaped.
**Compact,** referring to sediment in the form of a single fairly tenacious mass.
**Complement,** a nonspecific enzyme-like substance, destroyed if subjected to heat (56°C. or over for 30 min.) which occurs in blood serum, and is necessary, in conjunction with a specific antibody, in order to bring about cytolysis.
**Concentrically ringed,** marked with rings, one inside the other.
**Contoured,** having an irregular, smoothly undulating surface, like that of a relief map.
**Crateriform,** referring to a saucer-shaped liquefaction of the medium.
**Cuneate,** wedge-shaped.
**Curled,** composed of parallel chains in wavy strands, as in anthrax colonies.
**Cytolysin,** an antibody causing cytolysis.
**Cytolysis,** a dissolving action of cells.

**Diastatic action,** conversion of starch into simpler carbohydrates, such as dextrins or sugars, by means of diastase.
**Diphtheritic,** diphtheria-like.
**Dissociation,** separation of characters, usually referring to phase variation (*q.v.*).

**Echinulate,** showing a growth along the line of inoculation with toothed or pointed margins.
**Edema,** intercellular accumulation of fluid in a part of an animal body.
**Effuse,** of thin growth, veily, unusually spreading.
**Endospores,** thick-walled spores formed within the bacteria; *i.e.*, typical bacterial spores like those of *B. anthracis* or *B. subtilis*.
**Endotoxin,** a toxic substance produced within a microorganism and not excreted.
**Enzyme,** a chemical ferment produced by living cells.
**Erose,** irregularly notched.
**Excentric,** slightly to one side of the center, between the positions denoted central and subterminal.
**Exogenous,** originating outside the organism.
**Exotoxin,** a toxic substance excreted by a microorganism and hence found outside the cell body.

**Facultative anaerobe,** see anaerobic.
**Filamentous,** denoting growth composed of long, irregularly placed or interwoven threads.
**Filaments,** as applied to morphology of bacteria, refers to thread-like forms, generally unsegmented; if segmented, the organisms are enclosed in a sheath.
**Filiform,** in stroke or stab cultures, a uniform growth along line of inoculation.
**Flagellum** (pl. **-la**), a motile, whip-like attachment; an organ of locomotion.
**Flaky,** refers to sediment in the form of numerous separate flakes.
**Flocculent,** containing small adherent masses of various shapes floating in the field.
**Fluorescent,** having one color by transmitted light and another by reflected light.

**Gonidia,** asexual spores.
**Gonidial,** referring specifically to a bacterial phase producing gonidia-like bodies.
**Granular,** composed of small granules.

**Hemolysin,** a substance causing hemolysis either alone or in the presence of complement.
**Hemolysis,** a dissolving action on red blood corpuscles.
**Hemorrhage,** an escape of blood from the vessels.
**Histolysis,** breaking down of tissues.
**Hydrolysis of starch,** destruction of starch by the formation of a chemical union with water; includes diastatic action, but is a more general term.

**Immune serum,** an animal fluid containing an antibody.
**Inactivate,** to destroy complement by heat (at 56°C. for 30 min.).
**Infundibuliform,** in form of a funnel or inverted cone.
**Intraperitoneal,** within the peritoneum.
**Intravenous,** within a vein.
**Iridescent,** exhibiting changing rainbow colors in reflected light.

**Lesion,** a local injury or morbid structural change.
**Lobate,** having lobes, or rounded projections.

**Maximum temperature,** temperature above which growth does not take place.
**Membranous,** of thin growth, coherent, like a membrane.
**Metabolite,** a substance produced by metabolism.
**Microaerophilic,** growing best in presence of small quantities of oxygen.
**Minimum temperature,** temperature below which growth does not take place.
**Mucoid,** mucus-like, referring specifically to a bacterial phase producing slimy growth.
**Mycelioid,** colonies having the radiately filamentous appearance of mold colonies.

**Napiform,** denoting liquefaction in form of a turnip.

**Ontogenetic,** pertaining to the life history of an individual.
**Opalescent,** milky white with tints of color as in an opal.
**Opaque,** not allowing light to pass through.
**Optimum temperature,** temperature at which most growth occurs.

**Papillate,** denoting growth beset with small nipple-like processes.
**Parasitic,** deriving its nourishment from some living animal or plant upon which it lives and which acts as host; not necessarily pathogenic.
**Pathogenic,** not only parasitic (*q.v.*) but also causing disease in the host.

**Pellicle,** bacterial growth forming either a continuous or an interrupted sheet over the culture fluid.

**Peptonization,** rendering curdled milk soluble by the action of peptonizing enzymes.

**Peritrichiate,** applied to the arrangement of flagella, indicates that they are distributed over the entire surface of an organism.

**Peritrichic,** having flagella in peritrichiate arrangement.

**Per os,** through the mouth.

**Persistent,** lasting many weeks or months.

**Phase variation,** separation of a species into strains, having somewhat different characters.

**Photogenic,** glowing in the dark, phosphorescent.

**Polar,** at the end or pole of the bacterial cell.

**Precipitin,** an antibody having the power of precipitating soluble proteins.

**Pulvinate,** cushion-shaped.

**Punctiform,** very small, but visible to naked eye; under 1 mm. in diameter.

**Raised,** denoting thick growth, with abrupt or terraced edges.

**Reduction,** removal of oxygen or its equivalent from a chemical compound; or addition of hydrogen or its equivalent. Refers to the conversion of nitrate to nitrite, ammonia, or free nitrogen; also to the decolorization of litmus.

**Rennet curd,** coagulation of milk due to rennet or rennet-like enzymes, distinguished from acid curd by the absence of acid.

**Rhizoid,** growth of an irregular branched or root-like character, as *B. mycoides.*

**Ring,** growth at the upper margin of a liquid culture, adhering to the glass.

**Rugose,** wrinkled.

**Saccate,** liquefying in the form of an elongated sac, tubular, cylindrical.

**Saprophytic,** living on dead growth in the absence of organic matter, *i.e.,* neither autotrophic (*q.v.*) nor parasitic.

**Sensitize,** to render sensitive, usually to a foreign protein.

**Sepsis,** a state of infection.

**Sheath,** an envelope similar to a capsule (*q.v.*), but surrounding a filamentous organism.

**Spindled,** larger at the middle than at the ends. Applied to sporangia, refers to the forms frequently called "clostridia."

**Sporangium** (pl. **-ia**), cells containing endospores.

**Spreading,** denoting growth extending much beyond the line of inoculation, *i.e.,* several millimeters or more.

**Stratiform,** liquefying to the walls of the tube at the top and then proceeding downward horizontally.

**Strict aerobe,** see aerobic.

**Strict anaerobe,** see anaerobic.

**Subcutaneous,** under the skin.

**Subterminal,** situated toward the end of the cell but not at the extreme end, *i.e.,* between the positions denoted excentric (*q.v.*) and terminal.

**Synergism,** cooperative action of two organisms, resulting in an end product which neither could produce alone.

**Thermophilic,** growing best at high temperatures, *i.e.,* 50°C. or over.

**Toxic,** poisonous.

**Transient,** lasting a few days.

**Translucent,** allowing light to pass through without allowing complete visibility of objects seen through the substance in question.

**Trituration,** thorough grinding in a mortar.
**Truncate,** with ends abrupt, square.
**Turbid,** cloudy with flocculent particles, *i.e.*, cloudy plus flocculent.

**Ulcer,** an open sore.
**Undulate,** wavy.

**Villous,** having short, thick, hair-like processes on the surface, intermediate in meaning between papillate and filamentous (*q.v.*).
**Virulence,** degree of pathogenicity (referring to infectiousness).
**Virus,** a self-propagating cause of disease, often referring to one too small to be seen with a microscope.
**Viscid,** denoting growth that follows the needle when touched and withdrawn; or referring to sediment that on shaking rises as a coherent swirl.

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## CHAPTER VIII

## EFFECT OF ENVIRONMENT UPON BACTERIA

It is well known that the life activities of organisms are conditioned by their environment. Any marked change in the environment produces a corresponding change in the morphological and physiological characteristics of organisms. Bacteria quickly adapt themselves to the new conditions and, for this reason, are able to withstand great variations in the environment. In this respect, they differ markedly from higher plant and animal cells. By understanding the various physical factors controlling survival and multiplication, bacterial activity may be either increased, decreased, or destroyed completely.

Bacteria multiply normally by binary or transverse fission. The rate at which division takes place can be made to vary widely. Any alteration in the time between consecutive cell divisions (generation time) indicates that one or more environmental factors have changed.

Destruction of bacteria by physical agents, such as low temperature, heat, ultraviolet irradiation, osmotic pressure, desiccation, surface tension, and sonic waves, follows a monomolecular reaction, where only one substance undergoes change and in which the velocity of the reverse reaction is negligible. The disinfection process does not take place suddenly but is a gradual operation in which the number of organisms killed in unit time is greater at the beginning and becomes less and less as the action proceeds. If the numbers of survivors in unit time are plotted against time and lines are drawn, the points lie on smooth curves. On the other hand, if the logarithms of the numbers of survivors are plotted against time, the points fall on a straight line (page 198). This is a general rule applicable to all agents employed for the destruction of microorganisms.

### EFFECT OF LOW TEMPERATURE

Bacteria are able to survive wide limits of temperature, but the range in which they can grow and carry on their life activities falls between 0 and 90°C.

Most bacteria fail to multiply at temperatures 5 to 10°C. above the freezing point. Even though they are not frozen, they slowly die. When the water surrounding a suspension of cells changes to ice, the water inside the cells probably freezes also. In the frozen condition, all metabolic activity ceases. Hilliard and Davis (1918) found that all cells in a suspension

of *Salmonella typhosa* were destroyed by alternate freezing and thawing for five times. On the other hand, a suspension of the same organisms frozen to − 1°C. and kept at that temperature for 5 days showed many viable cells.

The most common cause of death by freezing is probably due to injury by ice crystals. Bacteria in the frozen state die at a very slow rate.

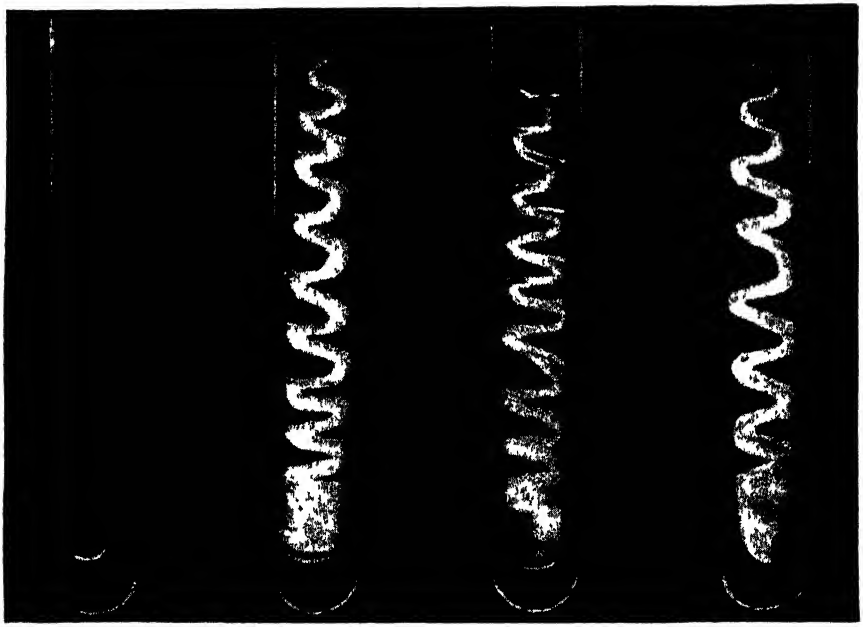


FIG. 98.—Effect of incubation at 15°C. upon growth. *A*, *Bacillus viridulus; B*, *Bacillus subtilis; C*, *Escherichia coli; D*, *Flavobacterium brunneum.*

Organisms survive for years when frozen, and it is not safe to attempt to sterilize foods, water, or liquids by freezing.

Luyet and Gehenio (1940) found that rapid freezing of bacteria to very low temperatures, *e.g.*, by immersion in liquid air, proved to be rather harmless. During freezing, the water is not changed to ice crystals but to a glass-like or vitreous, amorphous substance. If cells are vitrified without the formation of ice crystals, they can be held at low temperatures for long periods of time. During slow thawing, the water may change to ice crystals and cause death of the bacteria. However, rapid thawing may prevent the formation of ice crystals with the result that the bacteria are not killed.

Sherman and Naylor (1942) reported that young cells of *Escherichia coli* held at 1°C. remained physiologically young throughout an experimental period of 36 days. The death rate of such cells was faster than that

of more mature cells. On the other hand, young cells of *Streptococcus lactis*, kept at 1°C. progressively aged and exhibited the properties of mature cells after one week. Such cells, because of their ability to age at 1°C., showed greater viability than mature cells.

**Cold Shock.**—Young bacteria may be sensitive to sudden changes in temperature. If they are quickly cooled from 45 to 10°C., as many as

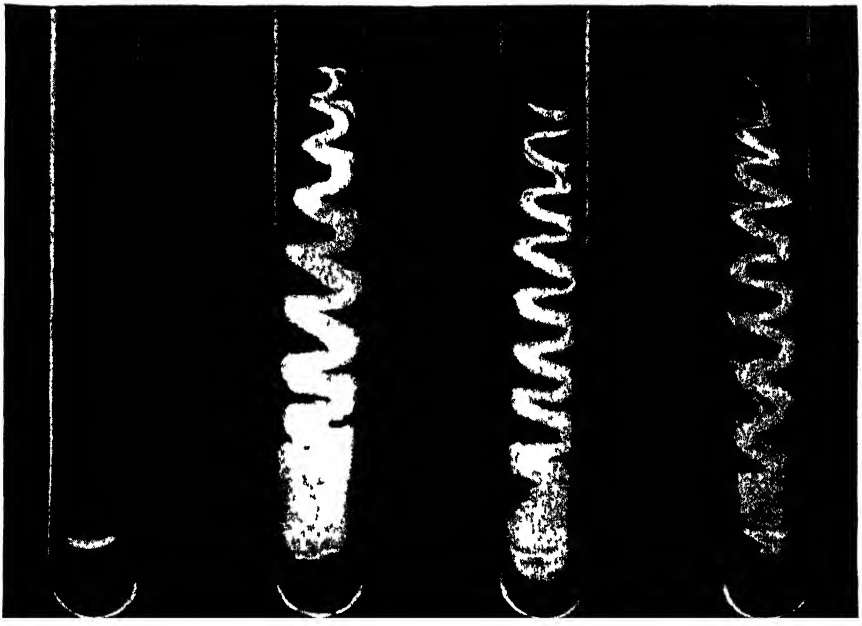


FIG. 99.—Effect of incubation at 25°C. upon growth. *A*, *Bacillus viridulus; B*, *Bacillus subtilis; C*, *Escherichia coli; D*, *Flavobacterium brunneum.*

95 per cent of the cells may be killed, whereas gradual cooling produces very little, if any, effect. Old cells are considerably less sensitive to a sudden drop in temperature. The cause of death from cold shock is not known.

For additional information, see Weiser and Osterud (1945) and Weiser and Hargiss (1946).

## EFFECT OF HEAT

There exists for every organism a maximum, a minimum, and an optimum temperature for growth.

**Maximum Growth Temperature.**—The maximum growth temperature may be defined as the highest temperature at which growth and multiplication occur, when the other environmental factors are kept constant. The psychrophilic organisms, *i.e.*, those that grow at low temperatures, do not

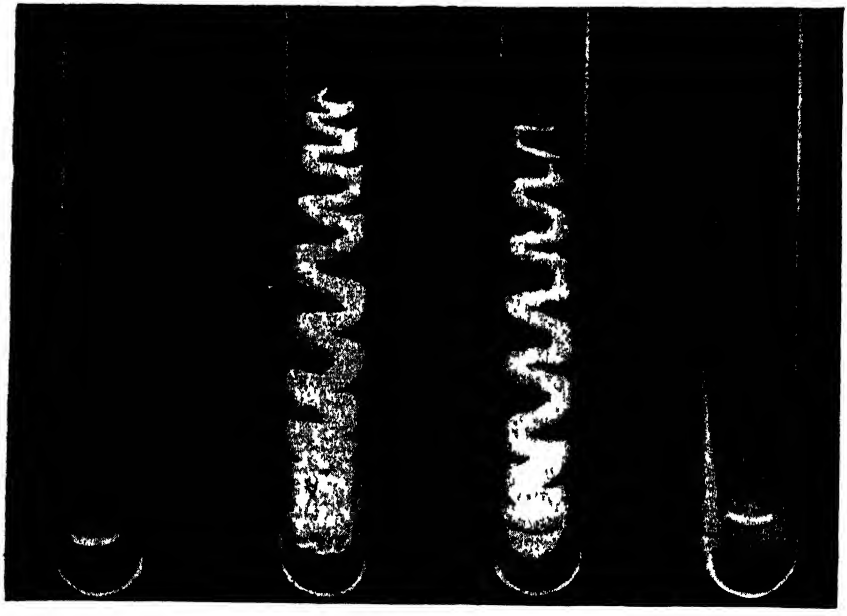


FIG. 100.—Effect of incubation at 37°C. upon growth. *A, Bacillus viridulus; B, Bacillus subtilis; C, Escherichia coli; D, Flavobacterium brunneum.*

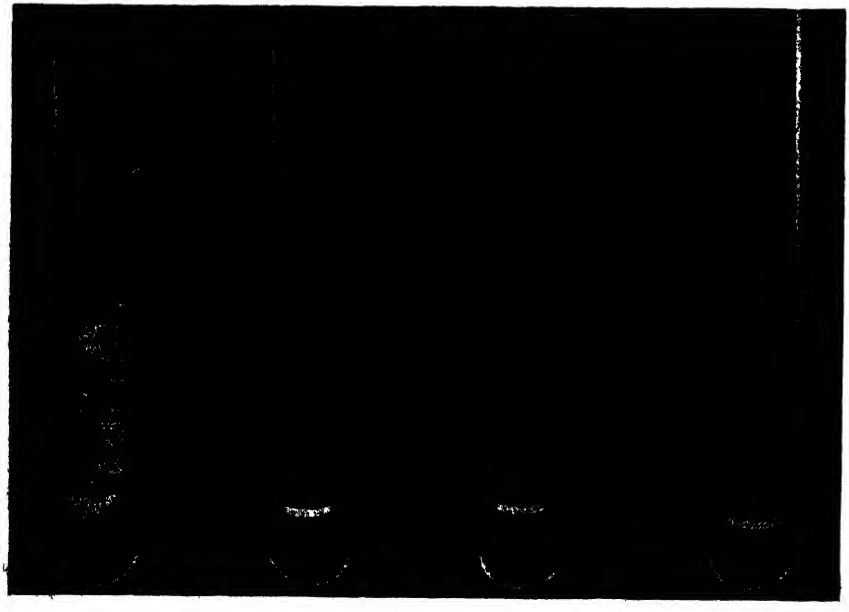


FIG. 101.—Effect of incubation at 55°C. upon growth. *A, Bacillus viridulus; B, Bacillus subtilis; C, Escherichia coli; D, Flavobacterium brunneum.*

develop well even at room temperature. Saprophytic mesophilic organisms show a maximum at about 30°C. A majority of the pathogenic forms for man fall between 40 and 50°C. The thermophilic or heat-loving bacteria may show growth at temperatures of 60 to 70°C., or even higher.

**Optimum Growth Temperature.**—The optimum temperature is the most favorable temperature for growth. The psychrophilic organisms have an optimum temperature below 20°C. These organisms are found in cold lake and spring waters and in brines kept under cold-storage conditions. This group includes many of the pigment-producing bacteria.

The mesophilic organisms have optimum temperatures of 18 to 45°C. The saprophytic mesophils grow best at temperatures of 18 to 25°C.; the parasitic mesophils grow best at the temperature of the host.

The thermophilic bacteria vary greatly in their temperature optima. Many possess an optimum temperature of about 55°C. They occur in soil, manure, excreta, decaying organic matter, etc. Owing to their great resistance to heat, they are the source of considerable trouble in the canning industry.

The effect of different temperatures of incubation on the growth of *Bacillus viridulus*, *B. subtilis*, *Escherichia coli*, and *Flavobacterium brunneum* is shown in Figs. 98 to 101.

**Minimum Growth Temperature.**—The minimum growth temperature is the lowest temperature at which growth and multiplication occur. This temperature will also show variation when one or more environmental factors are changed.

The multiplication rate of an organism is exceedingly slow at the minimum temperature. As the temperature is lowered from the optimum to the minimum, the rate of multiplication becomes progressively less and less. Beyond the minimum temperature, multiplication ceases entirely.

**Growth Temperature Range.**—This is defined as the number of degrees between the minimum and the maximum growth temperatures. With some organisms, this range is very narrow; with others, it is very wide.

The temperature relations of the three classes of organisms are as follows:

| | Minimum, °C. | Optimum, °C. | Maximum, °C. |
|---|---|---|---|
| 1. Psychrophilic | 0 | 15–20 | 30 |
| 2. Mesophilic | 5–25 | 37 | 43 |
| 3. Thermophilic | 25–45 | 50–55 | 60–90 |

**Thermal Death Rate.**—The thermal death rate may be defined as that temperature at which an organism is killed after a period of 10 min. under certain specified conditions. This temperature is generally referred to as

the thermal death point. Since organisms subjected to unfavorable conditions are not all killed in the same period of time, the term "thermal death rate" is more appropriate than "thermal death point."

The various factors that should be specified in reporting a thermal death rate include (1) the water content of the medium, (2) the hydrogen-ion concentration of the medium, (3) the composition of the medium, (4) the age of the cells, (5) the presence or absence of spores in a culture of a spore-forming organism, and (6) the incubation temperature of recovery cultures. If one or more of these factors is changed, the thermal death rate is also changed. Unless the above factors are mentioned, little importance can be attached to the results.

*Water Content of the Medium.*—Death of bacteria by heat is believed to be due to coagulation of the proteins of the protoplasm. Within limits, the greater the percentage of water in a medium, the lower will be the temperature required to kill bacteria. Moist heat is more effective as a sterilizing agent than dry heat. Dry egg albumin may be heated to a point where it decomposes without showing any appreciable coagulation. As the percentage of moisture is increased, the temperature of coagulation becomes progressively less. This may be seen in Table 5.

TABLE 5.—RELATION BETWEEN MOISTURE CONTENT AND TEMPERATURE OF COAGULATION OF EGG ALBUMIN

| Amount of Water, Per Cent | Temperature of Coagulation, °C. |
|---|---|
| 50 | 56 |
| 25 | 74– 80 |
| 18 | 80– 90 |
| 6 | 145 |
| 0 | 160–170 |

*Hydrogen-ion Concentration of the Medium.*—Most organisms are more easily killed in acid or alkaline solutions than in a neutral environment. In general, the greater the degree of acidity or alkalinity, the lower will be the temperature required to kill bacteria. A neutral medium should be used.

*Composition of the Medium.*—The composition of the suspending medium plays a very important role in the results obtained for the thermal death rate. Media containing high concentrations of proteins or albuminous substances usually increase the temperature required to destroy bacteria. The proteins form a film around the organisms protecting them from unfavorable influences.

The composition of the medium used for the recovery cultures is also very important. Curran and Evans (1937) and Nelson (1943) showed that the number of treated cells that would grow depended to a large extent upon the composition of the medium used for subculture. Stern

(1942) came to a similar conclusion on the time required for the germination of spores of a number of putrefactive and thermophilic anaerobic organisms.

*Age of the Cells.*—The age of the cells also influences the thermal death rate. Old cells are generally more resistant to adverse environmental conditions than very young cells. It is best to use 24-hr. cultures for the test.

*Presence of Spores.*—Nonspore-forming bacteria and the vegetative forms of the spore bearers are generally killed by moist heat at temperatures of 60 to 70°C. Spores can withstand a temperature of 100°C., or higher. Since spores are produced under unfavorable conditions, they will be more numerous in old cultures. In reporting the thermal death rate of a spore-forming organism, care should be taken to make sure spores are present in the culture.

Evans and Curran (1943) reported that sublethal preincubation of aerobic spores accelerated their germination. The spores were preheated at 65 to 95°C., incubated at 37°C. for 3 hr., then heated at 85°C. for 10 min., and plated. Greatest acceleration of spore germination was obtained with a preheating treatment of 85°C. for 8 to 10 min. In a later communication, Curran and Evans (1945) preheated a number of thermotolerant and thermophilic, aerobic spore-formers at 95°C. for 10 min. Subcultures showed that the rate of spore germination was greatly accelerated. Also, the number of spores that subsequently germinated was greater.

*Incubation Temperature of Recovery Cultures.*—Williams and Reed (1942) found that incubation temperatures of 24 and 27°C. were more favorable for the development of heated spores of *Clostridium botulinum* than was 37°C., and somewhat more favorable than 31°C. A temperature of 27°C. was also found to be more favorable for an unidentified anaerobe than was the higher temperature.

**Heat Resistance of Bacterial Cells and Spores.**—Lamanna (1942) found that the heat resistance of both vegetative cells and spores was related to the maximum temperature of growth. Members of the genus *Bacillus* (*B. subtilis, B. agri, B. vulgatus, B. mesentericus, B. cereus, B. mycoides,* and *B. megatherium*) were separated into three groups on the basis of heat tolerance of spores. The thermophils produced spores of greatest heat resistance and showed the highest growth temperature. The species with a maximum below 50°C. possessed spores of least resistance, whereas nonthermophilic types with maxima between 50 and 60°C. had spores of intermediate heat resistance.

**Thermal Death Time.**—In making a determination of the thermal death rate, the time is kept constant and the temperature is varied. In finding the thermal death time of an organism, the temperature is kept constant and the time required to kill all cells is determined. Workers in the

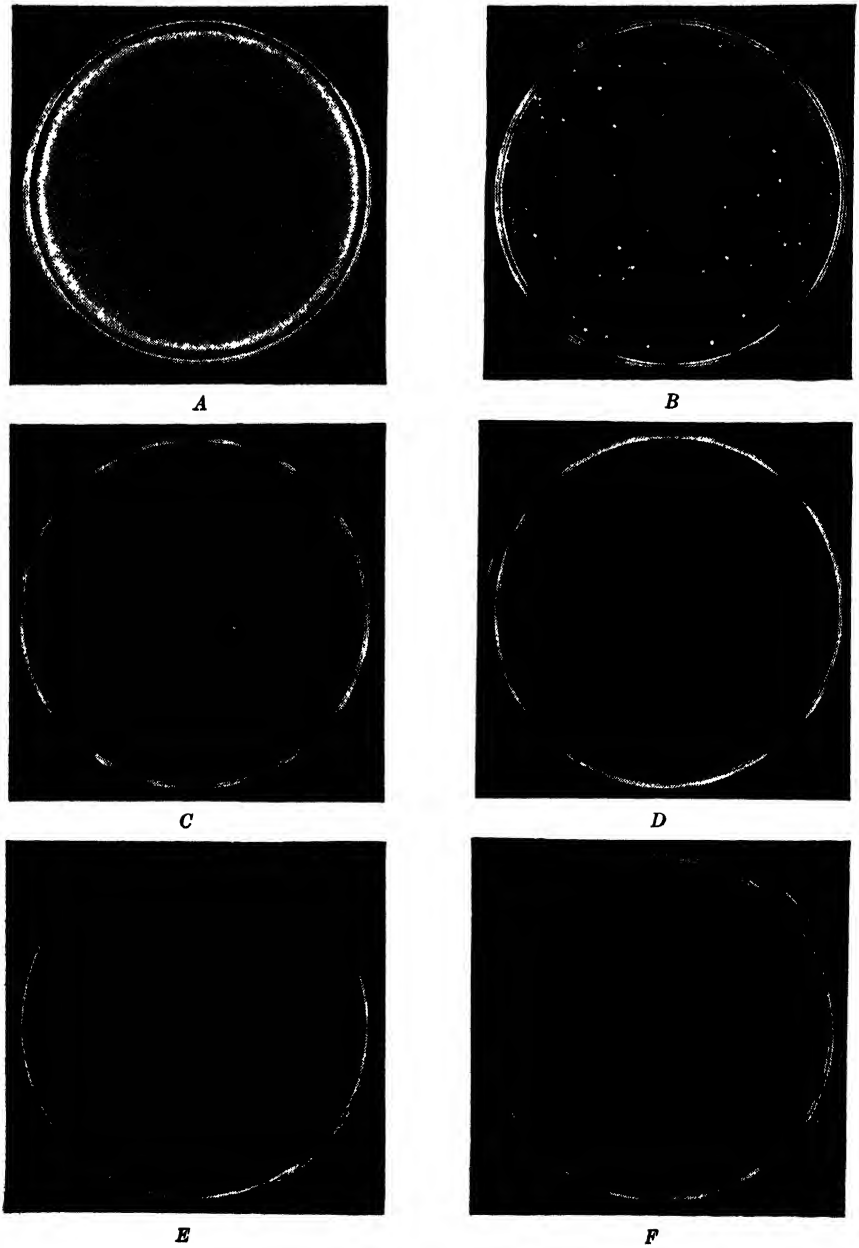



FIG. 102.—Thermal death rate of *Serratia marcescens*. Six tubes, each containing 1 cc. of culture, were heated for 10 minutes at the following temperatures: *A*, 50°C.; *B*, 55°C.; *C*, 60°C.; *D*, 65°C.; *E*, 70°C.; *F*, 75°C. Then the contents of each tube were transferred to Petri dishes and mixed with melted agar.

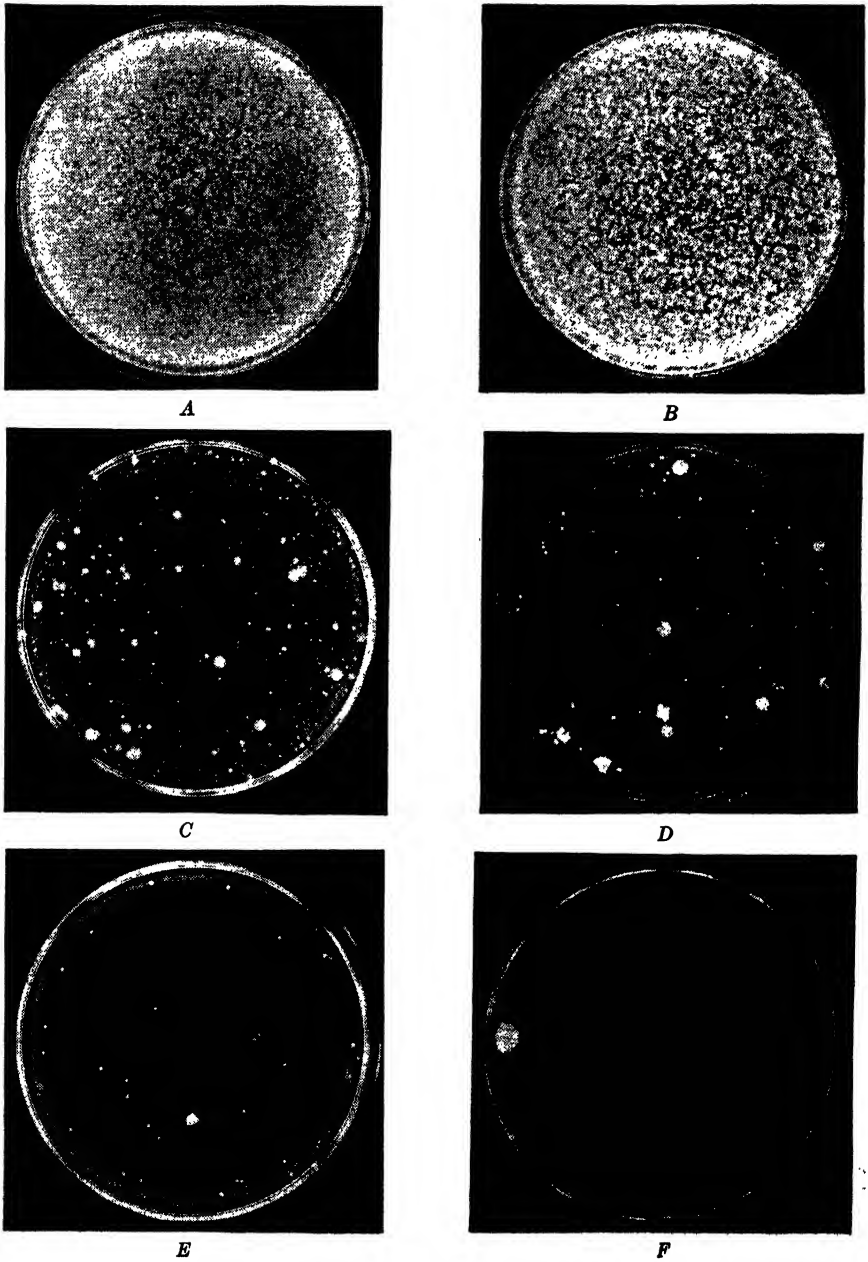



FIG. 103.—Thermal death rate of *Bacillus cereus*. Six tubes, each containing 1 cc. of culture, were heated for 10 minutes at the following temperatures: *A*, 75°C.; *B*, 80°C.; *C*, 85°C.; *D*, 90°C.; *E*, 95°C.; *F*, 100°C. Then the contents of each tube were transferred to Petri dishes and mixed with melted agar.

canning industry usually find it more suitable to keep the temperature constant and vary the time.

Results obtained from the determinations of thermal death rates and thermal death times are very valuable in applied bacteriology and especially in the canning industry. They aid the canner in determining the temperatures and times required to process certain canned foods.

It is the usual practice to isolate the organism or organisms causing the spoilage of a certain kind of food and determine their thermal death rates or thermal death times under similar environmental conditions. The results may then be used as a guide in determining the temperature and time required to process the food.

The thermal death rates of *Serratia marcescens* and *Bacillus cereus* are shown in Figs. 102 and 103.

For more information, see Rahn (1945).

## EFFECT OF ULTRAVIOLET IRRADIATION

A few of the higher sulfur bacteria elaborate a pigment known as bacteriochlorophyll which appears to function in these organisms in a manner similar to chlorophyll in plants. These organisms are benefited by light rays. However, the great majority of bacterial species known do not exhibit any photosynthetic action. These organisms are harmed by exposure to ultraviolet light.

Radiations are effective only if they are absorbed. Ultraviolet rays are readily absorbed and are employed in many ways for sterilization. The growth of an organism may be retarded or completely destroyed, depending upon the length of the rays and the period of exposure.

Most proteins in solution show marked absorption bands in the ultraviolet region. Blundell, Erf, Jones, and Hoban (1944) reported that ultraviolet rays exerted a negligible bactericidal action on bacteria suspended in blood. The proteins in the blood absorbed the light rays and prevented them from reaching the bacteria.

The germicidal lamps commonly employed radiate most of their ultraviolet energy in the spectral line 2537 Å. However, it has been definitely shown that the most germicidal rays have a wave length of 2650 Å. With longer or shorter wave lengths of light, the rate of destruction of organisms decreases.

Smithburn and Lavin (1939) irradiated the organism of tuberculosis (*Mycobacterium tuberculosis*) with sublethal doses of monochromatic light of 2537 Å. and found that the organisms gradually lost their virulence, and finally became avirulent without being killed. These avirulent organisms were still capable of inducing a demonstrable immunity whereas organisms killed by the same light rays did not induce a measurable immunity. Organisms killed by light rays still possessed acid-fast properties.

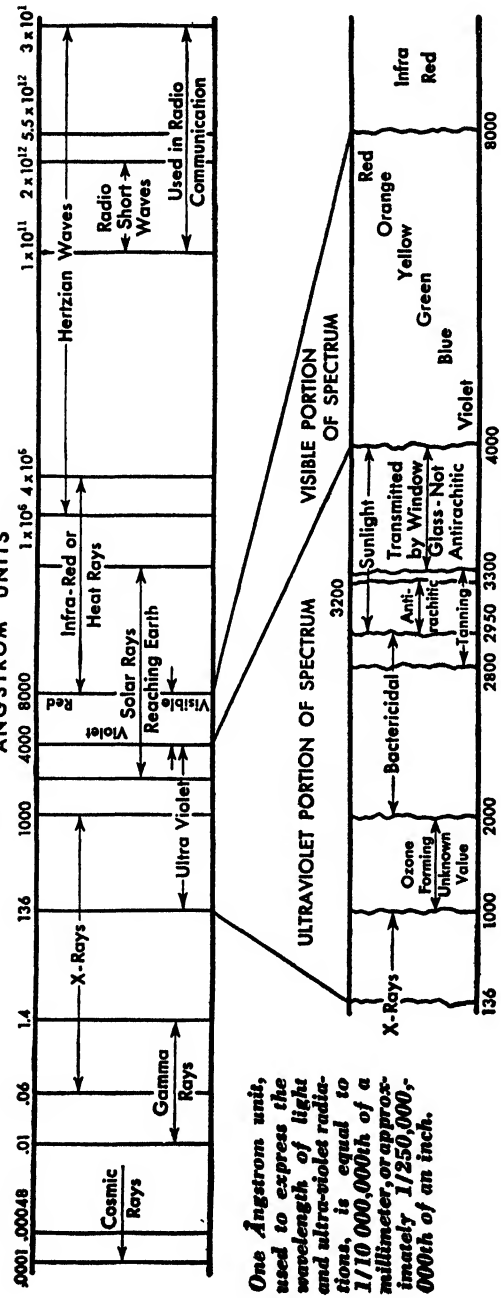



FIG. 104.—Spectrum charts. (*From The Westinghouse Sterilamp and the Rentschler–James Process of Sterilization, courtesy of the Westinghouse Electric & Manufacturing Company, Inc.*)

Hercik (1937) found that twice as much incident energy was required to destroy spores of *Bacillus megatherium* as the vegetative cells.

Sharp (1939) seeded agar plates from cultures of several species of nonspore-bearing organisms and one culture containing spores and vegetative cells of *B. anthracis*. Immediately after streaking, the plates were irradiated with light rays of 2537 Å. until the organisms were reduced to a 10 per cent survival (90 per cent killed). The results showed that spores of *B. anthracis* required approximately twice the exposure as vegetative cells to produce the same percentage reduction.

Spores of molds have also been treated with ultraviolet light and found to be susceptible to the same rays that are toxic to bacteria. Hollaender and Emmons (1939) reported that spores of *Trichophyton mentagrophytes* isolated from "athlete's foot" were destroyed by light rays of 2537 to 2650 Å.

Viruses and bacteriophages are also sensitive to light rays. Jungeblut (1937) and Toomey (1937) found that the virus of poliomyelitis (infantile paralysis) was destroyed by light rays in the ultraviolet region. Similar results were reported by Levaditi and Voet (1935) for herpes virus and *Escherichia coli* bacteriophage. Kendall and Colwell (1940) showed that bacteriophages specific for several strains of *E. coli*, *Shigella paradysenteriae*, and *Micrococcus pyogenes* var. *aureus* were destroyed within 1 min. by exposure to ultraviolet light emitted by a quartz mercury-vapor lamp exposed at a distance of 1 cm. from the face of the lamp. Ingredients of broth associated intimately with bacteriophage particles in the same medium, or interposed as a screen between pure phage and light source, prevented their destruction.

Ultraviolet rays are used commercially for the destruction of bacteria, yeasts, and molds in various foodstuffs, such as sugar, meats, and bakery products. Hall and Keane (1939) reported that spores of *Bacillus stearothermophilus*, which are commonly found in sugar and which are the cause of serious trouble in many food preparations containing sugar, may be destroyed by irradiation of the sugar crystals.

Arnold and Garrett (1943) found that ultraviolet lamps emitting radiations mainly in the narrow band of 2537 Å. were effective in destroying bacteria growing on the surfaces of such dairy equipment as milk bottles, tinned dippers, cans, and pasteurizing vats.

Many hospital rooms and operating rooms are irradiated to sterilize or greatly decrease the number of organisms in the air. Air sanitation is closely analogous to water sanitation (Robinson, 1939). Its purpose is substantially the same: to make the air in confined spaces more safe under the particular circumstances of its use, and to guard against the possibility that air-borne organisms may cause clinical infections. In hospital operating rooms, the primary objective is to reduce the risk from pathogenic

organisms settling from the air on the surface of open incisions, on instruments, or on the hands of the operating personnel from which they might be transferred to open wounds.

**Irradiation of Culture Media.**—The observations already reported are limited to the action of the light rays on microorganisms and their spores. It has been shown that culture media, when exposed to ultraviolet light, become less suited for bacterial growth. Bedford (1927), after a series of experiments, concluded that the irradiation of culture media caused the formation of hydrogen peroxide. The presence of this compound in media produced a toxic action on bacteria. The concentration of peroxide that accumulated in media depended upon the wave length of light and the period of exposure.

Pratt (1936) and Baumgartner (1936) showed that the irradiation of carbohydrate solutions and carbohydrate media caused a shift in the pH toward more acid conditions. The sequence of changes appeared to be polysaccharides → disaccharides → monosaccharides → alcohols, aldehydes, ketones, acids. A considerable portion of the acid formed was formic acid. Baumgartner found that neutralization of the acid restored the ability of the culture media to support growth of the bacteria.

The effect of ultraviolet light on the growth of *Bacillus subtilis* and *Micrococcus pyogenes* var. *aureus* is given in Fig. 105.

For information on the effect of ultraviolet irradiations on air-borne disease organisms see page 467 ff.

For further reading, consult Hollaender (1943), Luckiesh and Holladay (1944), Luckiesh, Taylor, and Kerr (1944), Miller and Schad (1944), Rahn (1945), and Taylor (1944).

## EFFECT OF OSMOTIC PRESSURE

Osmosis may be defined as a kind of diffusion that takes place between two miscible fluids separated by a permeable membrane where the conditions on the two sides of the membrane tend to become equal.

The term "osmotic pressure" refers to the unbalanced pressure that gives rise to the phenomena of diffusion and osmosis, as in a solution in which there are differences of concentration.

**Plasmolysis.**—The rate at which water passes into and out of cells is in part determined by the ratio that exists between the concentrations of electrolytes inside and outside of the cell membranes. Most cell contents exert a definite pressure on the cell membranes. If an organism is immersed in a solution having a higher osmotic pressure, water will leave the cell. This will continue until an equilibrium is established between the osmotic pressures inside and outside of the cell. If the initial difference in osmotic pressure between the inside and outside of the cell is sufficiently great, the cytoplasmic membrane will be drawn in with the cytoplasmic

contents and collect in the center of the cell. The cell is then said to be plasmolyzed, and the process is called "plasmolysis." The solution on the outside is hypertonic with respect to the solution on the inside of the cell.

**Plasmoptysis.**—If an organism is immersed in a solution having a

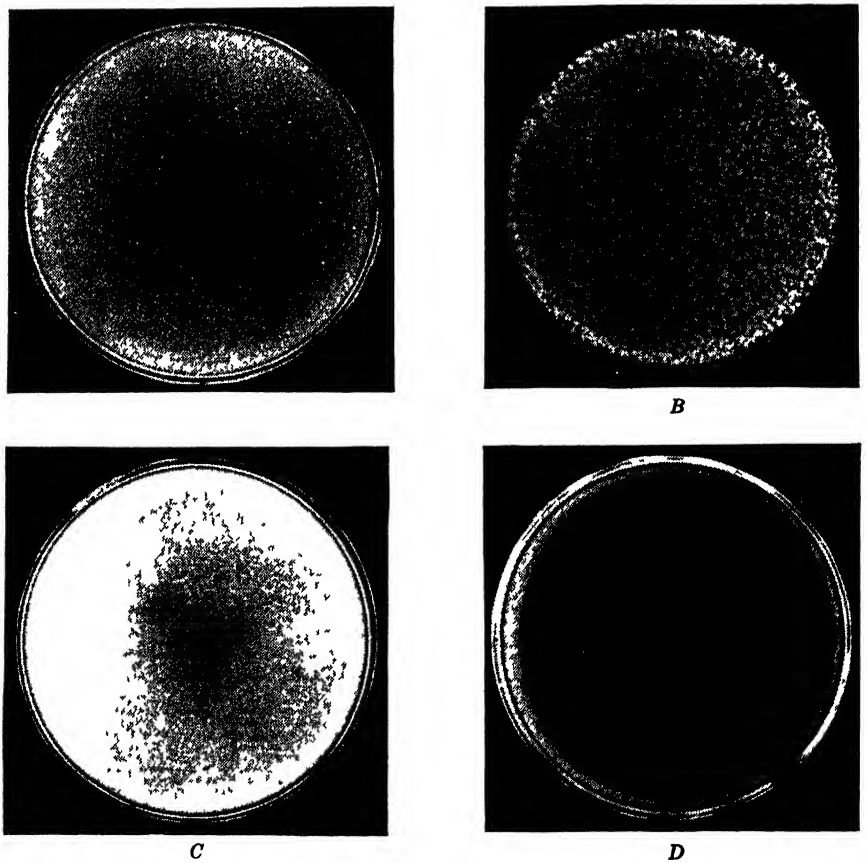



FIG. 105.—Effect of ultraviolet light. *A*, *Bacillus subtilis* exposed to an ultraviolet lamp for 1 hr.; *B*, same exposed for 2 hr. Since the culture contained many spores, a longer exposure was necessary to produce complete sterilization; *C*, *Micrococcus pyogenes* var. *aureus* exposed for 1 hr.; *D*, same exposed for 2 hr. Culture was completely sterilized after 2 hr.

lower osmotic pressure, water will enter the cell. This will continue until an equilibrium is established between the osmotic pressures inside and outside of the cell. If the initial difference in osmotic pressure between the inside and the outside of the cell is of sufficient magnitude, the cell membranes will burst, releasing their contents. The cell is then said to be plasmoptyzed and the process is called "plasmoptysis." In this case, the solution on the outside is hypotonic with respect to the solution on the inside of the cell.

**Isotonic Solutions.**—If the concentrations of ions and molecules on the inside and the outside of the cell membranes are equal, there will be no difference in their osmotic pressures. The result will be neither shrinking nor swelling of the cell contents. The two solutions are then said to be isotonic with respect to each other.

A great increase in the osmotic pressure of the surrounding solution is necessary before any toxic action is noted on bacteria. In this respect, bacteria differ markedly from higher plant and animal cells, which are very sensitive to relatively slight changes in the ionic concentration of the outside medium.

The use of high osmotic pressures finds a practical application in the preservation of some foods from attack by fungi. This principle is employed in the preservation of jams, jellies, and condensed milk by means of sugar; and salted meats, corned beef, fish, etc., by the use of salt (Fig. 106).

**Marine Bacteria.**—Marine bacteria differ from fresh-water organisms in that they are able to tolerate much greater concentrations of salt (ZoBell and Feltham, 1933; ZoBell, 1941, 1942). ZoBell and Feltham found that less than 10 per cent of the bacteria isolated from sea water were able to multiply in nutrient fresh-water media and that a smaller number of species isolated from fresh water could multiply in media prepared with undiluted sea water.

ZoBell and Michener (1938) acclimatized marine bacteria to hypotonic solutions by gradually diluting the sea-water medium with each successive transfer of the cultures. Of 12 species isolated, most of them could be acclimatized to 25 to 30 per cent sea-water medium. Below this concentration, considerable difficulty and delay were encountered in making the bacteria grow. All except three of the original cultures, which were kept in the refrigerator on undiluted sea-water agar, multiplied when transferred to fresh-water medium. The other three species multiplied when transferred to 10 per cent sea-water medium. The old stock cultures adapted themselves better to hypotonic solutions than did cultures of the same organisms gradually acclimatized to decreasing concentrations of sea-water medium.

This observation appears to be contrary to accepted beliefs with respect to the adaptability of organisms to changes in the environmental conditions. On second thought, however, other factors must be taken into consideration to make a correct interpretation of the facts. It is well established that young bacteria are more susceptible to adverse environmental conditions than old ones. This has been shown by Sherman and Albus (1923) and others. Cultures gradually acclimatized to decreasing concentrations of sea water tend to keep the organisms in a physiologically young condition. On the other hand, the parent stock cultures become

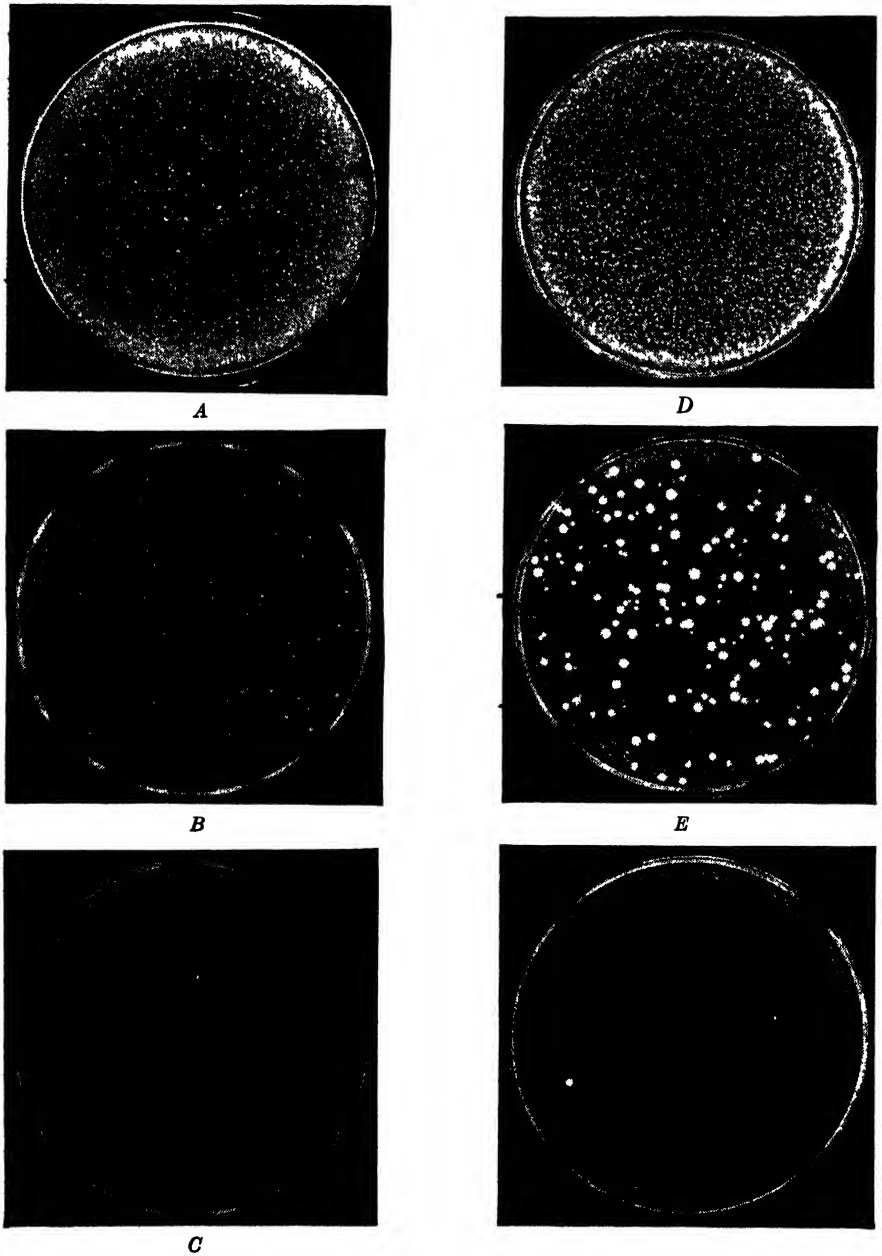



FIG. 106.—Effect of osmotic pressure. *Micrococcus pyogenes* var. *aureus* and *Escherichia coli* exposed to 30 per cent sodium chloride solution, then streaked over the surface of agar plates. *A*, *M. pyogenes* var. *aureus* streaked immediately; *B*, same after 24 hr.; *C*, same after 48 hr.; *D*, *E. coli* streaked immediately; *E*, same after 24 hr.; *F*, same after 48 hr.

physiologically old and senescent and less susceptible to changes in the environment. These cells are better able to adapt themselves to adverse conditions.

## PRESERVATION OF BACTERIA

Organisms in the desiccated state are not capable of multiplication. Moisture is absolutely necessary for this to occur. When organisms are dried, they gradually die, the rate of death being dependent upon several factors. Slow drying destroys bacteria more easily than rapid drying. The cause of death is not definitely known. It may be due to the denaturation of the proteins of the protoplasm, to the destruction of the essential enzymes, or to other causes

The percentage of survivors during desiccation may show wide variation, depending upon the species, the age of the culture, the surface on which the cells are dried, the temperature for drying, and the composition of the medium in which the organisms are suspended

Bacteria growing in culture media composed of broth, milk, and other albuminous fluids survive drying fairly well. The proteins of the medium are believed to protect the cells by acting as protective colloids, which make the drying process more gentle and less abrupt. On the other hand, bacteria suspended in water or saline are easily destroyed by desiccation.

Spores are more resistant to desiccation than the vegetative cells producing them. They are able to withstand adverse conditions for long periods of time. Likewise, capsulated organisms are more resistant to drying than noncapsule-producing bacteria. The mucilaginous deposit surrounding the organisms acts as a protective layer, decreasing the rate of desiccation.

Desiccated bacteria are more resistant to destructive agencies than the same organisms in the moist state. Much higher temperatures are required to coagulate the protoplasm of partly dried organisms than the same cells under normal conditions. Heat probably does not coagulate the protoplasm of completely dried bacteria. This explains why higher temperatures are required to sterilize glassware by the dry-air sterilizer than by the autoclave. Dried bacterial spores are also more resistant to adverse conditions than spores kept in the moist condition.

Death of bacteria by dry heat is believed to be due to oxidation. Protoplasm in the dry state does not coagulate at 100°C., and dry enzymes retain their activity. The death rate increases with temperature, owing merely to an increase in the rate of oxidation.

The effect of desiccation at 37°C. on survival of *Escherichia coli* and *Bacillus subtilis* is shown in Figs. 107 and 108.

For more information, see Rahn (1945).

**Maintenance of Stock Cultures.**—Bacteria for class use are generally preserved on nutrient agar slants. There are some exceptions, such as

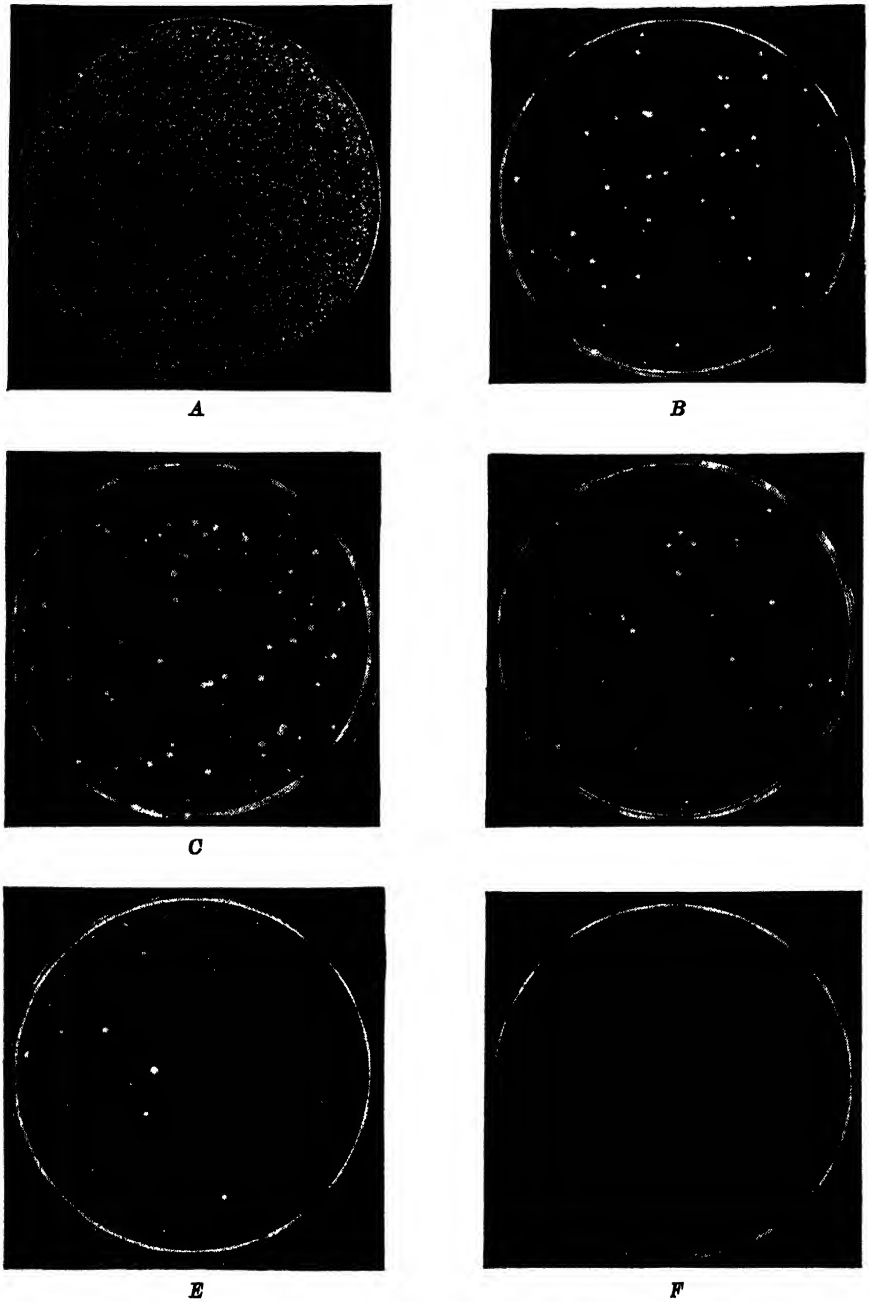



FIG. 107.—Effect of desiccation on *Escherichia coli*. *A*, control, not dried; *B*, dried for 24 hr. at 37°C.; *C*, dried for 48 hr.; *D*, dried for 72 hr.; *E*, dried for 96 hr.; *F*, dried for 216 hr.

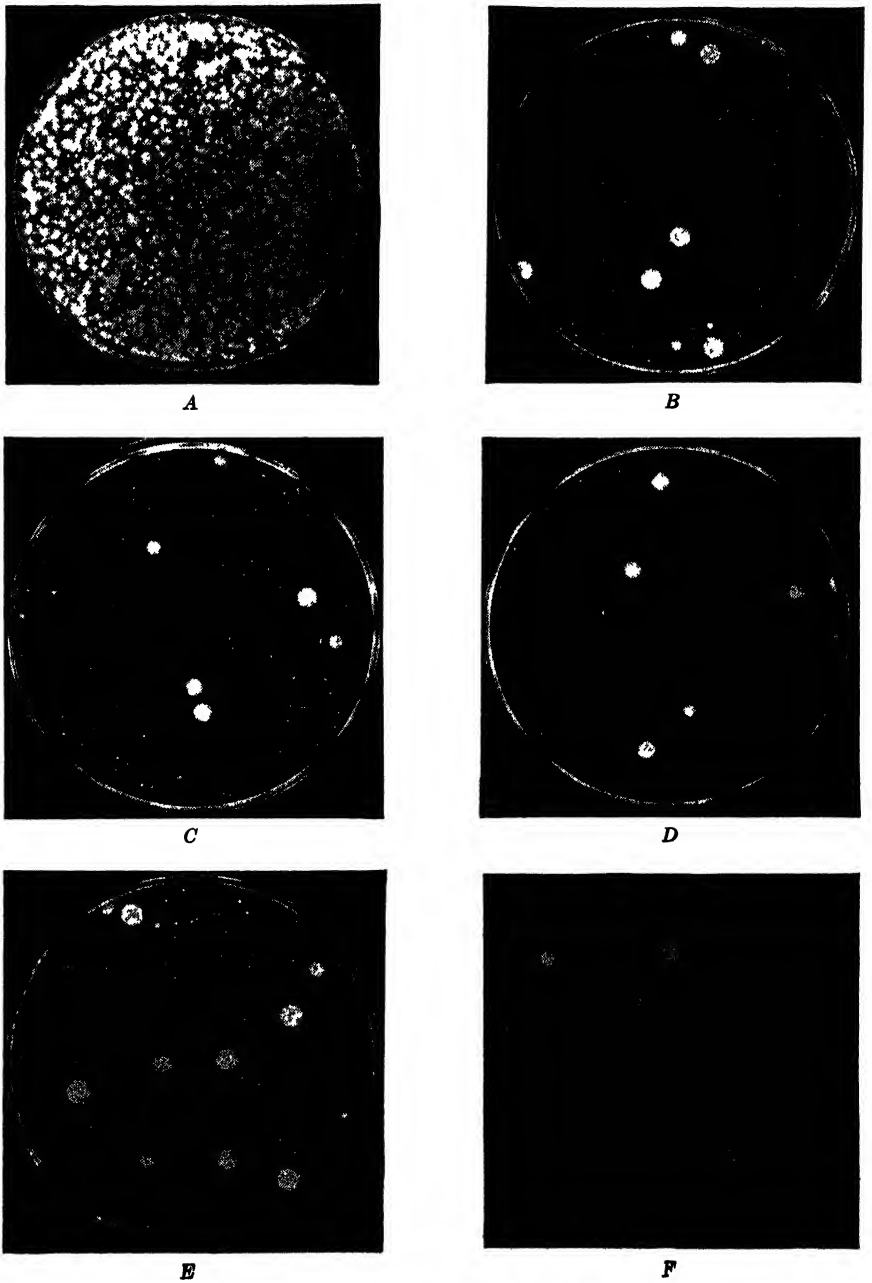



FIG. 108.—Effect of desiccation on *Bacillus subtilis*. *A*, control, not dried; *B*, dried for 24 hr. at 37°C.; *C*, dried for 48 hr.; *D*, dried for 72 hr.; *E*, dried for 96 hr.; *F*, dried for 216 hr. The organism produces spores which were not destroyed in that period of time.

certain pathogenic organisms that require the addition of serum, blood, and other body fluids to the medium; anaerobic organisms that require minced tissue, or a semisolid medium; certain soil organisms that require special inorganic media; etc. The toxic metabolic waste products secreted by organisms diffuse into the agar and away from the bacteria. Because of this property of agar, bacteria are able to survive longer than when grown in liquid media, where they are constantly bathed by the toxic substances dissolved in the surrounding liquid. Such cultures are referred to as stock cultures, because they are kept on hand by storing in a cool room or cupboard, and used as needed for the preparation of transplants.

The maintenance of a large number of stock cultures of bacteria requires frequent attention to prevent loss of the organisms. The method commonly employed for this purpose is to prepare transfers to suitable media at definite intervals before the media become too dehydrated and before the bacteria are destroyed by the accumulation of waste products of metabolism. This requires not only a considerable amount of time but involves also the possible loss of certain biological, immunological, and cultural characteristics of the organisms.

Various methods are employed to preserve bacteria and to maintain them as near as possible in their original state. According to Morton and Pulaski (1938) and Morton (1938), all the methods may be placed into either of two groups: in one, the bacteria are preserved by the prevention of slow drying; in the other, the organisms are preserved by rapid desiccation.

*Preservation by Prevention of Slow Drying.*—All the methods in this group attempt to preserve cultures by the application of some type of seal to the tubes to prevent or decrease drying. The most important methods in this group are the following:

1. Cultures may be preserved by impregnating the cotton stoppers with paraffin and then inserting them into the tubes. The stoppers are easily removed by gently heating them in a flame to melt the paraffin. Sometimes the tops of the stoppers and test tubes are covered with melted sealing wax. This is not so satisfactory as paraffin because sealing wax is brittle and must be replaced each time the culture tubes are opened.

There are disadvantages to the use of paraffin and sealing wax: (*a*) The sealing materials are difficult to remove during the cleaning process. (*b*) The cultures are not protected from mold contaminations. Mold spores are frequently present in cotton stoppers. When the stoppers are sealed, they become moistened by the evaporation of water from the medium. The water concentration soon becomes sufficiently great to permit germination of the spores. This results in the growth of hyphae, which penetrate through the cotton stoppers into the medium. (*c*) Many organisms are capable of dissociating when kept in sealed tubes. The or-

ganisms may undergo changes in colonial forms, in virulence, in their immunological specificities, and in other ways.

2. The tops of the tubes may be covered with paraffined paper, with tin or aluminum foil, or with rubber caps. The objections given under the first method also apply here, especially contaminations due to molds.

3. The culture tubes may be sealed off as ampules by heating the open end in a blast lamp and drawing out the melted tops with a pair of forceps. The disadvantages here are (*a*) the destruction of test tubes, (*b*) the difficulties encountered in opening the ampules, and (*c*) the cultures preserved in this manner also readily dissociate.

4. The culture media may be overlayered with sterile paraffin or mineral oil. The cultures may be in broth or on agar slants. The broth cultures should be overlayered with mineral oil to a height of 1 cm. Agar slant cultures are first incubated until good growth appears, and then covered with sterile mineral oil to a height of 1 cm. above the top of the slanted surface. Transplants are easily made by removing a loopful of the growth, touching the wire loop to the inner wall of the tube to drain off the excess oil, then streaking over the surface of fresh medium.

Ordinarily it is necessary to transfer recently isolated strains of *Neisseria gonorrhoeae* at least twice a week and older strains about once a week to keep the organisms viable. Hac (1940) made a study of the various methods for the preservation of over 400 strains of this organism. Those preserved by sterile paraffin oil seals remained viable after transfer at 6- and 12-month intervals.

Simmons (1942) preserved cultures of β-streptococci of human origin, *Corynebacterium diphtheriae*, micrococci, and *Salmonella* under mineral oil and found that the organisms remained viable after transfer periods of 12 to 24 months.

This method possesses distinct advantages over those already mentioned: (1) it is inexpensive, (2) the cultures do not evaporate, (3) dissociation is prevented, (4) transfers are easily made to fresh medium, (5) no special apparatus is necessary, such as desiccators, vacuum pumps, etc., and (6) the cultures are protected against mold contaminations.

Regardless of which method in this group is followed, no one of them will preserve bacteria for indefinite periods of time. All organisms slowly decrease in viability and finally die unless transfers are prepared to fresh medium at definite intervals.

*Preservation by Rapid Desiccation.*—Kitasato (1889) observed that the organism of cholera, *Vibrio comma*, survived longer when dried in a desiccator than when dried in air. Since that time, many workers have desiccator-dried other organisms and have reported similar results.

Shackell (1909) recommended freezing as a preliminary step to rapid desiccation. The cultures may be frozen in salt-ice mixture or by solid

carbon dioxide (dry ice), and then desiccated in vacuum over sulfuric acid as the desiccant. This method with its many modifications, especially those recommended by Swift (1937), Flosdorf and Mudd (1935, 1938), and more recently Rayner (1943), and Antoine and Hargett (1943), is widely used at present for the preservation of bacteria, sera, viruses, enzymes, and other biological products.

Bacteria may be preserved for longer periods by freezing and vacuum desiccation than is possible by the methods listed in the first group. Also, the organisms do not exhibit any appreciable differences in their morphological, biochemical, and immunological properties.

For an excellent review of the subject, see Flosdorf, Hull, and Mudd (1945).

### EFFECT OF HYDROGEN-ION CONCENTRATION

It is well known that the hydrogen-ion concentration of culture media is of prime importance for the successful cultivation of bacteria. Some organisms grow best in acid environments; others grow best in alkaline media; still others prefer substrates neutral in reaction. There exists for every organism an optimum concentration of hydrogen ions in which it will grow best. The hydrogen-ion concentrations above and below which an organism fails to grow are known as the minimum and maximum pH values, respectively. The values are true only if the other environmental factors are kept constant. Variations in such factors as composition of the medium, temperature of incubation, and osmotic pressure of the medium, even though slight, will produce changes in the minimum, the optimum, and the maximum hydrogen-ion values of an organism. The pH range of an organism is the difference between the maximum and the minimum values.

Michaelis and Marcora (1912) noted that *Escherichia coli* fermented lactose with the production of acids until the pH dropped to about 5.0. This acidity was sufficient to prevent further growth of the organisms. They stated that the final hydrogen-ion concentration of cultures of *E. coli* was a physiological constant. Similar results have been reported on other organisms. It may be concluded that the growth of any fermentative organism is inhibited upon reaching a rather definite hydrogen-ion concentration. The final degree of acidity will vary depending upon the species. These findings have been used as a basis for differentiating closely related groups of organisms (see page 486).

The effect of pH on the growth of *E. coli* is shown in Fig. 109.

### EFFECT OF SURFACE TENSION

Surface tension may be defined as that property, due to molecular forces, which exists in the surface film of all liquids and tends to bring the contained volume into a form having the least superficial area.

Molecules attract their neighbors and are attracted by them. A molecule situated in a liquid will be in equilibrium by virtue of the equal attractions on all sides. On the other hand, a molecule situated on the surface of a liquid will have equal horizontal attractions but unequal vertical attractions. This results in an unbalanced attraction toward the interior of the liquid. This resultant force reaches a maximum at the surface, and the mass of liquid behaves as if surrounded by an elastic membrane, tending to compress the liquid into the smallest possible volume.

The composition of the surface layer of a culture medium, inoculated

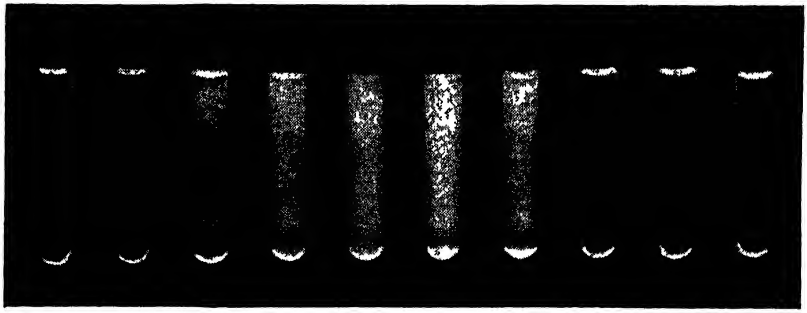


FIG. 109.—Effect of pH on the growth of *Escherichia coli*. From left to right, pH2.8 to 10 at intervals of 0.8 pH. Tubes 1 and 2, no growth; tubes 3 to 6 inclusive, increasing turbidity; tubes 7 to 10 inclusive, decreasing turbidity. Maximum turbidity occurred in tube 6 at pH6.8.

with an organism, may differ appreciably from the composition of the same medium taken as a whole. The surface tension of media may, therefore, play an important role in the growth of an organism. This is especially true in the case of those organisms which have a tendency to grow on the surface of culture media in the form of a film or pellicle.

Organisms growing on the surface of a medium in the form of a pellicle were at one time regarded as strict aerobes. The surface layer, exposed to air, gave the organisms more oxygen than could be obtained from the deeper portions of the medium. It is true that the pellicle-forming bacteria are aerobic but not obligately aerobic. They are capable of growth and multiplication under both aerobic and anaerobic conditions. Bacteria have a density slightly greater than that of the culture medium in which they are grown. If the pellicle produced by a pellicle-producing organism, such as *Bacillus subtilis*, is sedimented, the pellicle will not rise to the surface again but will remain at the bottom of the tube. A new pellicle will develop on the surface of the medium. It is obvious that the surface film is supported in this position by some force in the medium. This force is spoken of as surface tension.

The unit of force in the C.G.S. system of physical units is the dyne.

It is such a force that, under its influence, a particle whose mass is 1 gm. would experience during each second an acceleration of 1 cm. per second. The dyne is approximately the force exerted by a milligram weight under the influence of gravity.

The surface tension of the usual laboratory media varies between 57 and 63 dynes. The surface tension of pure water is 73 dynes. Some substances may be added to culture media to raise surface tension, among which may be mentioned charcoal and calcium chloride. Charcoal is effective by virtue of its ability to remove some surface-tension depressant from media. The growth of bacteria may, in some cases, raise the surface tension of the media in which they are cultivated. On the other hand, many substances may be added to culture media to lower their surface tensions. Among these may be mentioned ethyl alcohol, soaps, glycerol, and synthetic detergents. The soaps have been almost the only efficient detergents, but these are being gradually replaced by synthetic detergents or wetting agents. These latter compounds can be divided into anionic, cationic, and undissociated detergents (see page 214).

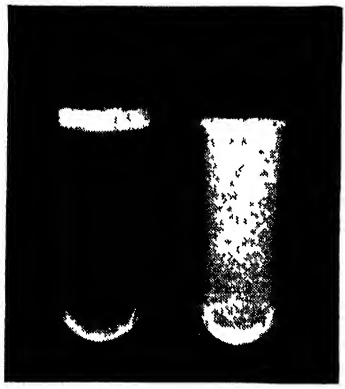


FIG. 110.—Effect of surface tension on the growth of *Bacillus subtilis*. Left, growth in nutrient broth. The growth is confined almost entirely to the pellicle. Right, nutrient broth containing 0.05 per cent sodium ricinoleate to lower the surface tension. The growth occurs as a uniform turbidity.

Larson, Cantwell, and Hartzell (1919) found that, if the surface tension of nutrient broth is depressed to some point below 40 dynes by means of soap and then inoculated with *B. subtilis*, the organism produced a diffuse growth rather than a pellicle on the surface. This same principle was found to apply to other pellicle-producing organisms. The formation of a pellicle apparently has nothing to do with the oxygen requirements of an organism, but is dependent entirely upon the surface tension of the medium (Fig. 110).

Wetting is a function of surface tension. If bacteria are not wetted by the medium, they will grow on the surface in the form of a film; if they are wetted, they will produce a uniform clouding of the medium. It has been shown that bacteria growing on the surface of media contain a higher content of lipoidal substances than nonpellicle-producing organisms. The organism of tuberculosis has been found to contain as high as 40 per cent lipoidal substances in contrast to nonpellicle producers, which contain about 7 per cent.

Larson and Larson (1922) showed that organisms that ordinarily produced a uniform turbidity of the medium could be made to grow in the

form of a pellicle if the lipoid content was increased. The fat content of *Micrococcus pyogenes* var. *aureus* was greatly increased if the organism was grown on a medium containing a carbohydrate or glycerol, neither of which is fermented. The organism produced a pellicle on the surface, which resembled very much the growth of the tubercle bacillus.

For additional information, consult Larson (1928).

## ANTAGONISTIC ACTION OF IONS

Winslow and Falk (1923*a*) reported that *Escherichia coli* maintained itself in distilled water at a pH of about 6.0 without material decrease in bacterial numbers for nearly 24 hr. An increase in numbers occurred during the first few hours. At pH5.0, the reduction was somewhat greater. The viability decreased as the solution was adjusted to more acid or alkaline conditions (Table 6). A heavy line is drawn to indicate the range of time and pH most nearly corresponding to a one-third reduction in bacterial numbers.

On the other hand, NaCl in a strength of 0.0145*M* exerted a distinctly favorable action on the viability of *E. coli*. Instead of a slight but definite decrease in numbers after 24 hr., as occurred in distilled water at pH6.0, the bacteria maintained themselves in undiminished numbers. Above a concentration of 0.0145*M* NaCl, the percentage of living organisms decreased with increasing concentrations of salt.

Similar results were obtained if $CaCl_2$ was substituted for the NaCl. The organisms maintained themselves better in the presence of this salt than in distilled water alone. Here again a pH of 6.0 appeared to be the most favorable for growth or maintenance of *E. coli*. The most favorable concentration of $CaCl_2$ appeared to be 0.00145*M*.

TABLE 6.—VIABILITY OF *Escherichia coli* IN DISTILLED WATER

| Hours | Per cent alive at pH | | | | | | |
|---|---|---|---|---|---|---|---|
| | 4.0 | 5.0 | 6.0 | 6.5 | 7.0 | 7.5 | 8.0 |
| 1 | 87 | 88 | 84 | 92 | 68 | 77 | 79 |
| 3 | 39 | 71 | 74 | 66 | 54 | 24 | 52 |
| 6 | 4 | 48 | 64 | 30 | 24 | 8 | 12 |
| 9 | 1 | 68 | 82 | 7 | 17 | 5 | 12 |
| 24 | 0 | 6 | 77 | 2 | 23 | 3 | 10 |
| Number of experiments | 2 | 2 | 4 | 2 | 10 | 2 | 4 |

In another communication, Winslow and Falk (1923*b*) reported that solutions of 0.725*M* NaCl and over and solutions of 0.435*M* $CaCl_2$ and

over exhibited distinctly toxic actions on *E. coli* at all hydrogen-ion concentrations. However, in a solution containing a mixture of these two salts in appropriate proportions, an antagonistic action was manifested, which tended to protect the bacteria against the toxic action of each salt if present alone (Table 7). This phenomenon is spoken of as the antagonistic action of ions.

TABLE 7.—VIABILITY OF *Escherichia coli* IN SOLUTIONS OF NaCl AND $CaCl_2$, SINGLY AND IN COMBINATIONS

| Total isotonic concentration* | Total molar concentration | Percentage of bacteria alive after 9 hr. in | | | |
|---|---|---|---|---|---|
| | | Pure NaCl | Pure $CaCl_2$ | NaCl + $CaCl_2$ | Ratio, Na/Ca |
| 0 | 0 | 89 | 89 | 89 | |
| 1 | 0.145 | 82 | 22 | | |
| 2 | 0.290 | .. | ... | 41 | 1:1 |
| 3 | 0.435 | 55 | 0+ | 28 | 2:1 |
| 4 | 0.580 | .. | ... | 40 | 3:1 |
| 5 | 0.725 | 46 | 0+ | 117 | 4:1 |
| 6 | 0.870 | 33 | ... | 30 | 5:1 |

* 1 tonicity = 0.145*M*.

In conclusion, it may be stated that the toxic effects exerted by salts may be of two distinct kinds: Very high concentrations of salts appear to exert a toxic effect that is apparent at all reactions and is additive when NaCl and $CaCl_2$ are mixed. At a lower concentration (0.145*M*), $CaCl_2$ exerts a different influence, manifest only in alkaline solutions and due to an inhibition of the power of the bacteria to reduce the alkalinity of the solution in which they are suspended. It is this latter type of toxic influence which is antagonized by NaCl. In alkaline solution, the mixture of these salts in the proportion of 4 parts NaCl to 1 part $CaCl_2$ is more favorable to viability than even distilled water.

A practical application of this phenomenon was the development of a physiological salt solution by Ringer before ionic antagonism was clearly understood. Ringer showed that when a beating heart was perfused with a 0.75 per cent solution of sodium chloride, pulsation stopped completely. On the addition of 0.0125 per cent calcium chloride to the solution, the heart beat was restored but not in a normal manner. On the further addition of 0.01 per cent potassium chloride and a small amount of alkali, such as sodium bicarbonate to adjust the pH of the solution, the heart beat became normal. This solution is known as Ringer's solution. Other physiological salt solutions of this type are Tyrode's and Locke's solutions.

## OLIGODYNAMIC ACTION OF HEAVY METALS

Naegeli (1893) noted that silver in very high dilutions produced a toxic action on certain organisms. He found that 1 part of silver in 100,000,000 parts of water killed algae of the genus *Spirogyra*. He believed that silver in such a high dilution could not produce a chemical action on living organisms. He, therefore, attributed its toxic effect to an oligodynamic action. The word "oligodynamic" is compounded from the two Greek words, ὀλίγος, *oligo*, few, little, small, and δυναμικός, *dynamic*, powerful. It may be defined as the toxic effect produced by heavy metals on living organisms in exceedingly minute quantities.

Other metals also exert a toxic action on organisms. Copper in a dilution of 1 part in 77,000,000 of water is toxic to certain algae. The spores of *Aspergillus niger* fail to germinate in the presence of 1 part of silver in 1,600,000 parts of water. Water distilled from a copper still is toxic to bacteria. This is due to the presence of traces of dissolved copper in the water. Water distilled from stills made of other heavy metals also exhibits this same phenomenon but to a lesser degree. This is not due to the greater toxicity of copper, as it is known that mercury is probably the most toxic metal, but to the fact that copper is more soluble in water than the other toxic metals. Therefore, metal stills should be avoided for the preparation of distilled water intended for biological use.

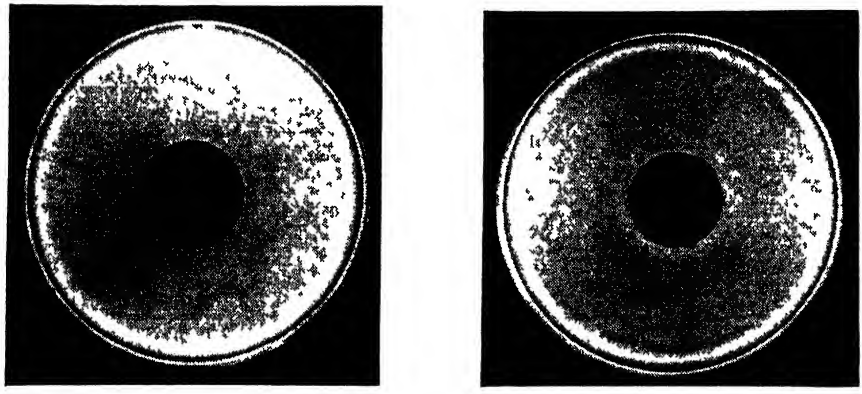
ZoBell (1941) found that containers made of copper, zinc, tin, or nickel alloys were not suitable for the collection of samples of sea water for bacteriological analysis owing to the inimical oligodynamic action of the metals. Under certain conditions, most of the bacteria in sea water were killed within a few minutes and the sea water itself was rendered bacteriostatic by exposure to the metals.

Burrows and Hemmens (1943) reported that bacteria swabbed on the polished surface of a silver chalice died off rapidly. Experiments on the transmission of test organisms from one person to another by common use of the chalice showed that approximately 0.001 per cent of the organisms was transferred even under the most favorable conditions. When the conditions approximated those of actual use, no transmission could be detected.

**Demonstration of Oligodynamic Action.**—Silver exerts a marked bactericidal or oligodynamic action on bacteria. This may be demonstrated by placing a piece of metallic silver or a silver coin in a Petri dish and pouring over it melted agar, previously inoculated with an organism such as *Sarcina lutea* or *Serratia marcescens*. After incubation for 24 hr. at 37°C., a clear zone will be seen immediately surrounding the silver metal or coin. This is the oligodynamic zone (Fig. 111). Beyond this will appear a narrower zone in which growth is stimulated. Minute amounts

of metallic ions stimulate growth whereas greater concentrations produce an inhibitory effect. Normal growth occurs in the remainder of the agar. The same result is obtained if a piece of copper or copper coin is substituted for the silver

Gibbard (1937) came to the following general conclusions regarding the action of silver on bacteria: (1) The width of the oligodynamic zone is increased by treating the silver with nitric acid and is decreased by careful cleaning of the metal. (2) Pure silver metal shows no bactericidal or



A B

FIG 111.—Oligodynamic action of silver *A*, agar inoculated with *Sarcina lutea* and poured into a dish containing a silver coin, *B*, same inoculated with *Serratia marcescens*. The zone immediately surrounding the silver coin is the oligodynamic zone This is followed by a narrow stimulating zone Normal growth occurs in the remainder of the agar

oligodynamic action and its toxic properties are probably due to silver ions coming from silver oxide. If silver oxide is prevented from forming, no inhibitory action occurs. This may be shown by melting silver, allowing a portion of it to cool in hydrogen, and another portion to cool in air. The silver cooled in hydrogen shows no bactericidal action while the metal cooled in air exhibits a pronounced bactericidal effect. (3) Silver nitrate, silver oxide, and electrically dispersed colloidal silver, when properly diluted to contain the same concentration of silver, all possess a similar bactericidal action. (4) The bactericidal properties of silver nitrate and silver oxide are greatly reduced in the presence of proteins. Silver proteinates are formed, removing the metal from solution.

**Application of Oligodynamic Action.**—Lisbonne and Seigneurin (1936*a*,*b*) showed that mercury could be used for the destruction of *E. coli* in drinking water.

Silver has been recommended for the treatment of water, milk, vinegar, wine, cider, fruit juices, liquors, etc. Its greatest application appears to be in the treatment of water for drinking purposes. According to Gibbard, three methods are generally used commercially for this purpose: (1) The

water is exposed to silver deposited on sand, porcelain, and other solid materials. (2) The silver is applied by electrolysis. (3) Use is made of the difference in E.M.F. that exists between nickel and silver electrodes kept in the material at different temperatures. In all three methods the objective is the same: to obtain a solution of silver.

**Silver Poisoning.**—There is still considerable discussion as to whether sufficient silver remains in treated water, foods, beverages, etc., to produce silver poisoning or argyria in man and animals. It is important to know how much of the silver ingested will be retained in the body. So far as is known, there are no available data on this point. More work is required before this controversy can be definitely settled.

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# CHAPTER IX

## STERILIZATION

Sterilization may be defined as the complete destruction of all living organisms in, or removal from, materials by means of heat, filtration, or other physical or chemical methods.

Plugged test tubes, flasks, bottles, etc., and Petri dishes must be sterilized before use to destroy all living organisms adhering to the inner surfaces. Pipettes are placed in containers and heated to sterilize both inner and outer surfaces. Likewise, all culture media must be sterilized previous to use in order to destroy all contaminating organisms present. Studies on single bacterial species or pure cultures could not be made if the glassware and culture media were contaminated with other organisms previous to use. When once sterilized, glassware and culture media may be kept in a sterile condition indefinitely if protected from outside contamination.

The usual methods employed for the sterilization of laboratory materials involve the use of heat. Three types of heat sterilizers are used in bacteriology for the destruction of living microorganisms: (1) the hot-air sterilizer, (2) the Arnold sterilizer, and (3) the autoclave.

**Hot-air Sterilizer.**—This is a dry-air type of sterilizer (Fig. 112). It is constructed with three walls and two air spaces. The outer walls are covered with thick asbestos to reduce the radiation of heat. A burner manifold runs along both sides and rear between the outside and the intermediate walls. Convection currents travel a complete circuit through the wall space and interior of the oven, and the products of combustion escape through an opening in the top.

The hot-air sterilizer is operated at a temperature of 160 to 180°C. (320 to 356°F.) for a period of 1½ hr. If the temperature goes above 180°C., there will be danger of the cotton stoppers charring. Therefore, the thermometer must be watched closely at first until the sterilizer is regulated to the desired temperature.

The hot-air sterilizer is used for sterilizing all kinds of laboratory glassware, such as test tubes, pipettes, Petri dishes, and flasks. In addition, it may be used to sterilize other laboratory materials and equipment that are not burned by the high temperature of the sterilizer. Under no conditions should the hot-air sterilizer be used to sterilize culture media, as the liquids would boil to dryness.

**Arnold Sterilizer.**—It is well known that moist heat is more effective as a sterilizing agent than dry heat. This is believed to be due to the following reasons: (1) Moist heat has greater penetrating power and (2) death of organisms is believed to be caused by a coagulation of the

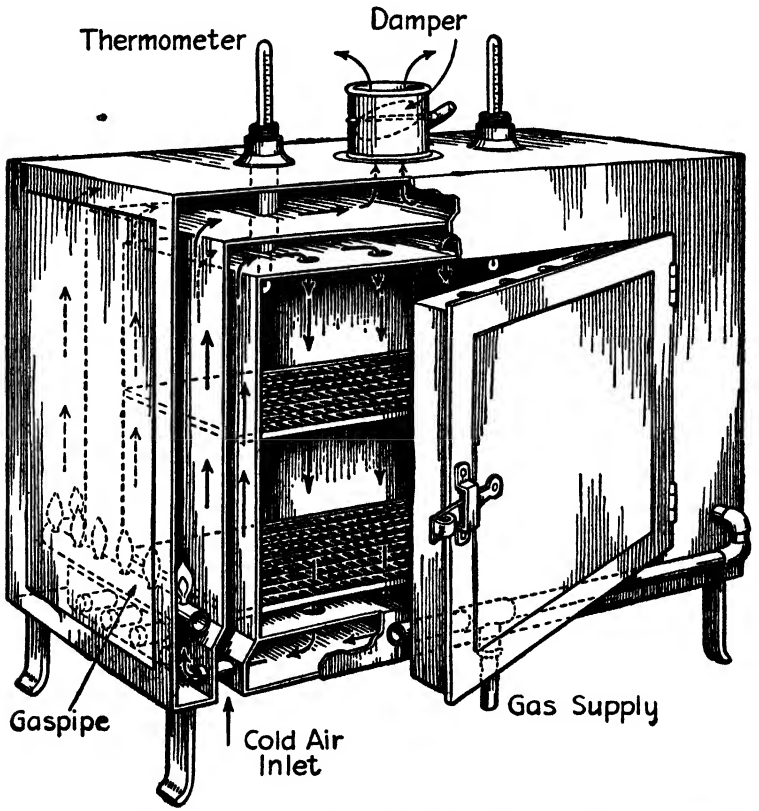



FIG. 112.—Hot-air sterilizer. (*From Belding and Marston, "A Textbook of Medical Bacteriology," D. Appleton-Century Company.*)

proteins of the protoplasm. An increase in the water content of the protoplasm causes the proteins to coagulate at a lower temperature.

The Arnold makes use of streaming steam as the sterilizing agent (Fig. 113). The sterilizer is built with a quick steaming base that is automatically supplied with water from an open reservoir. The water passes from the open reservoir, through small apertures, into the steaming base, to which the heat is applied. Since the base contains only a thin layer of water, steam is produced very rapidly. The steam rises through a funnel in the center of the apparatus and passes into the sterilizing chamber.

Sterilization is effected by employing streaming steam at a temperature of approximately 100°C. (212°F.) for a period 20 min. or longer on three consecutive days. The length of the heating period will depend upon the nature of the materials to be treated and the size of the container. Agar,

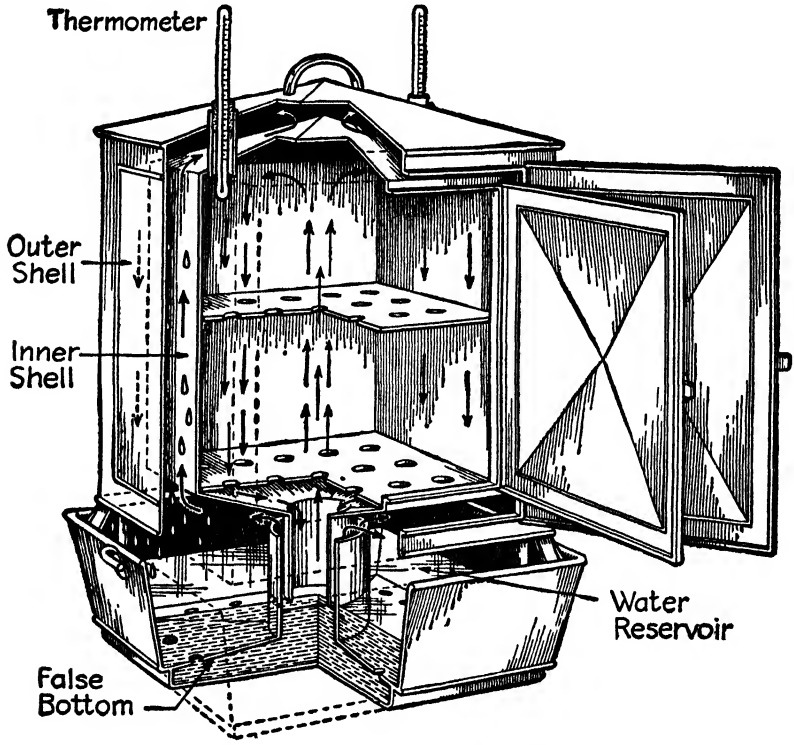



FIG. 113.—Arnold sterilizer. (*From Belding and Marston, "A Textbook of Medical Bacteriology," D. Appleton-Century Company.*)

for example, must be first completely melted before recording the beginning of the heating period.

It must be remembered that a temperature of 100°C. for 20 min. is not sufficient to destroy spores. A much higher temperature is required to effect a complete sterilization in one operation over a relatively short exposure period.

The principle underlying this method is that the first heating period kills all the vegetative organisms present. After a lapse of 24 hr. in a favorable medium and at a warm temperature, the spores, if present, will germinate into vegetative cells. The second heating will again destroy all vegetative cells. It sometimes happens that all spores do not pass into vegetative forms before the second heating period. Therefore, an addi-

tional 24-hr. period is allowed to elapse to make sure that all spores have germinated into vegetative cells.

It may be seen that, unless the spores germinate, the method will fail to sterilize. Failure may be due to the following causes: (1) The medium may be unsuited for the germination of the spores. Distilled water, for example, is not a favorable environment for the growth of bacteria. Therefore, it will not permit spores to germinate into vegetative cells.

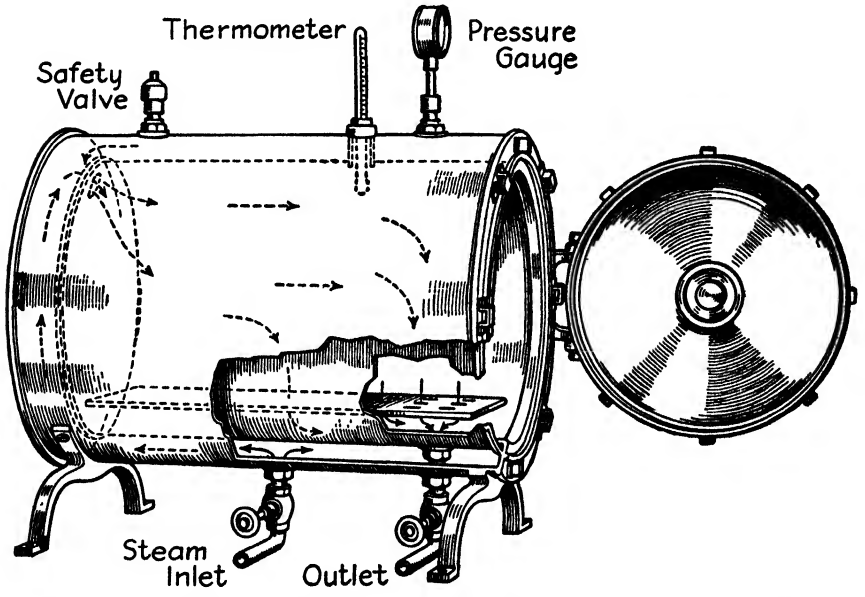



FIG. 114.—Autoclave sterilizer. (*From Belding and Marston, "A Textbook of Medical Bacteriology," D. Appleton-Century Company.*)

(2) Spores of anaerobic bacteria may be present, which will not germinate in a medium in contact with atmospheric oxygen.

The Arnold is used principally for the sterilization of gelatin, milk, and carbohydrate media. Higher temperatures or longer single exposures in the Arnold may hydrolyze or decompose carbohydrates and prevent gelatin from solidifying. Obviously, such media would then be unsatisfactory for use.

**Autoclave.**—The autoclave is a cylindrical metal vessel having double walls around all parts except the front (Fig. 114). It is built to withstand a steam pressure of at least 30 lb. per sq. in.

The principle of the method is that water boils at about 100°C., depending upon the vapor pressure of the atmosphere. If the atmospheric pressure is increased, the temperature will be increased. Therefore, if the steam pressure inside of the closed vessel is increased to 15 lb. per sq. in.

(2 atm.), the temperature will rise to 121.6°C. The relationship between pressure and temperature is shown in Table 8.

TABLE 8

| Pressure, lb. per sq. in. | Corresponding temperature | |
|---|---|---|
| | °C. | °F. |
| 5 | 107.7 | 227 |
| 10 | 115.5 | 240 |
| 15 | 121.6 | 250 |
| 20 | 126.6 | 260 |
| 25 | 130.5 | 267 |
| 30 | 134.4 | 274 |

The autoclave is usually operated at 15 lb. steam pressure for a period of 30 min., which corresponds to a temperature of 121.6°C. This temperature is sufficient to destroy both vegetative cells and spores in one operation.

Certain precautions must be observed to prevent sterilization failures. The most important single cause is due to incomplete evacuation of air from the chamber. Observation of the pressure gauge alone is not sufficient. The proper degree of temperature must also be taken into consideration. The temperature figures given in Table 8 are true only if all air is evacuated from the sterilizing chamber.

The temperature of a mixture of steam and air at a given pressure is less than that of pure steam alone. This means that, even though the autoclave is kept at the desired pressure, the temperature may not be sufficient to give complete sterilization. This is shown in Table 9 by Underwood (1937).

TABLE 9

| Degree of air evacuation | Pressure, lb. per sq. in | Ultimate uniform temperature in chamber, °C. |
|---|---|---|
| Complete | 15 | 121 |
| Two-thirds | 15 | 115 |
| One-half | 15 | 112 |
| One-third | 15 | 109 |
| No air evacuated | 15 | 100 |

Another important precaution to be observed is that the steam must have access to the materials to be sterilized. If the steam is prevented from penetrating the materials, the method will be of doubtful value. For example, suppose that it is desired to sterilize some cotton placed

inside of a bottle. If the bottle is closed with a rubber stopper, the steam cannot reach the cotton. The process will be no more effective than a hot-air sterilizer kept at 121.6°C. for a period of 30 min. It has already been seen that such a temperature and time interval is insufficient to destroy spores in a dry-air sterilizer. On the other hand, if the mouth of the bottle is covered with one or two thicknesses of muslin, permitting the steam to penetrate, then the cotton will be sterilized.

The autoclave is used to sterilize the usual noncarbohydrate broths and agar media, distilled water, normal saline solution, discarded cultures,

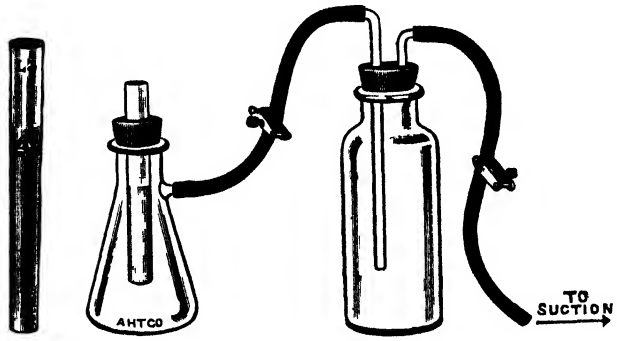



FIG. 115.—*A*, Chamberland-Pasteur filter. *B*, filter assembled in a filter flask and ready for filtration. (*Courtesy of A. H. Thomas Company.*)

contaminated media, aprons, rubber tubing, rubber gloves, etc. This is the type of sterilizer used commercially for processing canned foods.

For further reading, consult the report of Hoyt, Chaney, and Cavell (1938).

## STERILIZATION BY FILTRATION

Some solutions cannot be sterilized by heat without being greatly altered in their physical and chemical properties. Serum in culture media is easily coagulated by heat. If the serum content is high enough, the medium becomes changed from a liquid to a solid preparation. Certain physiological salt solutions containing the unstable compound sodium bicarbonate are ruined if heated. The bicarbonate easily loses carbon dioxide and is converted into the more alkaline sodium carbonate. Enzymes and bacterial toxins in solution are easily destroyed by heat. These are but a few examples of many that may be mentioned.

Preparations containing heat-sensitive compounds are best sterilized by the process of filtration. The types of filters employed for this purpose include: porcelain filters, Berkefeld filters, Mandler filters, plaster of Paris filters, fritted glass filters, asbestos filters, and collodion membranes or ultrafilters.

**Porcelain or Chamberland Filters.**—Porcelain filters are hollow, unglazed cylinders, closed at one end. They are composed of hydrous aluminum silicate or kaolin, with the addition of quartz sand, and heated to a temperature sufficiently low to avoid sintering. These filters are prepared in graduated degrees of porosity, from L1 to L13. Cylinders having the smallest pores are marked L13, those having the largest pores

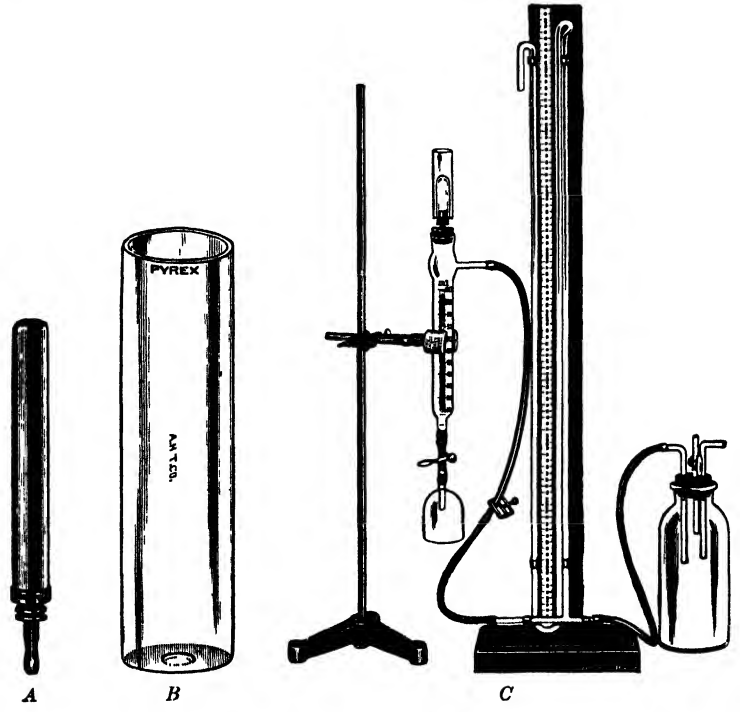



FIG. 116.—*A*, Berkefeld filter. *B*, glass mantle for use with either Berkefeld or Mandler filter. *C*, a convenient arrangement for assembling the filter for filtration. (*Courtesy of A. H. Thomas Company.*)

are designated L1. The finer the pores, the slower will be the rate of filtration. The L1 and L2 cylinders are preliminary filters intended for the removal of coarse particles and large bacteria. The L3 filter is probably satisfactory for all types of bacterial filtrations. A satisfactory method of assembling a Chamberland filter is shown in Fig. 115.

**Berkefeld Filters.**—Kieselguhr is a deposit of fine, usually white, siliceous powder composed chiefly or wholly of the remains of diatoms. It is also called "diatomaceous earth" and "infusorial earth."

Berkefeld filters are manufactured in Germany. They are prepared by mixing carefully purified diatomaceous earth with asbestos and organic matter, pressing into cylinder form, and drying. The dried cylinders are

heated in an oven to a temperature of about 2000°C. to bind the materials together. The burned cylinders are then shaped on machines into the desired shapes and sizes.

The cylinders are graded as W (dense), N (normal), and V (coarse), depending upon the sizes of the pores. The grading depends upon the rate of flow of pure filtered water under a certain constant pressure.

**Mandler Filters.**—These filters are similar to the Berkefeld type but are manufactured in this country. They are composed of 60 to 80 per cent diatomaceous earth, 10 to 30 per cent asbestos, and 10 to 15 per cent plaster of Paris. The proportions vary, depending upon the sizes of the pores desired. The ingredients are mixed with water, subjected to high pressure, and then baked in ovens to a temperature of 980 to 1650°C. to bind the materials together.

The finished cylinders are tested by connecting a tube to the nipple of the filter, submerging in water, and passing compressed air to the inside. A gauge records the pressure when air bubbles first appear on the outside of the cylinder in the water. Each cylinder is marked with the air pressure obtained in actual test.

A convenient arrangement of apparatus for filtering liquids through a Mandler or Berkefeld filter, as recommended by Mudd (1927), is shown in Fig. 116. The reduced pressure is indicated by the manometer. The liquid to be filtered is poured into the mantle and the filtrate is received into a graduated vessel, from which it may be withdrawn aseptically. Filtration may be interrupted at any time by stopping the vacuum pump and opening the stopcock on the trap bottle to equalize the pressure.

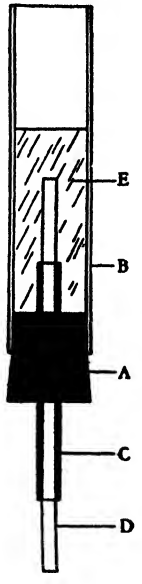



FIG. 117. Plaster of Paris filter.

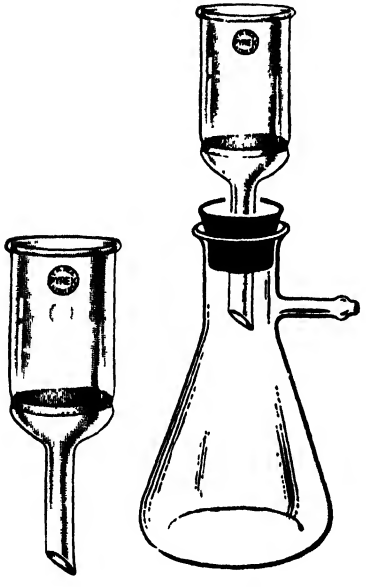
**Plaster of Paris Filters.**—These filters are shaped like Berkefeld and Mandler filters but are composed chiefly of plaster of Paris or calcium sulfate with some calcium carbonate (chalk) and magnesium oxide. It is probable that the calcium sulfate acts as a binder for the calcium carbonate and that the latter substance is the active absorbing component of the filter (Kramer, 1927).

Plaster of Paris filters have the advantage over the other types already discussed in that they may be easily prepared in the laboratory from cheap materials. They are attached to filter flasks and operated in the same manner as Berkefeld and Mandler cylinders.

A simple method for their preparation is shown in Fig. 117. A rubber stopper *A*, with a single perforation, is fitted into one end of a piece of heavy glass tubing *B*. Through the hole in the stopper is inserted a piece of glass tubing *C* containing a close-fitting glass rod *D*. A paste is pre-

pared, composed of 75 gm. of plaster of Paris (containing 2.5 per cent calcium carbonate), 25 gm. of magnesium oxide, and 75 gm. of water. The paste *E* is poured into the glass cylinder *B* and allowed to set. When it is set, the glass rod *D* is withdrawn, leaving a hole in the center of the filter for the filtrate to pass through.

**Fritted Glass Filters.**—Filters of this type are prepared by fritting



*A* *B*

FIG. 118.—*A*, fritted glass filter. *B*, filter assembled in a filter flask and ready for filtration.

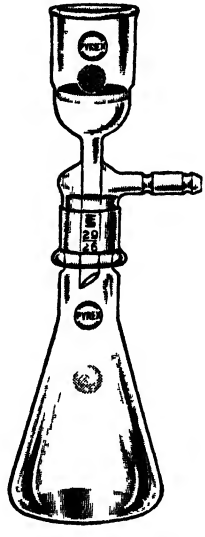


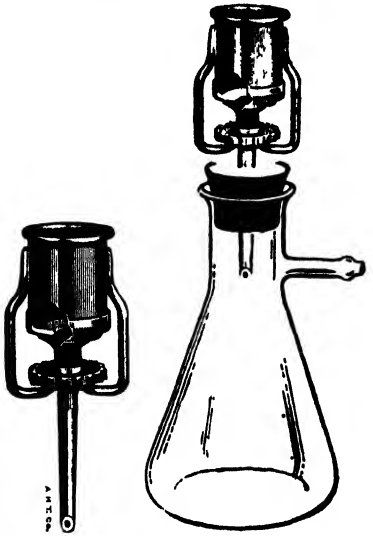
FIG. 119.—Fritted glass filter coupled to a flask through a ground-glass joint. (*Courtesy of Corning Glass Works.*)

finely pulverized glass into disk form in a suitable mold (Morton and Czarnetzky, 1937). The pulverized glass is heated to a temperature just high enough to cause the particles to become a coherent solid mass, without thoroughly melting, and leaving the disk porous. The disk is then carefully fused into a glass funnel and the whole assembled into a filter flask by means of a rubber stopper (Fig. 118). A better arrangement is the coupling of the filter to the flask through a ground-glass joint (Fig. 119), thus eliminating the use of a rubber stopper (Morton, 1944).

The filters are marketed in five degrees of porosity as follows: EC (extra-coarse), C (coarse), M (medium), F (fine), and UF (ultrafine).

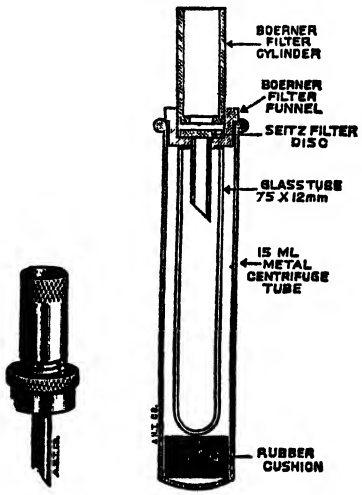
**Asbestos Filters.**—The best known filter employing asbestos as the

filtering medium is the Seitz filter (Fig. 120). The asbestos is pressed together into thin disks and tightly clamped between two smooth metal rims by means of three screw clamps. The liquid to be filtered is poured into the metal apparatus, in which the asbestos disk is clamped, and the solution drawn through by vacuum. The filtering disks are capable of effectively retaining bacteria and other particulate matter. At the end of the operation, the asbestos disk is removed, a new one inserted, and the



*A* *B*

FIG 120.—*A*, Seitz filter with asbestos disk in place *B*, filter assembled in a filter flask and ready for filtration (*Courtesy of A H Thomas Company*)




A B

FIG. 121.—*A*, Boerner centrifugal filter, *B*, Boerner filter assembled in a 15 cc metal centrifuge tube, with glass collecting tube inside (*Courtesy A H. Thomas Company*)

assembled filter sterilized. This feature makes the Seitz filter very convenient to use, since no preliminary cleaning is necessary.

A modification of the Seitz filter, utilizing centrifugal force instead of suction or pressure has been suggested by Boerner (Fig. 121). The filter consists of a cylinder and a funnel-shaped part with stem, which holds the filter pad supported on a wire gauze disk. The cylinder screws into the funnel with the filter disk pressed between them. The assembled filter fits closely into the top of a 15-cc. metal centrifuge tube, with the knurled collar of the funnel portion resting on the top of the metal tube. The filtrate is collected in a glass tube inside the cup. The filter can also be used for vacuum filtration in the conventional manner by inserting the stem through a rubber stopper fitted to a filter flask.

Webb, Irish, and Lyday (1944) found that washings through Seitz filters became more alkaline in reaction. The titration curves of the filtrates

indicated the presence of a weak base, probably magnesium, or a buffer substance with a basic pH. The soluble alkaline material probably could be eliminated by more thorough processing of the asbestos before preparation of the filters.

**Ultrafilters.**—Ultrafiltration generally means the separation of colloidal particles from their solvents and from crystalloids by means of jelly filters known as ultrafilters (Bechhold, 1926). The early jelly filters were composed of gelatin and of silicic acid but these have been replaced by collodion in membrane and sac form, or collodion deposited in a porous supporting structure. The supporting structure may be filter paper in sheet and thimble form, unglazed porcelain dishes and crucibles, Buchner funnels, filter cylinders, etc.

*Collodion.*—Several types of collodions are employed for ultrafiltration. The earliest type is prepared by dissolving pyroxylin or soluble guncotton in a mixture of 1 part of alcohol and 3 parts of ether. A more popular type is prepared by dissolving pyroxylin in glacial acetic acid. Pore size may be controlled by increasing or decreasing the pyroxylin content or by adding various liquids such as ethylene glycol and glycerol to alcohol-ether collodion. Elford (1931) recommended a new type of collodion for the preparation of a graded series of filters which he termed "Gradocol" membranes. The filters are prepared by incorporating a definite amount of amyl alcohol with an alcohol-ether collodion and then adding graded amounts of water or acetic acid to increase or decrease the permeability of the filters. Since membranes prepared by this procedure are quite strong, it is not necessary to deposit the collodion in a porous supporting structure.

Sacs may be prepared by pouring the collodion into a test tube or beaker, inverting and twirling continuously so that the excess drips out, until a thin even coating is formed. The tube or beaker is then plunged into cold water to jell the collodion. After most of the solvent has been washed away, the sac can be loosened from the glass mold and removed. Collodion membranes in sheet form may be prepared by cutting the sacs at right angles at the closed end and then lengthwise.

Membranes employing filter paper as the porous supporting structure are usually prepared with acetic collodion. Pore size of the membranes depends upon the strength of the collodion. A strong collodion gives finer pores than a weak collodion. It is a simple matter to prepare a graded series of filters. The paper foundation gives the membranes a strength that, under certain conditions, will withstand a pressure of 20 atm. or more.

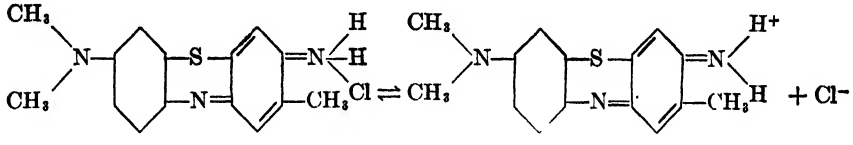
Collodion filters are extensively used for the isolation and determination of the diameters of ultramicroscopic viruses.

**Electrical Charge of Filters.**—The filtration of solutions of enzymes, toxins, immune bodies, viruses, etc., usually results in a loss of some of the active material. If the active material is present in very low concentration, it may be completely removed from solution.

Filters composed of porcelain (Chamberland), diatomaceous earth (Berkefeld, Mandler), fritted glass, and asbestos (Seitz), consist chiefly of metal silicates and carry negative electrical charges.

The metal ($Mg^{++}$, $Al^{+++}$, $Ca^{++}$, etc.) cations or positive ions are more soluble than the silicate anions or negative ions and show a greater tendency to pass into solution. When a liquid is filtered, positively charged particles will react with the negative silicate ions and negatively charged particles will react with the positive metal ions. Since the metal ions are soluble, they will react with the negatively charged particles and pass through the pores of the filter into the filtrate. On the other hand, the insoluble silicate ions will react with positively charged particles and remain fixed to the walls of the filter pores.

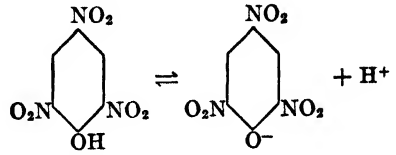
Adsorption of compounds from solution can be very effectively demonstrated by means of basic and acid dyes, such as toluidine blue and picric acid, respectively. Toluidine blue is a thiazine dye having the same chromophore as methylene blue and thionine. It ionizes as follows:



The cation is blue, and the chlorine anion is colorless. When a solution of this dye is filtered through one of the silicate filters, the blue cations will react with the negative silicate ions and remain fixed to the pores of the filter. The chloride anions will combine with the metal cations of the silicates to form soluble metal chlorides and pass into the filtrate. If more than sufficient dye is present in the solution to react with all the silicate ions in the filter pores, the excess will pass through imparting a blue color to the filtrate. If, on the other hand, the amount of toluidine blue is insufficient to take care of all the silicate ions, the dye will be completely removed from the solution and the filtrate will be colorless. The reaction is reversible, however, since the passage of distilled water through the filter saturated with dye results in a blue color in the filtrate.

If a solution of an acid dye, such as picric acid, is used instead of the basic toluidine blue, the results will be quite different. The dye will not be adsorbed by the filter material but will pass through the pores into the filtrate.

Picric acid is trinitrophenol, an acid dye, having the nitro group as the chromophore. It ionizes as follows:



In this case, the cation is colorless and the anion is yellow. When a solution of this dye is filtered through one of the silicate filters, not a trace of it will be adsorbed because an exchange of ions results in the formation of soluble picrates, which pass through in the filtrate.

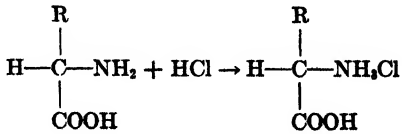
Michaelis (1925) found that collodion filters were nonionogenic but that they also carried a negative charge. Their negative charge was believed to be due to the adsorption of negative ions. Elford (1933) found that proteins in solutions, adjusted to different pH values by hydrochloric acid and sodium hydroxide, were most strongly adsorbed in the isoelectric zone (page 193). On the other hand, proteins in solutions buffered with $M/15$ phosphate instead of adjusting the pH with hydrochloric acid and sodium hydroxide are adsorbed on the acid side of the isoelectric zone. The negatively charged collodion now preferentially adsorbs the positively charged proteins. He concluded that the effect is probably associated with some specific influence of the phosphate ion.

Plaster of Paris filters, on the other hand, carry a positive electrical charge. They are capable of producing insoluble compounds with anions or negative ions, which remain adsorbed to the walls of the filter pores.

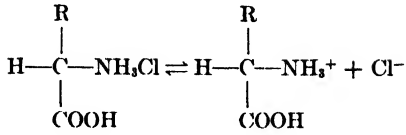
For an excellent discussion of the structure of the collodion membrane and its electrical behavior, see the series of articles by Abrams and Sollner (1943), Carr, Gregor, and Sollner (1945), Carr and Sollner (1943, 1944), Sollner, Abrams, and Carr (1941*a*, *b*), Sollner and Anderman (1944), Sollner and Beck (1944), Sollner and Carr (1942, 1943, 1944), and Sollner, Carr, and Abrams (1942).

**Amphoteric Nature of Proteins and Amino Acids.**—An important characteristic of proteins and amino acids is that they contain both acidic (COOH) and basic ($NH_2$) groups. In acid solutions, the compounds act as bases; in basic solutions, they act as acids. Representing the formula of an amino acid as $R{\cdot}CHNH_2{\cdot}COOH$, the reactions with acids and bases are as follows:

With an acid,

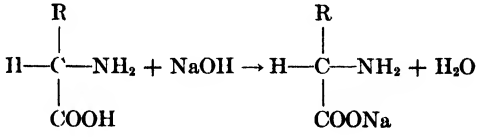


On ionization, this gives

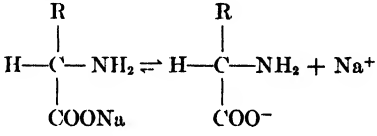


The acid reacts with the basic amino group. The amino acid molecule has a positive charge and, therefore, behaves as a base.

With a base,

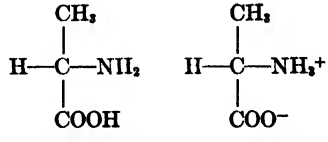


On ionization, this gives



The base reacts with the acid carboxyl group. The amino acid molecule has a negative electrical charge and, therefore, behaves as an acid. Compounds of this nature that are capable of reacting with both acids and bases are said to be amphoteric (from the Greek meaning both).

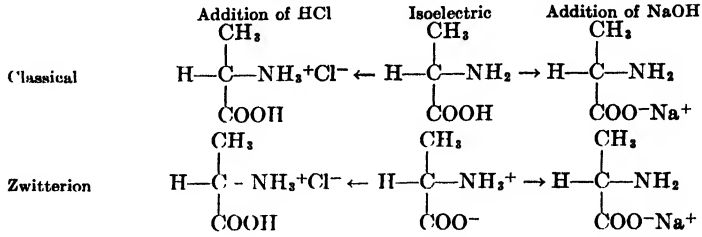
*Isoelectric Point.*—According to the classical theory, amphoteric compounds are supposed to dissociate into ions on either side of a pH point known as the isoelectric point. The isoelectric point has been defined as that point where the ionization of the amphoteric compound is at a minimum, expressed in pH. Opposed to this concept is the more recently developed view known as the "zwitterion" hypothesis, which states that the isoelectric point is that point where ionization is at a maximum. The difference in the two theories is indicated in the following formulas for isoelectric alanine:



I. Classical II. Zwitterion

Formula I represents a molecule that is not dissociated as either an acid or a base. The neutrality of the molecule is assumed to be due to the absence of dissociation. Formula II is also neutral, but the neutrality is assumed to be due to complete ionization of the acid and basic groups.

Regardless of which theory is correct, results obtained on the addition of an acid or a base are the same in both cases.




The isoelectric point of a protein is not necessarily the neutral point (pH7.0). As a matter of fact most proteins that have been studied have isoelectric points on the acid side of neutrality. The isoelectric points of a few of the common proteins are as follows:

| | Isoelectric Point (pH) |
|---|---|
| Casein (milk protein) | 4.7 |
| Egg albumin | 4.6 |
| Gelatin | 4.7 |
| Hemoglobin | 6.8 |
| Serum albumin | 4.7 |
| Serum globulin | 5.6 |

A knowledge of the isoelectric points is of considerable value in the filtration of solutions containing proteins, amino acids, bacterial toxins, enzymes, viruses, antitoxins, etc. If a solution is acid with respect to its isoelectric point, the active constituent will behave as a base and possess a positive electrical charge. The filtration of such a solution through a silicate filter, which has a negative charge, will result in the complete or partial adsorption of the active constituent on the walls of the filter pores. To avoid this, it would be necessary to use a filter, such as plaster of Paris, that possesses a positive charge, or to change the reaction of the solution to be filtered.

The adjustment of a solution to correspond to the acid or basic side of the isoelectric point can be carried out only provided the change in pH will not result in a destruction of the active material. After filtration, the pH of the filtrate should be readjusted to correspond to the optimum pH range of the active component. If the active material is very sensitive to slight changes in pH, a filter having an appropriate electrical charge should be selected instead.

**Cleaning Filters.**—All filters are intended to be cleaned after each filtration, or discarded and a new one employed. Collodion membranes and plaster of Paris filters are easily prepared and the Seitz asbestos disks can be purchased. These filters are intended to be used once and then discarded. On the other hand, porcelain, diatomaceous earth, and fritted glass filters are too expensive to be used only once, but are easily cleaned.

Porcelain filters are cleaned by placing them in a muffle furnace and raising the temperature to a red heat. This burns the organic matter in the pores and restores the filters to their original condition.

Filters of the Berkefeld and Mandler types are cleaned by placing the cylinders in a special metal holder connected to a faucet. The flow of water is reversed by passing through the cylinder from within outward. This should be continued until all foreign matter has been washed away from the filter pores. Albuminous or similar materials remaining in the pores of the filters are likely to be coagulated by heat during the process of sterilization with the result that the filters will be clogged. Filters in this condition are useless for further work.

Clogged filters may be cleaned in various ways but probably more conveniently by continuous suction of full-strength Clorox, or similar solution, for from 5 to 15 min. (Vaisberg, 1938). This treatment quickly dissolves the coagulated material and restores the usefulness of the filter. Thorough washing is necessary to remove the last traces of the oxidizing solution.

Fritted glass filters may be cleaned by treatment with concentrated sulfuric acid containing sodium nitrate. The strong acid quickly oxidizes and dissolves the organic matter. Thorough washing is necessary to remove the last traces of acid.

**Sterilization of Filters.**—With the exception of collodion membranes, the various filters are assembled in their appropriate holders, wrapped in paper, and autoclaved. Dry heat cannot be used because of the destruction of rubber fittings for the filter flasks.

Since acetic collodion solutions are sterile, it is not necessary to sterilize such membranes in which filter paper is used as the porous supporting structure, if aseptic precautions are observed in their preparation.

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## CHAPTER X

## DISINFECTION AND DISINFECTANTS

The destruction of bacteria by chemicals is generally discussed under disinfection. A number of terms are used to describe the process: germicide, bactericide, antiseptic, disinfectant, viricide, bacteriostatic agent, and sterilization. Considerable confusion exists as to their true meanings because popular interpretations are not always the same as their scientific definitions.

The terms are correctly defined as follows:

**Germicide.**—A germicide was originally defined as any agent that destroys disease organisms. It is now defined as any agent that destroys bacteria but not spores, irrespective of whether they are capable of producing disease.

**Bactericide.**—A bactericide refers to any agent that destroys bacteria but not spores. In practice, the term is synonymous with germicide.

**Antiseptic.**—The meaning of this term has caused probably more confusion than any of the others. Some define it synonymously with germicide and bactericide; others apply the term to any agent that prevents further bacterial action whether it does so by killing the organisms or by merely preventing them from multiplying. The term "antiseptic" should be limited to any agent that prevents or arrests the growth or action of organisms either by inhibiting their activity or by destroying them.

According to this definition, a germicide may be also an antiseptic, depending upon the strength of the solution, the period of action, and the nature of the organism. A germicide in high dilution may only inhibit growth and multiplication rather than kill the bacteria. Also, an agent that kills in a given period of time may only inhibit growth if the exposure time is shortened. In the former case, it would be classed as a germicide; in the latter, as an antiseptic. Some organisms are less resistant than others. This means that a substance may be a germicide against some organisms, and an antiseptic against others. Doubtless, other factors are also involved.

**Disinfectant.**—This term has been used rather loosely and defined in various ways, but it is generally agreed that it means the destruction of disease bacteria and other harmful organisms but not spores.

**Viricide.**—This term is applied to an agent that destroys or inactivates filterable viruses. Since viruses are of about the same order of resistance to chemical agents as bacteria, most germicides are also good viricides.

**Bacteriostatic Agent.**—This term was coined by Churchman (1912, 1928), in connection with his investigations on dyes, to denote a condition in which bacteria are not killed but merely prevented from multiplying. He noted that in certain concentrations dyes did not kill bacteria but kept them in a state of suspended animation. Further dilution of the bacteria-dye mixture resulted in growth of the organisms. The dyes were referred to as bacteriostatic agents and the phenomenon as bacteriostasis. Germicidal agents of a nondye nature, such as mercury and silver compounds, also exhibit the same phenomenon and are referred to as bacteriostatic compounds.

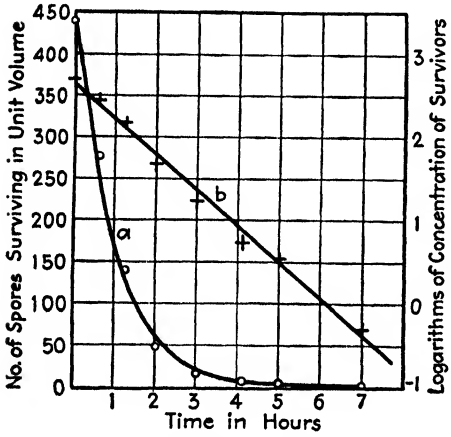



FIG. 122.—Disinfection of anthrax spores with 5 per cent phenol. *a*, the number of survivors in unit volume are plotted against time; *b*, logs of concentration of survivors are plotted against time.

**Sterilization.**—This is a more precise term, meaning the complete killing of all organisms present, including spores. The term "sterilization" should always be used where reference is made to complete killing of all organisms and spores.

## DYNAMICS OF DISINFECTION

Krönig and Paul (1897) were apparently the first to show that the disinfection process does not take place at once but is a gradual operation in which the number of organisms killed in unit time is greater at the beginning and becomes less and less as the exposure period is increased. If the numbers of survivors in unit volume are plotted against time, the points fall on smooth curves. Later, Madsen and Nyman (1907) and Chick (1908, 1910) showed that, if the logarithms of the numbers of bacteria surviving in unit volume are plotted against time, a straight line is obtained.

This may be shown in Fig. 122, which is the result of plotting the figures given in Table 10. Anthrax spores were treated with 5 per cent phenol and incubated at 33⅓°C. The curved line represents the numbers of anthrax spores surviving in unit volume, and the straight line represents the logarithms of the concentration of survivors.

The disinfection process, according to the results of Chick, appears to follow the mass-action law and to proceed in accordance with the monomolecular equation, or a reaction of the first order, provided the disinfectant is present in large excess:

$$-\frac{dN}{dt} = KN \quad \text{or} \quad \frac{1}{t_2 - t_1} \log \frac{N_1}{N_2} = K$$

where $N_1$ and $N_2$ represent the number of surviving bacteria in unit volume after times $t_1$ and $t_2$, respectively.

TABLE 10.—DISINFECTION OF ANTHRAX SPORES WITH 5 PER CENT PHENOL AT 33⅓°C.

| Sample No. | Size of sample, drops | Time of action, hr. | Number of organisms, average | Number of organisms present in 1 drop of disinfecting mixture, average | Log $N$ | Value of $K$ in the monomolecular equation |
|---|---|---|---|---|---|---|
| 1 | 1 | 0 | 439 | 439 | 2.64 | |
| 2 | 1 | 0.5 | 275.5 | 275.5 | 2.44 | 0.40 |
| 3 | 1 | 1.25 | 137.5 | 137.5 | 2.14 | 0.40 |
| 4 | 1 | 2 | 46 | 46 | 1.66 | 0.49 |
| 5 | 2 | 3 | 31.6 | 15.8 | 1.20 | 0.48 |
| 6 | 2 | 4.1 | 10.9 | 5.45 | 0.73 | 0.46 |
| 7 | 4 | 5 | 13.9 | 3.6 | 0.56 | 0.41 |
| 8 | 6 | 7 | 3 | 0.5 | −0.31 | 0.42 |

Average value of $K$ = 0.44.

**Importance of Logarithmic Survivor Curves.**—In the destruction of bacteria by various agents, the greater the number of cells present, the longer will be the time necessary for complete sterilization. This may be shown in the following example: A suspension contains 20,000 organisms per cubic centimeter. If the bacteria are destroyed at the rate of 90 per cent per minute, the number of survivors at the end of 6 min. will be

20,000 2,000 200 20 2 0.2 0.02

The last figure means that 2 living bacteria remain in 100 cc. of the suspension. If, instead of 20,000, the suspension contains only 200 organisms per cubic centimeter, the time required to reduce the count to 2 bacteria per 100 cc. will be only 4 min.:

200 20 2 0.2 0.02

The relationship between bacterial numbers and the times required to produce complete sterilization is of great value in the canning and dairying industries, in bacteriology, and in surgery.

Jordan and Jacobs (1944*a*, *b*), on the basis of their experimental results, found that the death rate of *Escherichia coli* was not constant, *i.e.*, did not follow the so-called logarithmic order of death. They cultivated the

organisms in a special flask to which was attached an automatic syringe mechanism for the continuous addition of culture medium and an opening for the removal of samples periodically. The bacterial culture was standardized with respect to age, medium, and temperature of growth. Within a few hours after inoculation, the viable population reached about 330,000,000 cells per cubic centimeter, at which level it was maintained constant by the continuous addition of broth at a standard rate. After

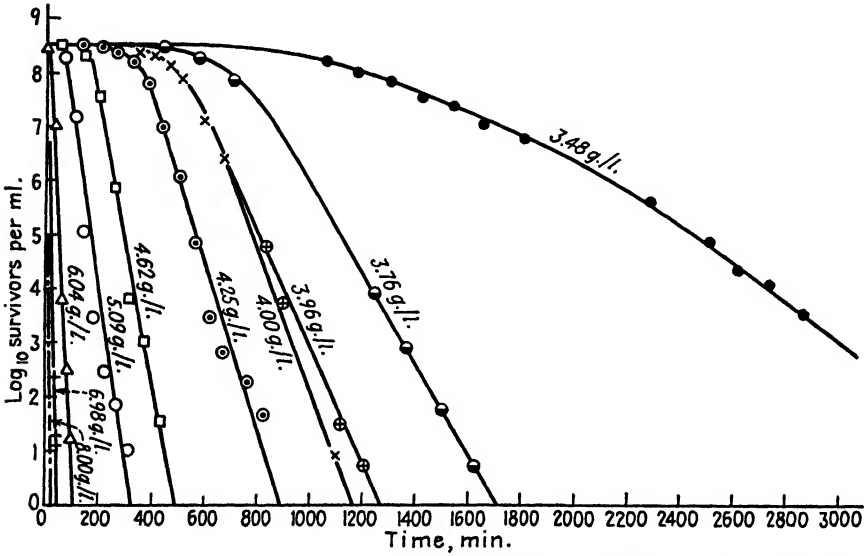



FIG. 123.—Survivor-time curves for *Escherichia coli* when exposed to various concentrations of phenol at 35°C. (*After Jordan and Jacobs.*)

an incubation period of 40 hr., a known concentration of phenol was added to the culture, and samples were removed at definite intervals for plate-count determinations. A typical set of survivor-time curves is shown in Fig. 123. It may be seen that the death rate was not constant. Two phases were definitely recognized: (1) a slow but increasing death rate which merged gradually into a phase (2) of approximately constant rate which was also the maximum for a given phenol concentration. There were indications that the death rate declined toward the extreme end of the disinfection process. A period of "no death" or lag occurred immediately after the addition of the phenol. This lag period became less and less as the concentration of phenol was gradually increased.

For more information see Jordan and Jacobs (1945*a*, *b*, 1946*a*, *b*).

## PHYSICAL AGENTS

The physical agents that have not been discussed in Chaps. VIII and IX are included here.

**Electricity.**—Both low-frequency and high-frequency currents have been employed for the destruction of microorganisms. Prochownick and Spaeth (1890) passed a low-frequency current through saline suspensions of *Micrococcus pyogenes* var. *aureus*, *Bacillus subtilis*, and *B. anthracis* with very little destructive effect. In another series of experiments, the electrodes were coated with agar, inoculated with the test organisms, immersed in saline solutions, and a current passed through. The organisms were destroyed at the anode but not at the cathode. A current of 60 ma. destroyed *M. pyogenes* var. *aureus* in 15 min. and 230 ma. destroyed *B. anthracis* in 30 min. Since the chlorine ions of the saline solution migrated to the anode, they concluded that death of the organisms was due to the toxic effect of the chlorine and not to the electric current.

Apostoli and Laquerrière (1890) passed a current through a broth culture of *B. anthracis* in which the electrodes were immersed a short distance apart. A current of 300 ma. destroyed the organisms in 5 min. whereas 250 ma. failed to destroy all the organisms in the same period of time. They concluded that the destructive action of the current depended more upon the intensity than upon the period of action, that the lethal effect occurred only at the anode or positive pole, and that the action of the current was due to the liberation of acids and active oxygen at the anode.

Beattie and Lewis (1920) employed a current of about 4000 volts and 2 amp. for 4 min. and reported the destruction of over 99.9 per cent of the organisms in milk.

Fabian and Graham (1933) noted the gradual destruction of *E. coli* by exposure to a high-frequency current of 10 megacycles per second and an intensity of 0.8 amp. However, the application of the current for 10 hr. failed to destroy all the organisms in a suspension. A frequency of 10 megacycles corresponds to a wave length of approximately 30 meters. Gale and Miller (1935) reported that they were unable to destroy *M. pyogenes* var. *aureus*, *S. typhosa*, *Diplococcus pneumoniae*, and streptococci when cultures were exposed to ultrashort waves of 10 meters and 200 watts for 1 hr. on each of three consecutive days.

Electric current has been employed for the pasteurization of milk and for the destruction of organisms in sewage, water, etc., but the results have been too unreliable to be of practical importance. Its use has been largely abandoned, preference being given to other germicidal agents.

**Sonic and Supersonic Waves.**—Sonic waves are waves of audible frequency of about 8900 cycles per second produced by a nickel tube vibrating in an electromagnetic field and in resonance with a 2000-volt, oscillating power circuit. Such waves are capable of destroying bacteria if exposed for sufficient time. Milk has been treated in this manner with a reduction of 99 per cent in the viable count after an exposure period of 40 to 60 min.

Supersonic waves are waves above audible frequency, of from 200,000

to 1,500,000 cycles per second, produced by connecting a piezoelectric crystal with a high-frequency oscillator. These waves have also been shown to exert a destructive effect on bacteria and other organisms.

Wood and Loomis (1927) employed sound waves of high frequency and great intensity generated by a piezoelectric oscillator of quartz operated at 50,000 volts and vibrating 300,000 times per second. They noted the fragmentation or the tearing to pieces of organisms such as *Spirogyra*, *Paramecium*, etc. Red blood cells suspended in saline were also broken into small fragments. On the other hand, bacteria were able to survive treatment with high-frequency sound waves of great intensity. Williams and Gaines (1930) employed high-frequency, audible sound waves of about 8800 cycles per second and reported the destruction of cells of *Escherichia coli*. They concluded that the lethal effects of the waves were probably due to a violent agitation set up within the cell. Harvey and Loomis (1929) worked with luminescent bacteria and reported that they were destroyed in 1½ hr. by supersonic energy.

Beckwith and Weaver (1936) treated a yeast and several species of bacteria by supersonic waves and reported destruction of the organisms. They concluded that the organisms were usually killed by sufficient application of supersonic radiation. The presence of protein interfered with the action of the sonic energy.

**X Rays.**—Haberman (1942) treated *Micrococcus pyogenes* var. *aureus* with both soft (> 1 Å.) and hard (< 1 Å.) X rays. When the logarithms of the percentage of surviving cells were plotted on semilogarithmic paper against time, a straight line was obtained, indicating that the lethal effects of the rays were dependent upon the number of bacteria present at any given time. The lethal action of the X rays was dependent more on the wave length emitted than on the intensity. Short wave lengths were more effective in killing *M. pyogenes* var. *aureus* than long wave lengths of the same intensity.

## CHEMICAL AGENTS

**Distilled Water.**—There appears to be a difference of opinion concerning the action of distilled water on the viability of bacteria and spores. Koch (1881) reported that anthrax spores were able to remain alive for more than 3 months in distilled water; vegetative cells were considerably more sensitive. Some have reported death in a few hours whereas others have stated that weeks were necessary to destroy all organisms. This discrepancy may be due, in part at least, to the vessel from which the water was distilled. It has already been shown that minute amounts of some metallic ions exert a toxic effect on bacteria. Wilson (1922) reported that water distilled from a copper vessel sterilized a suspension of *Salmonella aertrycke* in a few hours.

The number of organisms introduced in the inoculum has also been shown to be the cause of considerable discrepancy in results. Ficker (1898) showed that when distilled water was seeded with 60,000,000 cholera organisms per cubic centimeter, viability was present after several months, but when the number was reduced to 10,000 per cubic centimeter, all bacteria were dead after a period of 2 hr. He concluded that the inoculation of large numbers of organisms into distilled water resulted in the transfer of sufficient nutrients to prepare a dilute medium. Such a solution no longer possessed the properties of distilled water. Another cause of conflicting results may be due to variations in the pH of the distilled water. Winslow and Falk (1923*a*, *b*) adjusted distilled water to increasing hydrogen-ion concentrations and found that a pH of 6.0 gave the highest percentage of viable organisms after a period of 9 hr. Cohen (1922) showed that the stabilization of distilled water by the addition of buffers gave much more constant results. Spangler and Winslow (1943) reported that washed cells of *Bacillus cereus*, added to distilled water, died out very rapidly. However, the addition of NaCl in concentrations from 0.00001*M* to 0.3*M* protected the organisms for a time against the harmful effects of distilled water. Other factors that affect the final results include traces of alkali dissolved from soft glass, dissolved carbon dioxide from the air, percentage of dissolved oxygen, and temperature of incubation. Whipple and Mayer (1906) found that *Salmonella typhosa* remained viable in distilled water for 2 months under aerobic conditions but only 4 days in an anaerobic environment. Houston (1914) placed *S. typhosa* in distilled water kept at different temperatures. At 0°C. the organisms lived for 8 weeks; at 18°C. for 3 weeks; and at 37°C. for only 1 week.

There is no evidence to show that true bacteria, when inoculated into distilled water, are destroyed by the process of plasmoptysis, *i.e.*, the excessive intake of water resulting in the disruption of the cells. Bacterial cells are too resistant to osmotic changes for this to occur.

**Acids.**—Krönig and Paul (1897) were probably the first to show that the germicidal efficiency of acids is proportional to the hydrogen-ion concentrations of their solutions. A strong acid (HCl, $H_2SO_4$) is, therefore, more germicidal than a weak acid (lactic, acetic, benzoic). Winslow and Lochridge (1906) compared the strengths of HCl and $H_2SO_4$ required to produce a 99 per cent and a 100 per cent reduction, respectively, in the *E. coli* population in 40 min. They found that it required an 0.0077*N* solution of HCl or an 0.0096*N* solution of $H_2SO_4$ to produce a 99 per cent reduction in the number of *E. coli*. Since the degree of dissociation was greater with HCl than with $H_2SO_4$, the final hydrogen-ion concentrations of the two solutions were practically the same.

The hydrogen-ion concentration does not necessarily explain all of the disinfecting action of an acid solution. The action of some acids is also

dependent upon the nature of the molecule. Winslow and Lochridge (1906) showed that either an 0.0812*N* solution of acetic acid or an 0.0097*N* solution of benzoic acid was required to produce a 99 per cent reduction in the number of *E. coli* after an exposure period of 40 min. The amount of dissociated hydrogen in the acetic acid was equal to 1.2 parts per million but in the benzoic acid it was 0.1 part per million. On the other hand, 7.49 parts per million of HCl were required to produce the same result. It is evident that the acetic and benzoic acids produced a toxic effect in addition to that of the hydrogen ions. This action may be due either to the additional effect of the anions or to the undissociated molecules, or to all three.

Benzoic and salicylic acids are weak acids, almost completely dissociated in neutral or alkaline solution, but almost completely undissociated in strongly acid solution. Rahn and Conn (1944) found that benzoic, salicylic, and sulfurous acids were nearly a hundred times more germicidal in strongly acid solutions than in neutral solutions. Only the undissociated molecules were germicidal; the ions exhibited only a slight germicidal effect.

TABLE 11.—GROWTH RATE OF *Escherichia coli* IN VARIOUS CONCENTRATIONS OF NaCl AND AT VARIOUS pH VALUES

| NaCl concentration | Series 1 | | Series 2 | | Series 3 | | Series 4 | | Series 5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | pH | Hr. | pH | Hr. | pH | Hr. | pH | Hr. | pH | Hr. |
| Control | 5.3 | 36 | 6.3 | 10¾ | 7 | 7 | 7.7 | 6 | 8.3 | 7 |
| 0.05*M* | 5.3 | 6¾ | 6.3 | 8½ | 7.1 | 5¾ | 7.7 | 3¾ | 8.3 | 4 |
| 0.10*M* | 5.3 | 4 | 6.4 | 4 | 7.1 | 4 | 7.8 | 3¼ | 8.3 | 3½ |
| 0.20*M* | 5.3 | 4 | 6.5 | 3½ | 7.2 | 3½ | 7.8 | 3¼ | 8.3 | 3½ |
| 0.30*M* | 5.3 | 4½ | 6.5 | 3¾ | 7.3 | 3¾ | 7.9 | 3¾ | 8.3 | 3½ |
| 0.40*M* | 5.3 | 5¼ | 6.5 | 4½ | 7.3 | 4 | 7.9 | 4 | 8.3 | 4 |

**Alkalies.**—Krönig and Paul (1897) showed that the disinfecting action of alkalies was dependent upon the presence of $OH^-$ ions. The greater the degree of dissociation, the more effective the germicidal action. Alkalies that are especially toxic to bacteria include KOH, NaOH, LiOH, and $NH_4OH$. Of these, KOH shows the greatest germicidal action by virtue of its greater degree of dissociation; $NH_4OH$ shows the smallest because it is the least ionized.

There are some exceptions to the above rule. Barium hydroxide, $Ba(OH)_2$, for example, is less dissociated than KOH, yet it is considerably more toxic. This is due to the high toxicity of the barium ion. The combined action of the barium and hydroxyl ions produces a greater germicidal action than that exhibited by either ion acting alone.

Hydrogen ions exert a greater toxic effect than an equivalent number of hydroxyl ions.

**Salts.**—It has been shown that cations exert a peculiar and characteristic effect on the viability of bacteria. In general, cations in low concentration tend to stimulate bacterial growth, whereas cations in higher concentrations are inhibitory and ultimately toxic.

TABLE 12.—EFFECT OF NaCl UPON THE GROWTH OF *Escherichia coli* AT VARIOUS pH VALUES

| Medium | Time, hr., required to show turbidity at pH of | | | | | |
|---|---|---|---|---|---|---|
| | 4.8 | 5.2 | 6.2 | 7.6 | 8.2 | 9.2 |
| 1% peptone | No growth | 16 | 6 | 5½ | 8 | 32 |
| 1% peptone + 0.2*M* NaCl | 18 | 5½ | 4 | 3½ | 3½ | 14 |

Sherman and Holm (1922) found that low concentrations of NaCl produced an accelerating effect on the growth of *Escherichia coli*. The optimum stimulating action occurred at a salt concentration of about 0.20*M*. The optimum pH for growth in both the control and salt media appeared to be about 7.8. The results are given in Table 11. Similar results were reported by Winslow and Hotchkiss (1922).

Sherman and Holm found that *E. coli* rarely grew in a 1 per cent peptone medium at pH4.8, but grew quite readily in the same medium to which was added NaCl to make a 0.20*M* solution. The NaCl produced a widening effect upon the pH limit of growth. This widening effect was even more pronounced upon the growth of some other bacterial species. The results on *E. coli* are recorded in Table 12.

| Group 1 | Group 2 |
|---|---|
| NaCl | $NiCl_2$ |
| KCl | TlCl |
| LiCl | $CuCl_2$ |
| $NH_4Cl$ | $FeCl_3 \cdot 12H_2O$ |
| $SrCl_2 \cdot 6H_2O$ | $FeCl_2 \cdot 4H_2O$ |
| $MgCl_2 \cdot 6H_2O$ | $ZnCl_2$ |
| $CaCl_2$ | $CoCl_2 \cdot 6H_2O$ |
| $BaCl_2$ | $PbCl_2$ |
| $MnCl_2 \cdot 4H_2O$ | $AlCl_3$ |
| $TiCl_3$ | $CeCl_3$ |
| $SnCl_4 \cdot 5H_2O$ | $CdCl_2$ |
| | $HgCl_2$ |

Hotchkiss (1923) made a study of the effect of a series of inorganic compounds, in which different cations were combined with the same anion (chloride), on the growth of *E. coli*. The salts could be divided into two

groups on the basis of their toxicity. The salts in one group showed no growth of *E. coli* in concentrations of 2 to 0.05*M*; those in the other group prevented growth in dilutions of 0.01 to 0.00,001*M*. The salts in the first column are of common occurrence in the protoplasmic environment and are considered nontoxic. The salts are grouped as shown on page 205.

Results of studies on the chlorides of Na, K, $NH_4$, and Li showed that maximum growth occurred at a salt concentration of 0.25*M* after an incubation period of 3 days. Salt concentrations above or below 0.25*M* showed

TABLE 13.—COUNTS IN MILLIONS PER CUBIC CENTIMETER AFTER 3 DAYS OF INCUBATION AT 37°C.

| Concentration | NaCl | KCl | $NH_4Cl$ | LiCl |
|---|---|---|---|---|
| 4.0*M* | 0 | | | |
| 3.0*M* | 0 | 0 | | |
| 2.0*M* | 0 | 0 | 0 | 0 |
| 1.0*M* | 140 | 180 | 0 | 0 |
| 0.75*M* | 360 | 250 | 400 | 0 |
| 0.5*M* | 700 | 480 | 740 | 150 |
| 0.25*M* | 1700 | 1200 | 2700 | 500 |
| 0.125*M* | 1000 | 950 | 1100 | 600 |
| 0.05*M* | 1000 | 950 | 980 | 600 |
| 0.025*M* | 950 | 950 | 820 | 600 |
| 0.0125*M* | 820 | 950 | 700 | 450 |
| 0.005*M* | 850 | 950 | | |
| Control | 900 | 800 | 500 | 500 |

a decreased growth of the organisms. At the end of 10 days, the peak was obscured because the organisms in concentrations below 0.25*M* slowly increased in numbers until their turbidities were equal to those produced in the 0.25*M* concentration. The results are shown in Table 13.

The bivalent salts in Group 1 showed a greater toxicity than the monovalent salts. The optimum growth concentration ranged from about 0.05*M* to 0.025*M*. Manganese was the only notable exception. The results are included in Table 14.

Studies on the salts in Group 2 showed that they exhibited a greater degree of toxicity toward *E. coli*. In more dilute solutions, some of the salts, including $HgCl_2$, produced a definite stimulating action. The results are embodied in Table 15.

As has already been stated, salts that are stimulating in high dilutions become toxic in greater concentrations. However, the toxic effects have been studied to a greater extent. Koch (1881) was probably the first to

TABLE 14.—COUNTS IN MILLIONS PER CUBIC CENTIMETER AFTER 3 DAYS OF INCUBATION AT 37°C.

| Concentration | $SrCl_2$ | $CaCl_2$ | $MgCl_2$ | $BaCl_2$ | $MnCl_2$ |
|---|---|---|---|---|---|
| 1.0*M* | 0 | 0 | 0 | 0 | 0 |
| 0.75*M* | .... | 0 | 0 | ... | 0 |
| 0.5*M* | 400 | 0 | 0 | ... | 0 |
| 0.25*M* | 1450 | 1460 | 1130 | 0 | 0 |
| 0.1*M* | 1200 | 1530 | 2030 | 200 | 0 |
| 0.05*M* | 1570 | 2240 | 2200 | 800 | 0 |
| 0.025*M* | 1950 | 1500 | 1860 | 550 | 400 |
| 0.0125*M* | 700 | 1330 | 1700 | 400 | 400 |
| 0.005*M* | .... | 1700 | | | |
| Control | 1250 | 1100 | 1160 | 550 | 700 |

TABLE 15.—COUNTS IN MILLIONS PER CUBIC CENTIMETER AFTER 3 DAYS OF INCUBATION AT 37°C.

| Concentration | $AlCl_3$ | $CdCl_2$ | $CeCl_3$ | $CoCl_2$ | $CuCl_2$ | $FeCl_2$ | $FeCl_3$ |
|---|---|---|---|---|---|---|---|
| 0.005*M* | ... | ... | 0 | ... | 0 | 0 | |
| 0.001*M* | ... | ... | 0 | 0 | 200 | 0 | 0 |
| 0.0005*M* | 0 | ... | 0 | 200 | 700 | 200 | 550 |
| 0.0001*M* | 550 | 0 | 350 | 550 | ... | ... | 550 |
| 0.00005*M* | ... | 450 | 700 | 550 | ... | ... | 550 |
| 0.00001*M* | ... | 450 | 900 | | | | |
| 0.000005*M* | ... | 450 | 700 | | | | |
| Control | 700 | 700 | 700 | 700 | 700 | 200 | 700 |

| | $HgCl_2$ | $NiCl_2$ | $PbCl_2$ | $SnCl_4$ | $TiCl_3$ | $TiCl$ | $ZnCl_2$ |
|---|---|---|---|---|---|---|---|
| 0.01*M* | ... | ... | ... | 0 | 0 | ... | 0 |
| 0.005*M* | ... | 0 | 0 | 0 | 60 | 0 | |
| 0.001*M* | ... | 200 | 0 | 350 | 700 | 110 | 0 |
| 0.0005*M* | 0 | 450 | 0 | 500 | 1200 | 260 | 310 |
| 0.0001*M* | 0 | 700 | 300 | 600 | 700 | 330 | 400 |
| 0.00005*M* | 0 | 700 | 400 | 950 | ... | 330 | 700 |
| 0.00001*M* | 0 | 700 | ... | 950 | ... | 430 | 700 |
| 0.000005*M* | 430 | 700 | ... | 950 | | | |
| 0.000001*M* | 960 | | | | | | |
| Control | 820 | 400 | 300 | 660 | 550 | 500 | 540 |

show that the salts of heavy metals, particularly mercury and silver, were toxic to bacteria in low concentrations. Krönig and Paul (1897) found that the toxicity of solutions of $HgCl_2$ was due to the concentration of free Hg ions in solution. The greater the concentration of Hg ions, the more efficient the germicidal action. Later Paul and Prall (1907) arrived at the same conclusion. Mercury salts of the organic acids, *e.g.*, mercury acetate, which show a low degree of dissociation, exhibit a much weaker germicidal action.

The toxic action of salts of mineral acids may be due either to the cation, to the anion. to the molecule taken as a whole, or to all three.

TABLE 16.—CATIONS ARRANGED IN ORDER OF INCREASING TOXICITY
(Read down in columns from left to right)

| Eisenberg (1919), bacteria | | | Winslow and Hotchkiss (1922), *E. coli* | |
|---|---|---|---|---|
| Na | Yt | Zr | K | $Ti^{+}$ |
| K | Cr | Ni | Na | Zn |
| $NH_4$ | U | Cd | $NH_4$ | Cu |
| Li | Zn | Co | Li | $Fe^{++}$ |
| Mg | $Fe^{+++}$ | Au | Sr | $Fe^{+++}$ |
| Sr | Ti | Pt | Mg | Co |
| Ca | Be | Hg | Ba | Pb |
| Ba | Al | Ag | Ca | Al |
| Mn | Nd | | Mn | Ce |
| Ce | Pb | | $Ti^{+++}$ | Cd |
| Th | Cu | | Sn | Hg |
| $Fe^{++}$ | Tl | | Ni | |

In order to determine to which component the action is due, tests have been made in which one cation was combined with different anions and different cations combined with the same anion. Eisenberg (1919) and Winslow and Hotchkiss (1922) tested a number of cations on various bacteria and reported the results given in Table 16. It may be seen that, in general, the toxicity of cations increased with valence.

Holm and Sherman (1921) determined the growth rates of *E. coli* in a peptone solution to which were added various Na salts. Their results are recorded in Table 17. They concluded that the chloride ion showed the least toxicity and the fluoride ion the most. Cl, I, $NO_3$, $SO_4$, $PO_4$, and lactate ions accelerated growth of *E. coli* whereas the other ions showed an inhibitory effect.

Different species of bacteria vary considerably in their susceptibility to the same ion. Eisenberg (1919) showed that *B. anthracis* was quite resistant to the action of the fluoride, iodide, and oxalate ions; *Corynebacterium diphtheriae* to tellurates, tellurites, nickel, and copper; *Sal-*

*monella typhosa* to strontium salts; the pneumococcus to ferricyanides, tellurites; etc. In other words, organisms may be classified on the basis of their susceptibility to the various ions.

Another point to consider in making a study of the action of ions on bacteria is the composition of the culture medium. Salts exhibit a greater germicidal action in distilled water than the same concentration in a protein-containing medium. This is due to a chemical reaction between the salts and the proteins, resulting in a decreased concentration of ions in the medium.

TABLE 17.—GROWTH RATES OF *Escherichia coli* IN A PEPTONE MEDIUM COMBINED WITH VARIOUS ANIONS

| Medium | pH | First appearance of turbidity, hr. |
|---|---|---|
| 1% peptone | 7.2 | 4½ |
| 1% peptone + 0.20*M* NaCl | 7.3 | 3¼ |
| 1% peptone + 0.20*M* NaI | 7.3 | 3½ |
| 1% peptone + 0.20*M* $NaNO_3$ | 7.3 | 3½ |
| 1% peptone + 0.20*M* $Na_2SO_4$ | 7.0 | 4 |
| 1% peptone + 0.20*M* $NaHPO_4$* | 7.3 | 4¼ |
| 1% peptone + 0.20*M* Na lactate | 7.0 | 4½ |
| 1% peptone + 0.20*M* Na oxalate | 7.0 | 9½ |
| 1% peptone + 0.20*M* Na acetate | 7.0 | 10½ |
| 1% peptone + 0.20*M* Na citrate | 7.3 | 10½ |
| 1% peptone + 0.20*M* Na fluoride | 7.4 | 48 |

* Mono- and disodium phosphates were mixed to give a pH of about 7.0.

In general, Gram-positive organisms are more sensitive to various ions than Gram-negative bacteria. The same holds true for the action of various dyes on Gram-positive and Gram-negative bacteria (page 216).

**Mechanisms of Salt Actions.**—The following conclusions may be drawn concerning the action of salts on living organisms:

Most salts in high dilutions produce a stimulating action on bacteria; in more concentrated solutions, a toxic or germicidal effect occurs.

When the toxic range is reached, the germicidal effect is proportional to the concentration of the salt.

The toxic effect of monovalent salts can be neutralized by the addition of a divalent salt. Likewise, the toxic effect of a divalent salt can, in most cases, be neutralized by the addition of a monovalent salt.

In general, divalent cations are more toxic than monovalent cations. Also, the heavier cations are usually more germicidal than the lighter cations. For example, $HgCl_2$ is more toxic than $MgCl_2$.

Salts are more germicidal in distilled water than in solutions containing protein. This is probably true for all germicidal agents. The organic matter greatly inactivates the salts, probably by combining with them and reducing the ionic concentration.

Different bacterial species vary considerably in their susceptibilities to the same salt. In general, closely allied organisms behave in a similar manner.

The Gram-positive organisms are usually more sensitive to the toxic effect of salts than are the Gram-negative bacteria.

The organic acids show only slight dissociation, compared to the inorganic acids, yet some of them exhibit a strong germicidal action. The toxic effect is attributed to the action of the undissociated molecules and not to their ions. Probably the same holds true for the toxicity of the salts of organic acids.

*Reducing Agents.*—Some compounds produce a germicidal action by virtue of their powers of reduction. Sulfurous acid, sulfites, ferrous compounds, and formaldehyde act in this manner. Formaldehyde is a very efficient germicide, being effective against both vegetative cells and spores. A 5 per cent solution of formaldehyde (commercial formalin is a 40 per cent solution of formaldehyde gas in water) destroys anthrax spores in from 1 to 2 hr.

*Oxidizing Agents.*—Compounds that give up oxygen freely or are capable of releasing oxygen from other compounds have been used as germicidal agents. These agents produce their toxic effect by the process of oxidation. Krönig and Paul (1897) arranged the following oxidizing compounds in order of decreasing oxidizing ability: Nitric acid ($HNO_3$), dichromic acid ($H_2Cr_2O_7$), chloric acid ($HClO_3$), chlorine ($Cl_2$), persulfuric acid ($H_2S_2O_8$), and permanganic acid ($HMnO_4$). The halogens chlorine, bromine, and iodine, were found to be germicidal in the following order of decreasing toxicity: $Cl_2$, $Br_2$, and $I_2$.

Potassium permanganate is an oxidizing agent and was at one time employed to a considerable extent for the destruction of bacteria. Its action is increased in acid solution. Krönig and Paul found that a solution containing 1 per cent $KMnO_4$ and 1.1 per cent HCl in water destroyed anthrax spores in 30 sec. The salt reacts promptly with organic matter, being changed to inert $MnO_2$. For this reason, the use of $KMnO_4$ as a germicide has been largely discontinued.

Hydrogen peroxide is another active oxidizing agent, being easily decomposed into water and oxygen. The commercial solution of $H_2O_2$ (3 per cent) is said to be capable of destroying anthrax spores in 1 hr.

Chlorine-containing compounds such as hypochlorous acid (HOCl) and hypochlorites, bleaching powder CaCl(OCl), chloramine ($CH_3 \cdot C_6H_4 \cdot SO_2NNaCl \cdot 3H_2O$), and dichloramine ($CH_3 \cdot C_6H_4 \cdot SO_2NCl_2$) are effective germicides by virtue of their ability to produce vigorous oxidations.

Bleaching powder is probably the most important oxygen compound of the halogens. When this compound is dissolved in water it is said to break down as follows:

$$2CaCl(OCl) + 2CO_2 + 2H_2O \rightleftharpoons CaCl_2 + Ca(HCO_3)_2 + 2HClO$$

The HClO then breaks down to hydrogen chloride and oxygen:

$$2HClO \rightleftharpoons 2HCl + O_2$$

If chlorine gas is employed the reactions are

$$Cl_2 + H_2O \rightleftharpoons HCl + HClO$$
$$2HClO \rightleftharpoons 2HCl + O_2$$

Compounds containing active chlorine attached to a nitrogen atom of the general formula $R_2{=}N{-}Cl$ and $R{-}N{=}Cl_2$ are also strongly germicidal, the activity being directly proportional to the extent to which reactions of hydrolysis proceed in solution:

$$R_2{=}N - Cl + H_2O \rightarrow R_2{=}N{-}H + HClO$$

$$R{-}N{=}Cl_2 + H_2O \rightarrow R{-}N\begin{matrix}H\\Cl\end{matrix} + HClO$$

The active agent is HClO:

$$2HClO \rightleftharpoons 2HCl + O_2$$

Beckwith and Moser (1933) found chlorine, bromine, and iodine to be about equal in effectiveness in destroying *E. coli.* Tanner and Pitner (1939) reported that bromine was an effective agent against nonspore-forming organisms, particularly against *E. coli* and *Salmonella typhosa.* Similar results were reported by Hyatt and Piszczek (1943).

Bromine added to water reacts in the following manner:

$$2Br_2 + 2H_2O \rightleftharpoons 4HBr + O_2$$

or

$$Br_2 + H_2O \rightleftharpoons HBr + HBrO$$
$$2HBrO \rightleftharpoons 2HBr + O_2$$

In comparison with chlorine and bromine, iodine is not appreciably hydrolyzed in acid solution, hence, it acts as molecular $I_2$ or in the form of $I_3^-$ ion. These seem to be equally effective. The mechanism of action of iodine is apparently different from that of HClO and HBrO (Fair, Burden, Conant, and Morris, 1944).

**Phenols and Cresols.**—The phenols and cresols are very efficient germicides in fairly concentrated solutions. Phenol is soluble in water but most of the other members of the group are only slightly soluble. However, they may be held in suspension by mixing with soap, by which procedure colloidal solutions are obtained.

The emulsification of disinfectants only slightly soluble in water results in the formation of more potent germicidal preparations. In the emulsified

state, the particles of germicide are adsorbed onto the surface of the emulsifying agent (soap), resulting in an increased concentration in the vicinity of the bacteria. The emulsified disinfectants are more active when freshly prepared. After a few days, the activity decreases, probably owing to a change in their colloidal state. An important commercial disinfectant of this type is compound solution of cresol, known under the trade name of Lysol.

It is usually stated that phenols and cresols act on proteins with the formation of insoluble proteinates. This results in a precipitation of the proteins of the protoplasm. Kojima (1931) opposed the theory of direct

TABLE 18.—PHENOL COEFFICIENTS OF PRIMARY ALCOHOLS TO *Salmonella typhosa* AND *Micrococcus pyogenes* VAR. *aureus*

| Alcohol | Phenol coefficient | |
|---|---|---|
| | *Salmonella typhosa* | *Micrococcus pyogenes* var. *aureus* |
| Methyl | 0.026 | 0.030 |
| Ethyl | 0.040 | 0.039 |
| Propyl | 0.102 | 0.082 |
| Butyl | 0.273 | 0.22 |
| Amyl | 0.78 | 0.63 |
| Hexyl | 2.30 | |
| Heptyl | 6.80 | |
| Octyl | 21.00 | |

coagulation of the bacterial proteins. He found that the strength of phenol that was required to destroy bacteria failed to coagulate egg albumin. Reichel (1909) believed that the action was more physical than chemical, the phenol being capable of dissolving in coagulated proteins, in lipoids, fats, and the cytoplasm of bacteria. The germicidal action was due to its ability to penetrate the cell in the form of a colloidal solution.

**Alcohols and Ethers.**—Absolute alcohol is either not germicidal or only slightly so. On the addition of water, however, the compound shows a marked germicidal effect. Its maximum germicidal efficiency is exhibited in a concentration of 70 per cent by weight (77 per cent by volume).

Tilley and Schaffer (1926) reported that the toxicity of the primary alcohols to *Salmonella typhosa* and *Micrococcus pyogenes* var. *aureus* increased with an increase in molecular weight (Table 18). Since the alcohols decrease in solubility as their molecular weights increase, this explains why the higher members of the series are not generally employed as germicidal agents.

Koch (1881) and Krönig and Paul (1897) showed that the addition of absolute alcohol to $HgCl_2$ greatly reduced the germicidal potency of the latter. When 50 per cent alcohol was used instead of absolute, the toxic

action of $HgCl_2$ was found to be even stronger than a solution of the salt in water. This was also true of $AgNO_3$. Since these salts are toxic in proportion to the concentration of mercury and silver ions, water is necessary for ionization to occur. Other compounds, such as phenol and formaldehyde, are less germicidal in the presence of even small amounts of alcohol.

Topley (1915) found that ether ($C_2H_5 \cdot O \cdot C_2H_5$) was toxic to bacteria. Cultures of nonsporulating organisms exposed to an atmosphere of ether failed to grow after an exposure period of from 1 to 48 hr. *Escherichia coli* immersed in 50 per cent ether was destroyed in 3 min. at room temperature. The spores of *Clostridium oedematis maligni*, however, were not destroyed after being exposed to ether for 24 hr.

**Soaps.**—It has been known since the beginning of bacteriology that both soft and hard soaps are excellent germicidal agents. Soft soaps are prepared by boiling oils and fats with potassium hydroxide, whereas hard soaps are prepared with sodium hydroxide. The soft soaps are used in preparing liquid soaps and shampoos; the hard soaps are used in preparing soap powders, chips, and bars.

Soap has a number of important physical characteristics. When dissolved in water, it lowers the surface tension, forms colloidal solutions and gels, causes water to wet surfaces more rapidly, gives the solution a soapy or slippery feeling, and has the ability to emulsify and disperse oils and dirt in the solution and thus is able to cleanse.

Various chemicals, such as phenols, cresols, mercuric iodide, mercuric chloride, metaphen, and chloramine have been incorporated in soaps to enhance their germicidal value. It has been shown that these so-called "germicidal" soaps, when tested by the newer approved methods, are no more useful than the ordinary soaps for destroying bacteria. In fact, some materials may lose their germicidal effectiveness in the presence of soap and may even decrease the natural antiseptic properties of the soap. For example, soaps containing cresol and phenol are less antiseptic than either the cresol, or the phenol, or the soap alone when used in the same concentrations.

McCulloch (1940) tested a large number of commercial soaps for their action on several strains of *Streptococcus agalactiae* (Table 19) and concluded as follows:

> Solutions of commercial soaps and soap powders, at 40°C., and in the presence of 5 per cent skim milk and 5 per cent broth culture of the organisms, were found to be between two and three times as effective in killing mastitis streptococci in 1 min. as was phenol and were equally as effective as 100 parts per million of the most actively germicidal of several hypochlorites tested.
>
> A soap containing cresols was no more germicidal than were the nonmedicated soaps, and the soaps containing mercury compounds were only slightly more effective.

High-test household lye, which probably kills by the hydroxyl-ion concentration produced, was found to be effective in dilutions of 1:300 and 1:500, which gave a final alkalinity of pH11.22 to 11.18 but was much slower than the soaps in exerting germicidal action.

Soap solutions in the concentrations usually obtained in lathering the hands with soap in warm water are effective disinfectants against mastitis streptococci.

TABLE 19.—EFFECT OF SOAPS ON *Streptococcus agalactiae* IN THE PRESENCE OF 5 PER CENT SKIM MILK AT 40°C.

| Soap | Highest dilution killing in 1 min. | pH of solution |
|---|---|---|
| Lifebuoy | 1:220 | 9.01 |
| Mercuric potassium iodide soap (1 per cent mercury) | 1:270 | 8.78 |
| Neko germicidal soap (2 per cent mercuric iodide in combination) | 1:250 | 8.81 |
| Nu Bora powder | 1:240 | 8.76 |
| Borene | 1:200 | 8.75 |
| Drene (regular) | 1:280 | 6.78 |
| Liquid soap (coconut oil base) | 1:50 | 8.91 |
| Woodbury's soap | 1:250 | 9.01 |
| Phenol (carbolic acid) | 1:100 | |
| Soft soap, Lilly (cottonseed-oil base) | 1:120 | 8.81 |
| Tincture of green soap, U.S.P. | 1:65 | 9.13 |
| Fels Naptha chips | 1:220 | 8.94 |
| Lux flakes | 1:340 | 8.71 |
| Oxydol | 1:250 | 9.13 |
| Peets granulated soap | 1:250 | 9.24 |
| Rinso | 1:250 | 9.31 |
| White King Powder | 1:240 | 9.05 |
| Ivory | 1:250 | 8.97 |

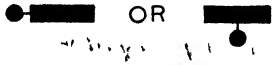
**Surface Active or Wetting Agents.**—Wetting agents are compounds that possess soap properties but to a greatly intensified degree.

Like soaps, they consist of a hydrophobic (water-repelling) group and a hydrophilic (water-attracting) group. Wetting agents can be divided into two classes: (1) cationic, or positively charged compounds and (2) anionic, or negatively charged compounds. The cationic compounds are generally substituted ammonium salts; the anionic agents are generally substituted sulfates or sulfonates. Soaps, like the anionic agents, are also negatively charged, but they may be distinguished by the nature of the polar salt-forming groups:

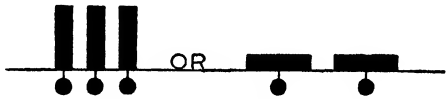
| Soaps | Anionic Agents |
|---|---|
| R—COONa | R—$SO_3$Na |
| Carboxyl group | Sulfonic group |

fates and sulfonates are derivatives of much stronger acids than the soaps. This explains why wetting agents are neutral and remain active in acid solutions where soaps cannot be used. Wetting agents are not precipitated by hard water (calcium and magnesium).

Wetting agents have a tendency to localize in the surface layer or interface of liquids. A wetting agent molecule may be diagrammatically represented by a bar for the hydrophobic (fat-soluble) group and a disk for the polar (water-soluble) group, depending upon whether the polar group is at the end or somewhere along the carbon chain:




Then the surface film can be represented as follows:

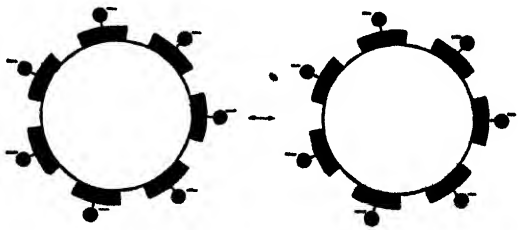



The surface of a solution containing a wetting agent is actually altered, being covered with a hydrocarbon film having the thickness of one molecular layer. When an aqueous solution containing a wetting agent is in contact with a lipoidal surface, the hydrophobic group of the wetter is absorbed and the polar group protrudes:

Such a surface is now capable of being wetted by water. For this reason wetting agents lower the surface tension of water. Such solutions can penetrate into openings and cracks, very small spaces, or even into the center of clumps of bacteria. The same solutions without wetters would simply bridge over openings without showing any appreciable penetration.

Wetting agents are of great importance as additions to germicidal solutions intended for clinical application. They make it possible for such solutions to penetrate into infected tissues, pus, necrotic debris, bacteria, etc.

A cell surface covered with adsorbed molecules of a wetting agent may be represented as follows:



The number of negative charges on the cell surface is much higher in the presence of a wetting agent than in its absence. This results in immediate dispersion and exposure of living organisms that previously were protected from the action of the bactericidal agent.

For additional information on soaps and wetting agents, see Fair, Chang, Taylor, and Wineman (1945), Fishbein, *et al.* (1945), Hoogerheide (1945), Miller, Abrams, Huber, and Klein (1943), Morton (1944), Ordal and Deromedi (1943), Ordal, Wilson, and Borg (1941), Rahn (1945*a*), and Valko and DuBois (1944, 1945).

## DYES

It has long been known that certain coal-tar dyes, notably those of the triphenylmethane group, possess the power of affecting the viability of bacterial organisms. This action was first described as bactericidal because it was believed that, in the absence of growth, the organisms were killed. It was shown later that the organisms were not always killed but merely prevented from multiplying. Churchman (1912, 1928) applied the term "bacteriostasis" to describe this condition. This term appears to be quite appropriate in describing the toxic action of these compounds for bacteria.

In most cases, selective bacteriostatic action parallels the Gram reaction. This means that those organisms which retain the Gram stain (Gram+) are more susceptible to the action of the above dyes than are the Gram-negative bacteria. Conversely, those organisms which do not retain the Gram stain (Gram−) are more resistant to the action of the above dyes than are the Gram-positive bacteria. Notable exceptions to the parallelism between bacteriostatic action and Gram reaction are the acid-fast organisms *Mycobacterium tuberculosis*, *M. paratuberculosis*, *M. avium*, etc. These organisms are Gram-positive but comparatively resistant to the action of the triphenylmethane dyes.

An increase in basicity of the solution of a basic dye results in an increase in its germicidal power. A decrease in basicity results in a decrease in its germicidal power. Likewise, an increase in acidity of the solution of an acid dye results in an increase in its germicidal power. A decrease in acidity results in a decrease in its germicidal power (Tables 20 and 21 by Stearn and Stearn, 1926).

The effect of crystal violet on *Escherichia coli* and *Bacillus subtilis* is shown in Fig. 124.

Ingraham (1933) believed that the bacteriostatic effect of crystal violet was due to its property of poising the oxidation-reduction potential in a range too high for cell multiplication to occur. Hoffmann and Rahn (1944) agreed with the findings of Ingraham and extended on the work. They found that, above a certain concentration, the dye acted like any

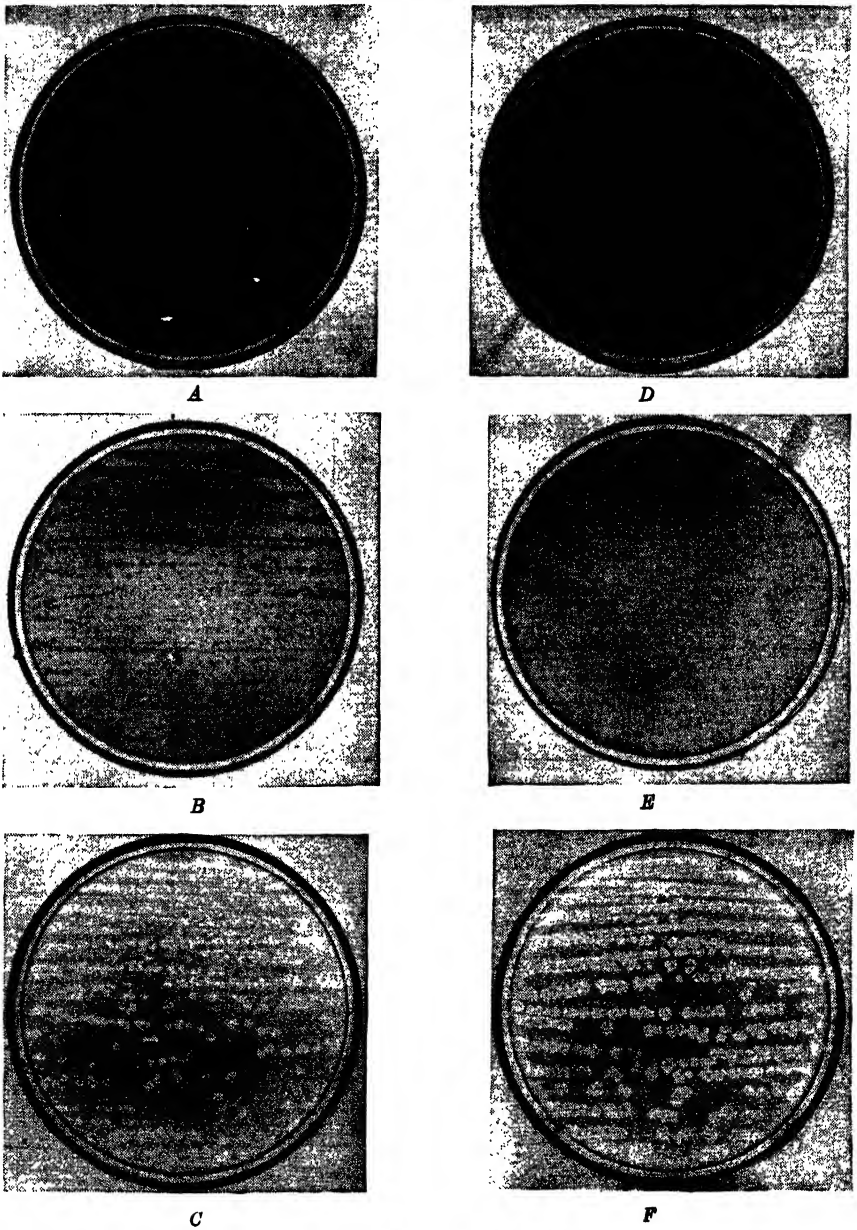



FIG. 124.—Effect of crystal violet. *A*, *B*, *C*, *Escherichia coli* streaked over the surface of nutrient agar containing respectively 1:5000, 1:100,000, and 1:2,000,000 concentration of the dye. *D*, *E*, *F*, same streaked with *Bacillus subtilis*. A 1:5000 concentration of crystal violet inhibited both organisms; a 1:100,000 concentration inhibited *B. subtilis* (Gram +) but not *E. coli* (Gram −). A 1:2,000,000 concentration was not sufficient to prevent growth of *B. subtilis*.

other germicide. The cells died in logarithmic order and in proportion to the dye concentration. The dye was more toxic to young than to old cells, and its toxicity increased only slightly with an increase in pH. This strict germicidal action was probably due to the combination of dye with some indispensable cell constituents. At lower concentrations, the dye did not give a logarithmic survivor curve and was not influenced by cell

TABLE 20.—EFFECT OF pH ON THE RESISTANCE OF *Aerobacter aerogenes* TO GENTIAN VIOLET, A BASIC DYE

| Dilution of gentian violet | pH of dye solution | | | |
|---|---|---|---|---|
| | 5 2 | 6 23 | 7 1 | 7.7 |
| 1:5,000 | − | − | − | − |
| 1:10,000 | + | − | − | − |
| 1:30,000 | + | + | − | − |
| 1:50,000 | + | + | + | − |
| 1:100,000 | + | + | + | + |

TABLE 21.—EFFECT OF pH ON THE RESISTANCE OF *Escherichia coli* TO ACID FUCHSIN

| Dilution of acid fuchsin | pH of dye solution | | | |
|---|---|---|---|---|
| | 5 28 | 6 23 | 7.16 | 7.73 |
| 1:25 | − | − | − | + |
| 1:50 | − | − | + | + |
| 1:75 | − | − | + | + |
| 1:100 | − | + | + | + |

age or pH or the dye concentration. Perhaps this unusual effect was due to the unfavorable oxidation-reduction potential poised by the dye. In this range, the cells usually overcame the dye action and multiplied. The dye produced an abnormally long lag period which increased with the dye concentration. The length of the lag phase was inversely proportional to the logarithm of the number of inoculated cells.

For additional reading on dyes, see Albert (1942), Fischer, Hoffmann, Prado, and Boné (1944), and Tilley (1939).

**Photodynamic Sensitization.**—Raab (1900) found that certain fluorescein dyes, such as eosin and acridin, sensitized paramecia with the result that no action occurred in the dark but, when the suspensions were exposed to diffuse sunlight, destruction of the organisms occurred. Tappeiner (1900) repeated the experiment using monochromatic light and

found that only the green rays were effective. This is of interest because (1) the green rays are probably the least germicidal and (2) eosin fluoresces most strongly in green light. When acridin was used, destruction of the organisms was most rapid by exposure to violet light. It was shown that acridin fluoresced most strongly in the violet region.

Schmidt and Norman (1920) stated that photodynamic action of dyes was not due simply to their ability to fluoresce. They mixed red blood cells with a solution of eosin and reported that no change occurred in the dark but, in the presence of light, hemolysis took place, even though the rays that caused eosin to fluoresce were filtered out. In the presence of a protective substance, such as tyrosine, and a strongly fluorescent solution hemolysis did not occur.

During the past few years, a considerable literature has developed on the photodynamic action of dyes on bacteria, viruses, and protozoa. Only a few of the investigations are reviewed here. T'ung (1935) mixed bacteria with different concentrations of methylene blue in Petri dishes and exposed them to a 100-candle-power lamp at a distance of 10 cm. for 30 min. The organisms were then plated and incubated. The results indicated that the Gram-positive organisms were, in general, more easily destroyed than the Gram-negative bacteria. Employing the same procedure T'ung and Zia (1937) showed that other dyes, including eosin, mercurochrome, and trypaflavine gave similar results. He concluded that eosin exhibited a strong photodynamic action, being 10,000 times more effective in the presence of light than in its absence. In the case of methylene blue, this difference was only a hundredfold. The bactericidal action of mercurochrome was increased by exposure to light. The dyes were ineffective against Gram-negative organisms as the maximal difference between the bactericidal action of the dyes in the presence and absence of light was at most only tenfold. In a later communication, T'ung (1938*a*) reported that, whereas several dyes were shown to be effective against Gram-positive but not Gram-negative bacteria, safranine exhibited an opposite effect, namely, a greater degree of toxicity to Gram-negative than to Gram-positive organisms.

In an attempt to determine the effective photodynamic rays, Ch'in (1938) suspended *Neisseria gonorrhoeae* in solutions of various dyes and exposed them to both unfiltered and filtered light (Table 22). Unfiltered light was found to be more effective than filtered light. Also, each dye showed a special absorption band in the spectrum where it exhibited a greater action than at any of the other bands.

Perdrau and Todd (1933*a*, *b*) reported the photodynamic action of methylene blue on bacteriophage and a number of viruses including herpes, smallpox, and canine distemper. Rosenblum, Hoskwith, and Kramer (1937) found that the virus of poliomyelitis was destroyed by methylene

blue in a concentration of from 1:50,000 to 1:100,000 when exposed to a 100-watt bulb at a distance of 20 cm. for 60 min.

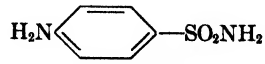
T'ung (1938*b*), working with *Trypanosoma brucei*, found that the flagellates were completely immobilized when a suspension of the organisms was mixed with methylene blue and exposed to a 100-watt lamp at a distance of 10 cm. for 30 min The immobilizing effect of the dye paralleled its lethal action Similar results were reported by Hawking (1938) working with the dye acriflavine.

TABLE 22.—PHOTODYNAMIC ACTION OF SEVERAL DYES ON *Neisseria gonorrhoeae* EXPOSED FOR 60 MIN IN THE PRESENCE OF FILTERED AND UNFILTERED LIGHT

| Dye | Dilution | Filtered light | | | | | | | Unfiltered light | Control |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Red | Orange | Yellow | Green | Light blue | Blue | Violet | | |
| Methylene blue | 1 10⁵ | − | − | − | − | − | − | − | − | ++ |
| | 1 10⁶ | − | − | − | ++ | ++ | ++ | − | − | ++++ |
| | 1 10⁷ | ++ | − | ++ | ++++ | ++++ | ++++ | ++++ | − | ++++ |
| | 1 10⁸ | ++++ | +++ | +++ | ++++ | ++++ | ++++ | ++++ | + | ++++ |
| Eosin | 1 10⁵ | − | − | − | − | − | − | − | − | +++ |
| | 1 10⁶ | − | + | − | − | − | + | +++ | − | +++ |
| | 1 10⁷ | +++ | ++++ | ++++ | − | − | ++ | +++ | − | ++++ |
| | 1 10⁸ | ++++ | ++++ | ++++ | ++ | ++++ | ++++ | ++++ | + | ++++ |
| Trypaflavine | 1 10⁵ | − | − | − | − | − | − | − | − | + |
| | 1 10⁷ | − | − | +++ | − | ++ | ++ | − | − | ++ |
| | 1 10⁸ | ++ | − | ++++ | ++ | ++++ | ++++ | ++++ | − | ++++ |
| | 1 10⁹ | ++++ | +++ | ++++ | ++++ | ++++ | ++++ | ++++ | − | ++++ |
| Mercurochrome | 1 10⁶ | − | − | − | − | − | − | − | − | +++ |
| | 1 10⁷ | +++ | − | − | ++ | ++ | ++ | − | − | ++++ |
| | 1 10⁸ | ++++ | ++++ | ++++ | ++ | ++ | ++ | ++++ | + | ++++ |
| | 1 10⁹ | ++++ | ++++ | ++++ | +++ | +++ | +++ | ++++ | ++ | ++++ |

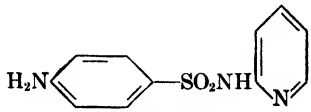
## SULFONAMIDES

The sulfonamides is a name given to a group of drugs that exhibit bacteriostatic activity in vitro and a bactericidal effect in vivo. The first important member of this group to be widely used clinically is para-amino benzene sulfonamide (the amide of sulfanilic acid) commonly known as sulfanilamide:



Sulfanilamide

A large number of derivatives of sulfanilamide have been prepared by substituting the hydrogen atoms of the amino radicals for other groups or radicals. For example, sulfapyridine is prepared by substituting the pyridine ring for a hydrogen atom of an amino group:



Sulfapyridine

Other commonly used sulfonamide compounds include sulfathiazole, sulfaguanidine, sulfadiazine, sulfamerazine, and sulfasuxidine. Hundreds of others have been synthesized in an attempt to find better ones or to extend on those now in use.

**Clinical Uses.**—The drugs are useful in a number of diseases and infections, some of which at one time produced a very high mortality rate. Some of these are pneumonia, meningitis, gonorrhoea, infections due to micrococci and hemolytic streptococci, gas gangrene, wounds, and urinary tract infections. The derivatives vary in their usefulness to a certain disease. Some may prove to be of great value; others may be useless. Therefore, it is necessary to select the proper derivative for the disease or infection to be treated. As an example, sulfanilamide proved to be of tremendous value to the troops in the Second World War as a dusting powder to wounds to prevent infection. Each soldier carried a supply at all times as an emergency measure.

**Mode of Action.**—From the many theories advanced to explain the mode of action of the sulfonamides, the one advanced by Fildes (1940) appears to be the most logical. Woods (1940) showed that *p*-aminobenzoic acid (PABA) in high dilutions antagonized the action of sulfonamides. Fildes showed that PABA is an essential metabolite normally associated with an enzyme. The sulfonamides displace PABA from its enzyme and thereby stop this essential line of metabolism.

Unless a large enough dose of sulfonamides is administered, the organisms are likely to develop a resistance or fastness to the drug, making it necessary to give much larger doses. Landy, Larkum, and Ostwald (1943) and Landy, Larkum, Ostwald, and Streightoff (1943), working with *Micrococcus pyogenes* var. *aureus*, showed that the development of resistance to sulfonamides resulted in an increased synthesis of PABA.

For additional reading, consult Clifton and Loewinger (1943), Ekstrand and Sjögren (1945). Henry (1943), Kohn (1943), Lamanna and Shapiro

(1943), Landy and Dicken (1942), Lockwood (1943), Poth and Knotts (1942), Poth, Knotts, Lee, and Inui (1942), Rantz and Kirby (1944), Roblin and Bell (1943), Sevag and Green (1944), Shannon (1943), Spink, Wright, Vivino, and Skeggs (1944), and Wilson (1945).

## EVALUATION OF GERMICIDES

The method generally employed for the evaluation of germicides is to rate them according to their phenol coefficients. In addition to this, a number of newer methods have been proposed, which are designed to measure the toxicity of germicides for tissue as well as for the test bacteria.

**Phenol-coefficient Method.**—The phenol coefficient test was first proposed by Rideal and Walker (1903) for comparing and rating substances intended for the destruction of bacteria. Since that time, many modifications of the original method have been recommended. At the present time, the standard procedure in this country is that proposed by Ruehle and Brewer (1931) of the Food and Drug Administration, U.S. Department of Agriculture. The test, in one form or another, is universally employed for examining and rating disinfectants.

*Definition.*—The phenol coefficient may be defined as the killing power of a germicide toward a test organism as compared to that of phenol under identical conditions. The conditions that must be specified include (1) time of action of germicide, (2) temperature of the test, (3) presence and amount of organic matter, (4) organism used in the test, (5) age of the culture, (6) composition and reaction of the culture medium, (7) proportion of disinfectant to culture, and (8) temperature and time of incubation of the transfer tubes or flasks. Variations in one or more of the conditions will affect the final result. It is, therefore, of utmost importance to specify the conditions of the test, otherwise the final result will be worthless.

*Time and Temperature.*—In general, germicidal action is increased with time. This means that a higher dilution may be employed with an increase in the period of action. This applies also to temperature. An increase in temperature increases the effectiveness of a germicide, making higher dilutions possible. Germicides are not affected to the same degree by an increase in time and temperature and, for this reason, no general rule can be made.

An important exception to the rule that germicidal action is increased with time is iodine. This germicide is a vigorous oxidizing agent and acts almost immediately when placed in contact with bacteria.

*Organic Matter.*—Probably all germicides are largely reduced in activity in the presence of organic matter, although some are affected more than others. This is especially true in the presence of proteins, amino acids, and compounds of a similar nature. Results of the evaluation of germicides in aqueous solutions are quite different from those obtained when organic

matter is added. The kind and amount of organic matter must be mentioned in reporting the efficiencies of germicidal substances.

*Organism.*—Germicides vary considerably in their action on different bacterial species. Some are more effective against Gram-positive than against Gram-negative organisms, and vice versa. Still others display approximately the same degree of toxicity toward both groups of organisms. The name of the organism used in the test must be mentioned.

*Age of the Culture.*—In general, old organisms are more resistant to adverse environmental conditions than young ones. In practically all procedures for evaluating germicides, 24-hr. cultures are specified. This precaution must be observed in order that constant and comparable results be obtained.

*Composition and Reaction of Medium.*—Variations in composition and pH of culture media also affect the final results. In general, an organism is more resistant to adverse conditions at its optimum pH. A change in the reaction of the medium on either side of the optimum pH increases the susceptibility of the organism to a germicide.

*Proportion of Disinfectant to Culture.*—A parallelism exists between the number of organisms employed in the test and the smallest amount of germicide required to destroy them. If the number of organisms is increased or decreased, the concentration of germicide required to destroy them is likewise increased or decreased.

*Method.*—The Food and Drug Administration (FDA) method of Ruehle and Brewer (1931) is as follows: A series of dilutions of phenol and germicide to be compared are prepared in sterile distilled water contained in test tubes measuring 25 mm. in diameter and 150 mm. in length. Each tube must contain not more than 5 cc. of germicidal dilution. The tubes are placed in a rack in a water bath, previously adjusted to a temperature of 20°C., and allowed to remain for at least 5 min. in order to bring the temperature of the germicidal dilutions to that of the water bath.

The test organism should be transferred daily for five successive days previous to use. A 24-hr. culture must be employed in the test. The culture is shaken vigorously to break up small clumps of bacteria and then placed in the water bath for 15 min. to permit large suspended particles to settle out. One-half cubic centimeter of culture is pipetted into each dilution of the germicide. At intervals of 5, 10, and 15 min. a 4-mm. loopful of material is removed from each tube and transferred to a corresponding tube containing 10 cc. of broth. The subculture tubes are incubated at 37°C. for 48 hr. If the germicide is suspected of being bacteriostatic, the subculture tubes should be incubated for a longer period of time.

If mercurials, silver preparations, dyes, or other compounds exhibiting strong bacteriostatic properties are tested, it is necessary (1) to make

secondary subcultures from the first subculture tubes immediately after the test has been completed, or (2) to make the first transfers to 100-cc. amounts of broth contained in flasks, or (3) to make transfers to broth containing substances that combine with or destroy the germicidal agent. For example, bacteria treated with mercuric chloride contain $Hg^+$ ions adsorbed to their cell walls. In this condition, the bacteria are not necessarily killed but merely prevented from multiplying. The numbers of $Hg^+$ ions may be insufficient to produce death but sufficient to produce a

TABLE 23.—KILLING DILUTIONS OF PHENOL AND GERMICIDE *A* FOR *Salmonella typhosa* AT DIFFERENT TIME INTERVALS

| Germicide | Dilution | Time interval, min. | | |
|---|---|---|---|---|
| | | 5 | 10 | 15 |
| Phenol | 1:70 | − | − | − |
| | 1:80 | − | − | − |
| | 1:90 | + | − | − |
| | 1:100 | + | + | + |
| Germicide *A* | 1:325 | − | − | − |
| | 1:350 | + | − | − |
| | 1:375 | + | + | − |
| | 1:400 | + | + | + |

bacteriostatic effect. Sodium thioglycollate contains a sulfhydryl group that is capable of reacting with $Hg^+$ ions. If mercury-treated organisms are transferred to a broth medium containing sodium thioglycollate, the germicide is removed from the bacteria by the sulfhydryl groups. This destroys the bacteriostatic effect of the mercury and permits growth of the organisms.

The phenol coefficient is calculated by dividing the highest dilution of germicide killing the test organism in 10 min. but not in 5 min. by the corresponding dilution of phenol. For example, in Table 23 the phenol coefficient would be $\frac{350}{90} = 3.89$. This means that germicide *A* is 3.89 times more effective than phenol.

Since *Salmonella typhosa* was used as the test organism, the value is referred to as the *S. typhosa* phenol coefficient.

**Limitations of the Test.**—The phenol-coefficient test was originally designed to be used for comparing the toxicity of phenol with phenol-like com-

pounds. However, the method has been used to test compounds that are totally unlike phenol in composition and mode of action leading to considerable variation in results. As Reddish (1937) stated,

> Germicides have been tested by this method which are not chemically related to phenol and whose chemical and germicidal activity differ so greatly from that of this standard germicide that they should not be compared in this way, for example, chlorine compounds, mercury compounds, formalin, hydrogen peroxide, iodine, picric acid, certain essential oils, etc., and a large number of organic compounds.
>
> Another departure has been the liberties which certain investigators have taken with the test itself, changing it to suit the compounds tested. Many germicides which are soluble in alcohol but not in water, or only slightly so, have been tested by this method in dilutions of alcohol instead of water, although water is specified as the diluting agent in all the standard phenol coefficient tests. Certain other germicides which are soluble in alkali but not in water, or only slightly so, have been tested in dilutions made in alkali solutions instead of water, and the figures obtained have been designated as phenol coefficients. As a result, so-called phenol coefficients have been reported in the chemical literature which do not represent a true comparison of the germicidal activity of such compounds with phenol which is water soluble. The test itself is often blamed when discordant results are reported whereas the unwarranted modifications made by certain investigators are entirely responsible.

Another limitation of the test is that it is of no value in determining the efficiency of a germicide intended for clinical application. A phenol coefficient attempts to compare the toxicity of a germicide for a given organism with that of phenol but gives no information as to its effect on living tissue. It can be seen that a germicide having a high phenol coefficient and a proportionately high toxicity to tissue would have no advantage over one having a low phenol coefficient and a proportionately low toxicity to living tissue.

**Tissue Toxicity Method.**—A number of methods have been proposed for determining the effects of germicides on living tissue cells as well as for their ability to kill bacteria.

Nye (1937), Welch and Hunter (1940), Welch and Brewer (1942), Welch, Slocum, and Hunter (1942), and Hirsch and Novak (1942) tested germicides by using the inhibition of phagocytosis as a criterion of tissue toxicity. Witlin (1942) and Green and Birkeland (1942, 1944) tested the effect of germicides on the infected chorioallantoic membrane of the developing chick embryo.

Salle and Lazarus (1935), Salle, McOmie, and Shechmeister (1937), Salle, McOmie, Shechmeister, and Foord (1938, 1939), Shechmeister and Salle (1938), and Foord, McOmie, and Salle (1938) tested germicides for their effect on the viability of chick heart tissue fragments cultivated in

vitro. Paff, Lehman, and Halperin (1945) employed a modification of the method. A number known as the toxicity index may be calculated which is defined as the ratio of the highest dilution of germicide required to kill the tissue in 10 min. to the highest dilution required to kill the test organism in the same time and under identical conditions. Theoretically, an index less than 1 means that the germicide is more toxic to bacteria than to tissue; an index greater than 1 means that the germicide is more toxic to

TABLE 24.—TOXICITY OF GERMICIDES FOR TISSUE AND BACTERIA

| Germicide | Toxicity index * | |
|---|---|---|
| | *Micrococcus pyogenes* var. *aureus* | *Salmonella typhosa* |
| Iodine | 0.2 | 0.2 |
| Azochloramid | 0.3 | 0.08 |
| Chloramine | 0.45 | 0.45 |
| Silver picrate | 0.71 | 1.1 |
| Hexylresorcinol | 0.9 | 0.8 |
| Amphyl | 1.2 | 1.2 |
| Metaphen | 1.5 | 0.4 |
| Silver nitrate | 1.8 | 0.11 |
| Silver protein strong, U.S.P. | 1.7 | 0.14 |
| Phenol | 2.0 | 1.2 |
| Mercurochrome | 7.2+† | 0.6 |
| Merthiolate | 169+† | 1.6 |

* Ratio of the highest dilution of germicide required to kill tissue in 10 min. to the highest dilution required to kill the test organism in the same period of time under identical conditions.

† Failed to kill. A more concentrated solution could not be prepared.

tissue than to bacteria. The smaller the toxicity index, the more nearly perfect the germicide.

A group of germicides and their corresponding toxicity indexes are given in Table 24. The halogens (iodine and chlorine) exhibited the highest degree of germicidal efficiency of the compounds tested by this technique, combining low tissue toxicity with high germicidal potency against *Micrococcus pyogenes* var. *aureus* (Gram-positive) and *Salmonella typhosa* (Gram-negative).

For further reading, consult Dubos (1945), Hunter (1943), Lovell (1945), Salle, Shechmeister, and McOmie (1940), and the monographs by McCulloch (1945) and Rahn (1945*b*).

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# CHAPTER XI

## NUTRITION OF BACTERIA

Culture media (singular, medium) are solid, semisolid, and liquid nutrient preparations employed for the cultivation of microorganisms. They are artificial environments prepared to simulate natural conditions as closely as possible.

The strict autotrophic bacteria cannot utilize organic matter and may even be harmed by its presence. These organisms are able to synthesize complex compounds composing their protoplasm from simple inorganic salts. They obtain their carbon from carbon dioxide and their energy from the oxidation of certain inorganic compounds or even elements. Because of this fact, they are independent of vegetable or animal life. The strict or obligate heterotrophic organisms cannot synthesize their complex protoplasm from simple inorganic salts but must have organic compounds, such as proteins, peptones, amino acids, and vitamins for growth. The facultative heterotrophic organisms show characteristics intermediate between the two, being able to utilize both inorganic and organic compounds. This latter group comprises the great majority of bacteria that have been studied and classified. At one end of the scale, the organisms exhibit complete independence; at the other end, they show complete parasitism. Fildes (1934) advanced the theory that parasitism involved the loss of enzymes essential for the synthesis of bacterial protoplasm, making it necessary to add certain complex ingredients to the culture medium.

The various ingredients employed for the preparation of the common laboratory media and their uses are as follows:

**Water.**—Water is absolutely necessary for the existence of all living cells. Distilled water is generally preferable to tap water because it is of definite composition. Uniform media cannot always be prepared from tap water. The calcium and magnesium in tap water react with the phosphates present in peptones, beef extract, and other ingredients of culture media to give insoluble calcium and magnesium phosphates. During sterilization, such media throw down considerable precipitate, which usually proves objectionable.

For an interesting discussion on the commercial uses of water, see Lythgoe (1943).

**Peptones.**—Peptones are intermediate products of hydrolysis formed by the action of certain proteolytic enzymes (usually trypsin) on native

proteins. As hydrolysis proceeds, the large colloidal protein molecule is broken up into a number of fragments called, respectively, "proteoses," "peptones," "peptides," and finally "amino acids." The proteoses still exhibit colloidal properties, and it is customary to consider them the last hydrolytic product still possessing true protein characteristics. In other words, the protein nature of the molecule disappears on further hydrolysis.

The commercial peptones are not the same as the peptones of the chemist who uses the term in its narrow, chemical sense. The commercial preparations employed by the bacteriologist are composed of proteoses or albumoses, peptones, peptides, and amino acids. The proportions vary, depending upon the type of peptone. The usual commercial preparations are composed largely of peptones and amino acids, with smaller amounts of proteoses; others contain less peptones but more proteoses; still others contain all the components in more or less well-balanced proportions. Some organisms prefer one type of peptone; others grow better in another type.

Whole proteins, such as casein and egg albumin, are probably not attacked by bacteria, when used as the only source of nitrogen and carbon. They are indiffusible compounds and cannot enter the cell unless their molecules are broken up into smaller units. If a trace of commercial peptone is added to a protein, it will be readily attacked and utilized. The organisms utilize the amino acids of the peptone and, in so doing, elaborate the proteolytic enzymes required to attack the protein molecules. Amino acids readily pass through the cell membranes of bacteria where they are used for structure and energy.

The most important function of peptones in culture media is to furnish an available source of nitrogen. Since amino acids are amphoteric compounds, peptones are also excellent buffers.

For information on chemical and bacteriological studies on various peptones, see Hook and Fabian (1943).

**Meat Extract.**—Meat extract is prepared by boiling lean beef in water, removing the liquid by filtration, and concentrating in vacuo. It is a dark-colored, thick, pasty mass and is prepared in such a manner that almost all food constituents are removed. For this reason, it is not a good food in itself.

The constituents removed from muscle by boiling in water are known as extractives. The total amount of extractives, including both inorganic salts and organic matter, obtained from fresh muscle tissue by boiling in water, amounts to about 2 per cent of the weight of the muscle. Two classes of extractives are obtained from meat: the nitrogenous and the nonnitrogenous. The nitrogenous extractives include creatine, xanthine, hypoxanthine, uric acid, adenylic acid, inosinic acid, carnosine, carnitine, glycocoll, urea, glutamine, $\beta$-alanine, etc. The nonnitrogenous extractives

include glucose, hexosephosphate, lactic acid, succinic acid, fat, inositol, inorganic salts, etc.

The use of beef extract in culture media was introduced by Loeffler (1881) and has been a routine procedure in bacteriology ever since. Meat extract is added to media to supply certain substances that stimulate bacterial activity. It contains enzyme exciters, which cause accelerated growth of microorganisms. McIlwain, Fildes, Gladstone, and Knight (1939) showed that glutamine, a constituent of beef extract, was a necessary nutrient for the growth of *Streptococcus pyogenes*. Williams (1941*a*) believed that β-alanine was present in beef extract in small amounts, since it may arise from the hydrolysis of carnosine or in traces from pantothenic acid. Stokes, Gunness, and Foster (1944) analyzed beef extract for the presence of eight members of the B complex, namely, thiamine, riboflavin, pantothenic acid, nicotinic acid, biotin, pyridoxin, folic acid, and *p*-aminobenzoic acid, and found all of them to be present (see page 242ff. for a discussion of growth factors).

**Gelatin.**—Gelatin is a protein and is prepared by the hydrolysis of collagen with boiling water. Gelatin is not soluble in cold water but swells and softens when immersed in it. It is quite soluble in boiling water. On cooling, it solidifies to form a transparent jelly.

Gelatin is rarely used as a substitute for agar for the preparation of solid media because (1) it is attacked and decomposed by many bacteria and (2) it melts at 37°C. Gelatin is added to media principally to test the ability of organisms to liquefy it. Some organisms can liquefy it; others cannot. It is of importance in the identification and classification of bacteria.

**Agar.**—Koch (1881) recommended the use of gelatin as a solidifying agent to obtain pure cultures of organisms. The disadvantages to its use for this purpose have already been given. Later, Koch overcame these disadvantages quite satisfactorily by the substitution of agar for the gelatin.

Agar is the dried mucilaginous substance extracted from several species of *Gelidium* and closely related species growing chiefly along the coasts of Japan, China, Ceylon, Malaya, and Southern California. Agar is a galactan and belongs to the compounds known as polysaccharides. It dissolves in water at a temperature of about 98°C. and does not solidify until the temperature drops to about 45°C.

Agar is attacked and liquefied by a number of terrestrial and marine forms. Stanier (1941, 1942) described seven well-recognized marine species belonging to the genera *Vibrio*, *Pseudomonas*, and *Cytophaga*, and several strains of an *Actinomyces* capable of liquefying agar (Fig. 125). The enzyme responsible for agar digestion has been shown to be extracellular.

For additional reading on agar-digesting species see Humm (1946), and Humm and Shepard (1946).

For information on the history, collection, manufacture, and uses of agar, consult Tseng (1944*a*, *b*)

**Sodium Chloride.**—Sodium chloride is commonly added to culture media to increase their osmotic pressures, although this is usually not necessary

Red blood cells are hemolyzed when added to water or to media having low osmotic pressures Therefore, a satisfactory base for the preparation

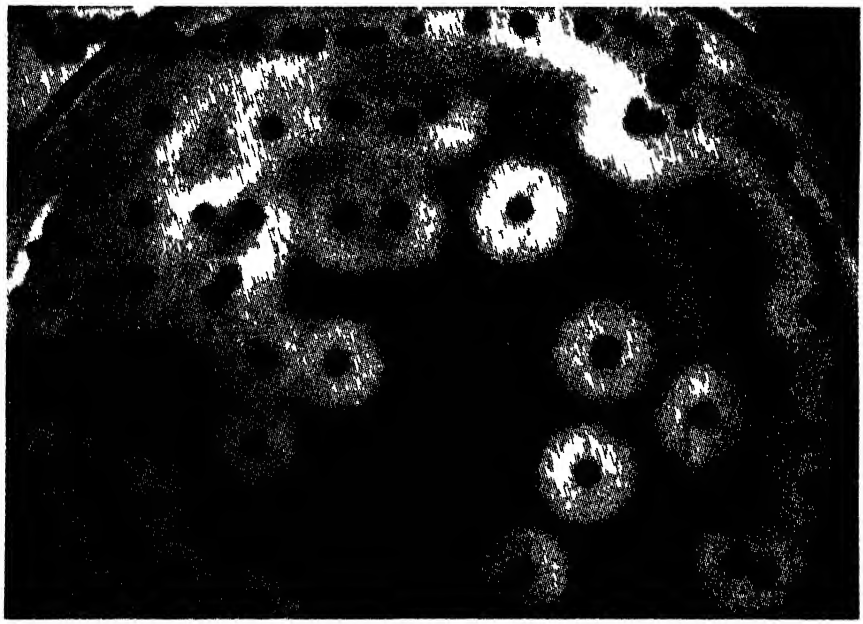


FIG 125.—Liquefaction of agar A plate culture of *Actinomyces coelicolor* was flooded with a solution of iodine to bring out the liquefied areas surrounding the colonies (*After Stanier*)

of a blood medium is one that contains not only the necessary food substances in proper proportions but an osmotic pressure approaching that of an isotonic salt solution

Sodium chloride does not act as a buffer. Salts such as phosphates and carbonates do possess a strong buffering action and are frequently added to culture media for this purpose (see page 261).

**Inorganic Requirements.**—The inorganic requirements of bacteria are not known. In the absence of accurate information, the following elements are usually supplied: Na, K, Mg, Fe, $SO_4$, $PO_4$, and Cl, C, N, and H are usually obtained from organic matter. Young, Begg, and Pentz (1944), working on the inorganic nutrient requirements of *Escherichia coli*, found that the organism required Mg, Fe, Na, K, $PO_4$, $SO_4$, Cl, N, and H.

The Fe requirements of bacteria have been studied by Waring and Werkman (1942, 1943, 1944). They employed an iron-deficient medium and found that cells of *Aerobacter indologenes* required a minimum of 0.025 part per million of iron in the culture medium for optimal growth. The organism grown on a medium without the addition of iron contained 0.0031 per cent iron. When grown in the same medium with the addition of its minimal requirement (0.025 p.p.m.), it contained 0.0073 per cent iron. When grown with a large excess, it contained 0.1049 per cent iron. The effect of iron deficiency on the enzyme systems of *A. indologenes* showed that catalase, peroxidase, formic dehydrogenase, hydrogenase, and cytochrome systems were depressed.

Perlman (1945) prepared a carbohydrate medium low in metallic cations by passage of the nonionic portion of the medium over a cationic exchange column. When this purified medium was fermented by *A. aerogenes*, a redistribution of the fermentation products occurred. The quantities of acetic acid, formic acid, and ethyl alcohol formed were decreased, whereas the quantities of lactic acid and carbon dioxide increased. A reversion to the "normal" fermentation was accomplished by the addition of manganese and chromium ions to the purified medium. The addition of zinc, copper, aluminium, and iron caused a partial reversion.

Some salts play an important part in the vital functions of protoplasm and enter into the more intimate structure of the cell. Guilleman and Larson (1922) showed that when *E. coli* was killed by heat, about one-half of the inorganic salts diffused out of the cells, while the other half remained fixed inside of the cells. They suggested that the half that remained fixed inside the cells was exercising some vital function. The fixed salts showed a high K/Na ratio as compared to the free salts. Also, practically all of the iron remained inside the cell, indicating that the element was necessary for protoplasmic activity.

**Fermentable Compounds.**—Fermentable compounds are frequently added to culture media. These compounds serve two functions: (1) They furnish readily available sources of energy, provided the organisms elaborate the enzymes necessary to ferment the compounds, and (2) fermentation reactions are of great help in identifying and classifying organisms.

The important fermentable substances added to culture media include

Monosaccharides:
  Pentoses: Arabinose, xylose, rhamnose.
  Hexoses: Glucose, levulose, mannose, galactose.
Disaccharides:
  Sucrose, lactose, maltose, trehalose, melibiose.
Trisaccharides:
  Raffinose, melezitose.
Polysaccharides:
  Starch, inulin, dextrin, glycogen, cellulose.

Alcohols:
Trihydric: Glycerol.
Pentahydric: Adonitol.
Hexahydric: Mannitol, dulcitol, sorbitol.
Glucosides:
Salicin, amygdalin.
Noncarbohydrate Compounds:
Inositol.

## NUTRITIONAL REQUIREMENTS

Culture media employed for the cultivation of bacteria may be divided into two groups on the basis of the character of the compounds making up their composition: (1) synthetic media and (2) nonsynthetic media.

**Synthetic Media.**—Synthetic media are composed of compounds of known chemical composition. They may be composed entirely of inorganic salts, or mixtures of inorganic salts and organic compounds, or inorganic and organic compounds with added growth factors. The exact chemical make-up of all components is known so that two batches of the same medium can be duplicated with a high degree of accuracy. Synthetic media are employed where it is desired to ascertain what effect an organism will have on certain compounds. The nutritional requirements of bacteria can be accurately determined only by the use of synthetic culture media.

McCullough and Dick (1943) cultivated a number of species of *Brucella* in a synthetic medium composed of inorganic salts, glucose, and growth factors. Johnson and Rettger (1943) made a study of the nutritional requirements of *Salmonella* species in a synthetic medium composed of phosphate, amino acids, and growth factors. Smiley, Niven, and Sherman (1943) cultivated *Streptococcus salivarius* in a chemically defined medium composed of inorganic salts, glucose, sodium thioglycollate, amino acids, and growth factors. Barker and Peterson (1944) found that *Clostridium acidi-urici* would grow on a medium containing inorganic salts, sodium thioglycollate, and uric acid. All growth factors and amino acids were excluded. Gould, Kane, and Mueller (1944) grew *Neisseria gonorrhoeae* on an agar medium containing amino acids, glucose, starch, glutathione, and salts. Youmans (1944) obtained subsurface growth of *Mycobacterium tuberculosis* in a synthetic medium composed of inorganic salts, asparagine, and glycerol. Stokes and Larsen (1945) cultivated *Acetobacter suboxydans* in a synthetic medium composed of amino acids, glycerol, salts, and growth factors. These are a few of the many uses of synthetic media that have been reported in the literature.

**Nonsynthetic Media.**—The nonsynthetic media are composed of ingredients of unknown chemical composition. Some of these are peptones, beef extract, meat infusion, blood, and serum. It is practically impossible to prepare two identical lots of the same medium from different batches

of the ingredients. Some of the more fastidious bacteria either do not grow or grow very poorly on synthetic media. However, these organisms are the exception rather than the rule. The usual culture media are of the nonsynthetic type.

It is now known that the presence of growth factors in culture media is absolutely necessary for the successful cultivation of most bacteria (page 242). The failure of an organism to grow on a certain medium is probably due to the absence of one or more of the essential growth accessory substances. Media are usually selected for their ability to produce results rather than because they are known to contain the necessary growth substances. It seems highly probable that a few media containing all the necessary accessory substances can be successfully employed for the cultivation of bacteria. Until such investigations are made, the bacteriologist will continue to employ many kinds of media, each more or less specific for a particular purpose.

**Use of Nitrogen Compounds.**—Koser and Rettger (1919) worked with a synthetic medium containing glycerol as the only source of carbon. To this medium were added various amino acids and other nitrogen-containing compounds, such as valine, glycocoll, asparagine, glutamic acid, phenylalanine, tyrosine, tryptophane, lysine, leucine, histidine, and urea. Of a total of 39 organisms studied, 21 were able to grow and multiply on such media. All the amino acids employed were utilized as sources of nitrogen, with no marked differences in their availability. Asparagine, lysine, and the cyclic amino acid histidine apparently possessed no advantage over the monoamino acids. The compounds, urea, taurine, creatine, hypoxanthine, and uric acid were inferior to the amino acids as available sources of nitrogen. Combinations of amino acids, or of amino acids and other nitrogenous compounds, were found to possess little advantage over any one of the amino acids. Dibasic ammonium phosphate yielded results only slightly different from those obtained from the amino acids. Compounds furnishing nitrogen as ammonia, whether amino acids or inorganic ammonium salts, were of equal value as immediately available sources of nitrogen.

Braun and Cahn-Bronner (1922*a*, *b*) employed ammonium lactate and other organic ammonium salts instead of ammonium phosphate and obtained essentially the same results. They found that all organisms that were capable of growing on ammonium lactate grew also if the ammonium salt was replaced by an amino acid. Their results confirmed those of previous workers, namely, that ammonia and amino nitrogen were interchangeable as sources of nitrogen for the nutrition of the organisms studied.

Fildes, Gladstone, and Knight (1933) showed that the amino acid tryptophane was necessary for the growth of *Salmonella typhosa*. They employed a basal medium containing sodium citrate, magnesium sulfate,

phosphate buffer of pH7.4, and glucose. To this medium they added 14 amino acids and ammonium chloride as sources of nitrogen. The acids tested included alanine, glycine, leucine, valine, glutamic acid, asparagine, tyrosine, phenylalanine, proline, histidine, arginine, lysine, cystine, and tryptophane. The typhoid organism grew very well on this medium. By a process of elimination, they found that the organism could derive its nitrogen requirement from a mixture of amino acids containing tryptophane, but ordinarily the organisms would not grow in the absence of this essential amino acid. Some strains of *S. typhosa*, which initially required tryptophane, could be trained to grow without it; others could not. From a nutritive standpoint, three varieties of the typhoid organism existed: (1) those which could grow with ammonia as the only source of nitrogen, (2) those which required tryptophane but could be trained to synthesize the compound, and (3) those which could not be trained to synthesize the compound. They concluded that tryptophane was probably an essential constituent of protoplasm and, if the organisms could not synthesize the compound, it must be added to the culture medium.

Fildes and Knight (1933) placed organisms into three groups on the basis of their action toward tryptophane:

1. This group includes those organisms which ordinarily cannot grow unless tryptophane is present in the medium. Some of the organisms in this group are, *Clostridium sporogenes*, *C. botulinum*, *C. tetani*, *Bacillus anthracis*, *Corynebacterium diphtheriae*, *Salmonella typhosa*, and *Micrococcus pyogenes* var. *aureus*.

2. This group includes those organisms which do not require the presence of tryptophane in the culture medium. Some of the organisms in this group are, the autotrophic bacteria, species of *Escherichia* and *Aerobacter*, *Pseudomonas acruginosa*, *S. aertrycke*, and *Mycobacterium tuberculosis* var. *hominis*. These organisms contain tryptophane in their protoplasm, being capable of synthesizing the amino acid.

3. This group includes those organisms intermediate between groups 1 and 2 in their requirements for tryptophane. The organisms are unable to synthesize tryptophane on first transfer to a medium in which it is absent but they can be trained to do so by gradual elimination of the compound. Organisms that behave in this manner include *S. typhosa*, *C. diphtheriae*, *M. pyogenes* var. *aureus*, and probably *M. tuberculosis* var. *hominis*.

Burrows (1939*a*, *b*) obtained essentially the same results as reported by Fildes, Gladstone, and Knight and on the basis of further studies came to the following conclusions:

> . . . the function of tryptophane in the growth of the strains of typhoid bacilli which apparently require it, is not that of supplying a molecular configuration

that the organism is unable to synthesize. It would appear, rather, that it exerts a stimulatory effect on growth, a kind of "trigger" action. Not only is tryptophane not "essential" in the original sense, but it is actually synthesized by the bacteria during the growth process.

Contrary to usual belief, the typhoid organism produced indole but in extremely small amounts. This would suggest that some of the biochemical differences between bacteria may be a matter of degree rather than of kind, quantitative rather than qualitative, and in view of this implication, evidence which suggests the acquiring of new physiological characters by bacteria should be subjected to rigorous test before drawing conclusions.

Since *S. typhosa* is known to require tryptophane, Fildes (1940*a*) tested a number of related but less complex compounds for their ability to support growth of the organism. The compounds tested included indoleacrylic acid, indoleacetic acid, indolepropionic acid, indolecarboxylic acid, indolepyruvic acid, indole-ethylamine, indolealdehyde, skatole, and indole. Indole stimulated the growth of the organism and, since indolecarboxylic acid contained a trace of indole as an impurity, this accounted for the slight growth obtained in the presence of the compound. The other substances were found to be entirely inactive. This observation suggested that indole was a stage in the synthesis of tryptophane by the typhoid bacillus, according to the scheme: $NH_3$ or amino N $\rightarrow$ indole $\rightarrow$ tryptophane. If the scheme is correct, it should be possible to find an organism that can synthesize tryptophane when supplied with any of the stages given above. Results showed that some bacteria were capable of synthesizing tryptophane from any of its precursors whereas others required the finished amino acid. Fildes concluded that tryptophane was synthesized from ammonia in stages, one of which is indole or a closely related substance. Failure of an organism to grow was due to inability to synthesize tryptophane. In a later report Fildes (1945) demonstrated the synthesis of indole by bacteria and showed that it was a stage in the formation of tryptophane probably by a condensation of indole with serine.

**Use of Carbon Compounds.**—Braun and Cahn-Bronner tested a large number of carbon compounds including formic, acetic, oxalic, lactic, succinic, malic, tartaric, and citric acids, glycerol, glucose, and arabinose. They found that glucose, glycerol, lactic, and citric acids were utilized more than any of the other carbon compounds when tested against *Salmonella schottmuelleri*, *S. enteritidis*, and *S. typhosa*. Acetic and oxalic acids ranked next. Formic and probably tartaric acids were not available as sources of carbon. The amino acids ranked lower than the organic acids, carbohydrates, and glycerol from the standpoint of availability. Den Dooren de Jong (1926) tested about 250 organic compounds for their availability as sources of carbon for a number of organisms. The compounds were added to a synthetic medium containing ammonia as the

only source of nitrogen. His conclusions were similar to those of Braun and Cahn-Bronner. He found that carbohydrates and related compounds were most generally utilized; these were followed by malic, citric, succinic, and lactic acids; next came the fatty acids; and last the monohydric alcohols.

Braun and Cahn-Bronner found that anaerobic growth was entirely absent when *S. schottmuelleri* was inoculated into an inorganic medium containing ammonium lactate and glucose. Koser (1923) found the same to be true when the members of the *Escherichia* and *Aerobacter* groups were inoculated into media containing various organic acids as carbon sources. Citric acid and its salts were utilized by *A. aerogenes* but not by *E. coli* (see page 487).

**Formation of Lipids.**—The formation of lipids by bacteria is dependent upon the nature of the carbon compounds added to media. Stephenson and Whetham (1922) employed an inorganic medium containing ammonium salts to which were added (1) lactic acid, (2) lactic and acetic acids, (3) glucose, and (4) glucose and acetic acid. The media were inoculated with *Mycobacterium phlei*, an acid-fast organism. Their results are shown in Table 25. The addition of acetate to the various media produced no increase in protein formation, but did increase the lipid concentration.

TABLE 25.—EFFECT OF COMPOSITION OF MEDIUM ON FAT FORMATION BY *Mycobacterium phlei*

| Constituents of medium | Period of maximum lipid formation, days | Milligrams per 100 cc. medium | | Ratio, lipids/nitrogen |
|---|---|---|---|---|
| | | Nitrogen synthesized | Lipids synthesized | |
| 0.68% lactic acid | 10 | 19 | 16 | 0.84 |
| 1.4% lactic acid | 11 | 26 | 20 | 0.78 |
| 1.2% lactic acid + 0.4% acetic acid | 7 | 20 | 35 | 1.78 |
| 1% glucose | 12 | 18 | 28 | 1.6 |
| 1% glucose + 1% acetic acid | 21 | 18 | 42 | 2.34 |
| 1% glucose | 15 | 15 | 19 | 1.3 |
| 2% glucose | 15 | 34 | 21 | 0.62 |

Increased concentrations of lactate and of glucose increased both protein and lipid, usually the former. An organism that normally synthesizes sufficient lipid material to become acid-fast could be made to grow acid-sensitive by omitting from the culture medium an appropriate carbon source.

Larson and Larson (1922) found that those organisms which ferment glucose or glycerol were unable to utilize the compound for the synthesis

of lipid material. Since *Escherichia coli*, *Micrococcus pyogenes* var. *aureus*, and *Clostridium mucosum* ferment glucose, they did not synthesize additional lipid. On the other hand, those organisms which do not ferment glucose or glycerol utilized the carbonaceous material for the synthesis of lipids. *E. coli*, *M. pyogenes* var. *albus*, and *Bacillus megatherium* do not ferment glycerol. These organisms showed a marked increase in fat content over either the glucose broth or the nutrient broth controls.

Geiger and Anderson (1939) inoculated *Agrobacterium tumefaciens* into two synthetic media, one containing glycerol and the other sucrose. The organisms grown on the glycerol-containing medium yielded only 2 per cent of total lipid, whereas those grown on the sucrose-containing medium gave 6 per cent of lipid. The nature of the fatty material synthesized by the organisms on the two media also showed considerable difference (Table 26).

TABLE 26.—YIELD OF LIPIDS FROM DRIED *Agrobacterium tumefaciens*

| | Medium 1, grams | Medium 2, grams |
|---|---|---|
| Bacteria used for extraction | 364 | 276 |
| Alcohol-ether-soluble lipids | 4.85 | 16.23 |
| Chloroform-soluble lipids | 2.46 | 0.63 |
| Total phosphatide | 3.21 | 10.90 |
| Total acetone-soluble fat | 3.35 | 5.92 |
| Ether-insoluble substance | 0.64 | 0.03 |

## VITAMINS AND OTHER GROWTH FACTORS

Hopkins (1906) was probably the first to point out that compounds other than fat, protein, carbohydrate, minerals, water, and oxygen are necessary in human nutrition. This observation led to the discovery of the vitamins by Funk (1912). Wildiers (1901) reasoned that, since certain factors not known at that time were required in human nutrition, a comparable situation existed in the requirements of fungi. He employed an inorganic medium containing cane sugar and ammonia as sources of carbon and nitrogen, respectively, and found that yeast cells failed to grow unless a certain number of organisms (size of inoculum) were transferred to fresh medium. A larger inoculum carried more growth factors to the new medium resulting in multiplication. The addition of a boiled suspension of yeast produced the same growth-promoting effect on small inocula as the addition of an emulsion of living yeast. Wildiers named the growth-promoting factor "bios."

It is now known that bios is a mixture of several of the water-soluble members of the vitamin B complex including: thiamine (vitamin $B_1$), pantothenic acid, pyridoxin (vitamin $B_6$), biotin, and inositol.

A large number of growth-promoting substances have been studied, some of which have been synthesized and their chemical constitution determined. Some are simple in structure, others are quite complex. These compounds are referred to by various terms such as growth factors, growth accessory substances, growth determinants, vitamins, and coenzymes Williams (1941*a*) coined the term "nutrilites" to include all

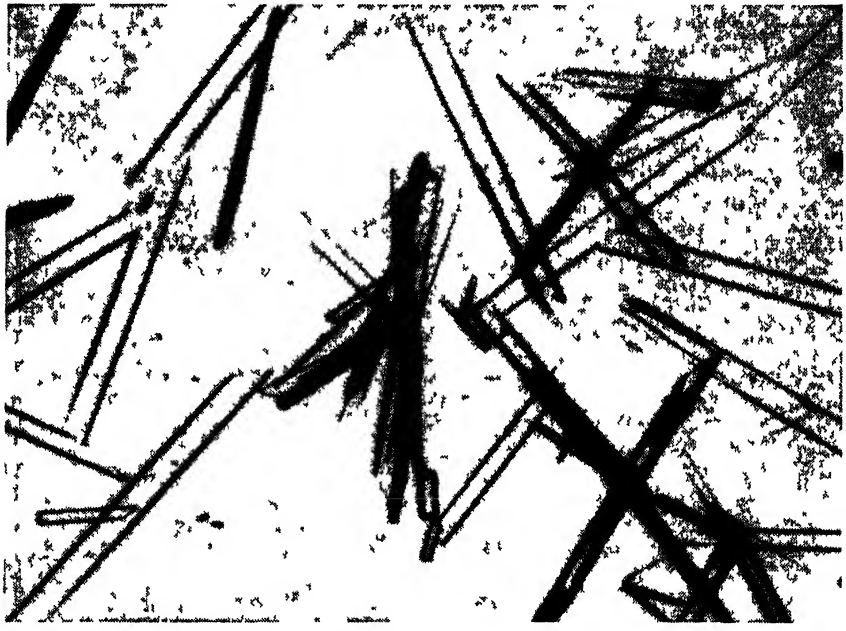


FIG. 126.—Thiamin chloride crystals (*Courtesy Merck & Company, Inc*)

organic substances, regardless of their nature which in minute amounts are of importance in the nutrition of microorganisms

**Response of Bacteria to Growth Factors.**—The fact that an organism does not require a particular growth factor does not mean necessarily that it is not necessary. Some organisms can synthesize one or more factors whereas others are unable to do so Therefore, it is erroneous to conclude that an organism does not require a certain factor because it is not added to the medium in which the organisms are growing

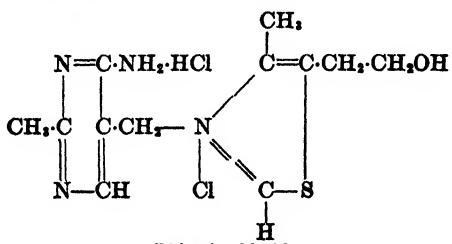
O'Kane (1941) cultivated micrococci on a medium free from riboflavin and found that the organisms were capable of synthesizing the growth factor. Burkholder and McVeigh (1942) demonstrated that certain intestinal species could synthesize a number of vitamins of the B complex. Actinomycetes were shown by Herrick and Alexopoulos (1943) to be capable of synthesizing thiamine. Miller (1944) found that *Escherichia coli* synthesized folic acid.

Some bacteria can be adapted to dispense with certain vitamins by repeatedly subculturing to fresh media. For example, strains of *Propionibacterium* can be adapted to grow without riboflavin and thiamine. Koser and Wright (1943) isolated variants of dysentery bacilli capable of growing without added nicotinamide.

## GROWTH FACTORS REQUIRED BY BACTERIA

The factors most frequently reported as promoting growth of bacteria include thiamine, biotin, pantothenic acid, pyridoxin, nicotinic acid, riboflavin, and *p*-aminobenzoic acid. In addition to these, there are a number of miscellaneous compounds that have been shown to be indispensable for some but not for all bacteria. Some of these are folic acid, inositol, codehydrogenases I and II, pimelic acid, β-alanine, glutamine, purines, pyrimidines, glutathione, hematin, nicotinamide, and betaine.

**Thiamin (Vitamin $B_1$).**—This factor is probably necessary for the growth of all bacteria (Fig. 126). A number of organisms have been shown to be capable of synthesizing the compound. Chemically, it is a pyrimidine-thiazole compound having the following structural formula:

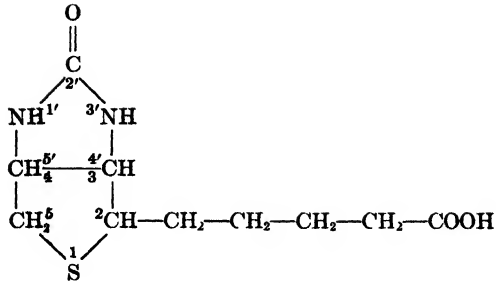


Thiamin chloride

**Biotin.**—This growth factor was first isolated by Kögel (1935). It is identical with vitamin H. It has been crystallized as pure biotin or as the biotin methyl ester (Fig. 127).

Biotin in extraordinarily small amounts stimulates the growth of yeast. According to Kögel, an amount as small as 0.00004 μg. added to 2 cc. of culture caused a 100 per cent increase in growth. This is equivalent to 1 part in 50,000,000,000 parts of medium. It is also necessary for the growth of molds and bacteria. The nutrilite has been shown to be present in plant and animal tissues. Du Vigneaud, Melville, Folkers, Wolf, Mozingo, Keresztesy, and Harris (1942) worked out the structure of the compound and believed it to be 2-keto-3,4-imidazolido-2-tetrahydrothiophene-*n*-valeric acid.

For more information, consult Koser and Wright (1942), Landy (1941), Melville, Moyer, Hofmann, and du Vigneaud (1942), S. M. A. Corporation (1943), Stokes and Gunness (1943), Wright (1942), and Wright and Skeggs (1944*a*).



Biotin

**Pantothenic Acid.**—Williams and Bradway (1931) were the first to show that a growth factor, which they called "pantothenic acid," was

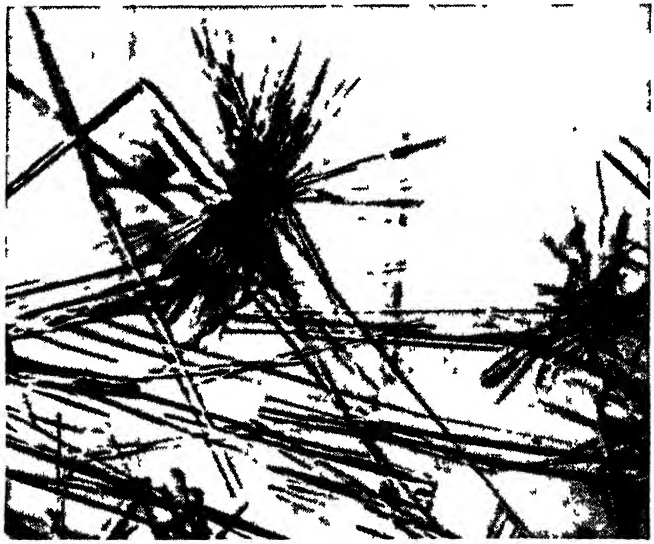
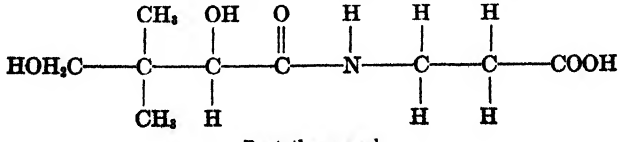


Fig. 127.—Biotin methyl ester. (*Courtesy S.M.A Division, Wyeth Inc.*)

necessary for the nutrition of yeast (Fig. 128). In a later report Williams, Lyman, Goodyear, Truesdail, and Holaday (1933) stated that pantothenic acid was a growth determinant of universal biological importance, being present in bacteria, yeasts, molds, algae, protozoa, plants, and animals. It was shown to consist of $\beta$-alanine united to a saturated dihydroxy acid by a peptid-like combination and having the following formula:



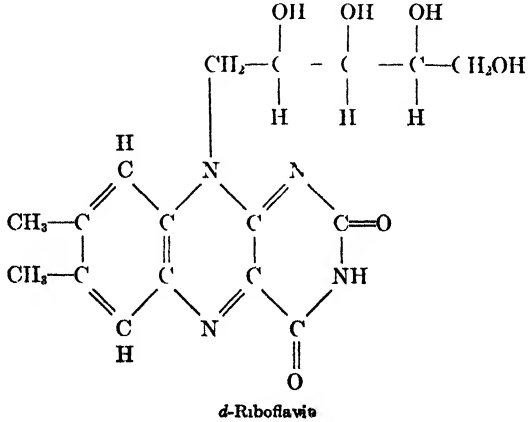
Pantothenic acid

For more information on the pantothenic acid requirements of bacteria, see Cheldelin, Hoag, and Sarett (1945), Dorfman, Berkman, and Koser (1942), and Hills (1943).

**Riboflavin.**—This factor has been studied extensively and found to be necessary for the growth of a large number of organisms (Fig. 129).

FIG 128.—Pantothenic acid crystals (*Courtesy Merck & Company, Inc.*)

It is sometimes referred to as lactoflavin, vitamin $B_2$, and G. Chemically, it is a *d*-ribose derivative of isoalloxazine having the following formula:



*d*-Riboflavin

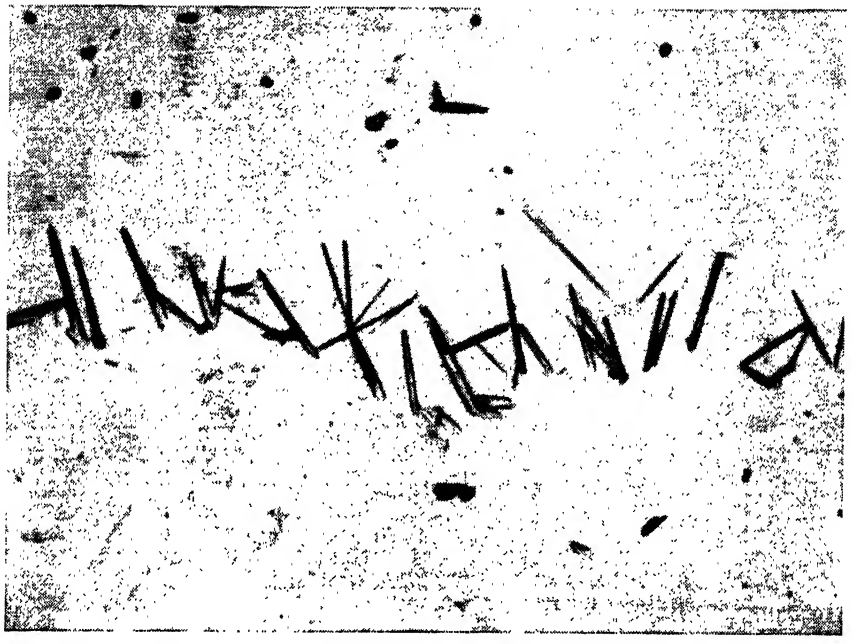


FIG. 129.—Riboflavin crystals. (*Courtesy Merck & Company, Inc.*)

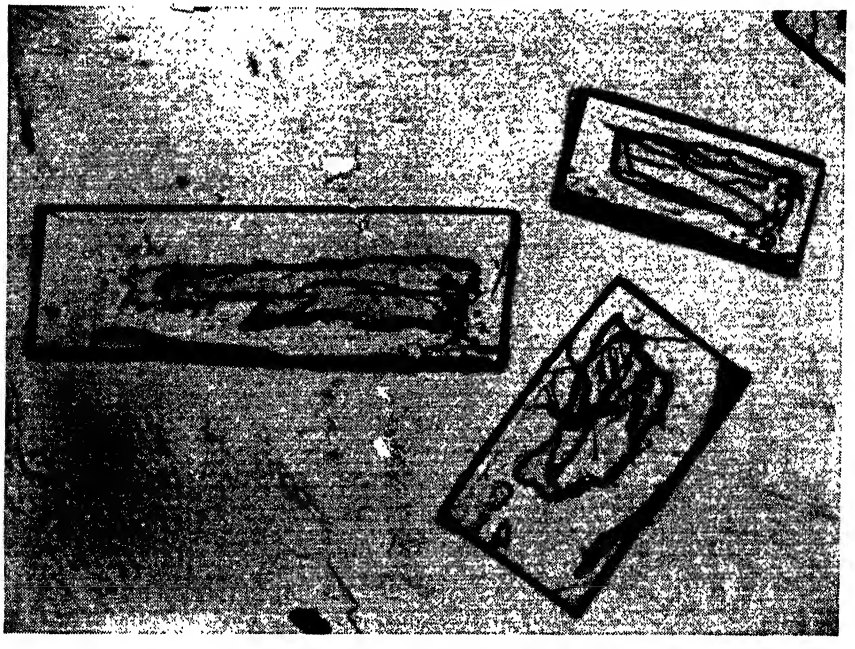
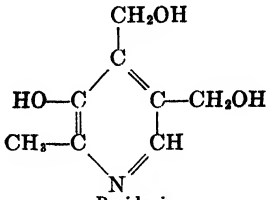


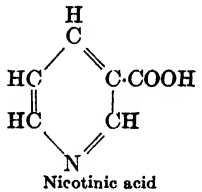
FIG. 130.—Pyridoxin crystals. (*Courtesy Merck & Company, Inc.*)

**Pyridoxin.**—This compound was first recognized as a vitamin by György (1935). Keresztesy and Stevens (1938) and Lepkovsky (1938), working independently, isolated the vitamin in crystalline form (Fig. 130). Harris and Folkers (1939) synthesized the vitamin and showed it to be 1-methyl-2-hydroxy-3,4-dihydroxy-methyl pyridine having the following structural formula:

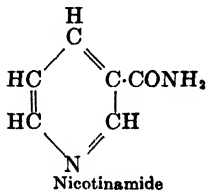


Pyridoxin

**Nicotinic acid and Nicotinamide.**—Nicotinic acid was first prepared in 1867, but the nutritional importance of the compound was not recognized until 1937 by Elvehjem, Madden, Strong, and Woolley (Fig. 131). The structural formulas for nicotinic acid and its amide are as follows:



Nicotinic acid

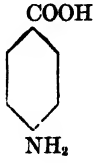


Nicotinamide

***p*-Aminobenzoic Acid.**—Woods (1940) and Fildes (1940*b*) found that sulfanilamide competes with *p*-aminobenzoic acid (PABA) for an enzyme and thus interferes with some essential metabolic reaction. Landy, Larkum, Oswald, and Streightoff (1943) found that sulfonamide-resistant strains of *Micrococcus pyogenes* var. *aureus* produced greater amounts of PABA than their parent strains. The quantity of PABA synthesized by resistant strains appeared sufficient to account for their resistance to sulfonamide drugs.

*p*-Aminobenzoic acid has been shown to be a nutrilite for a number of bacteria but its function in cell metabolism is obscure.

It has the following structural formula:



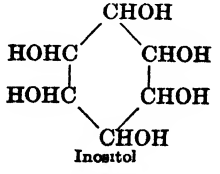
*p*-Aminobenzoic acid

**Inositol.**—This was the first pure substance isolated that was found to contribute to bios activity. The compound was isolated in pure form by

Eastcott (1928) from tea. Inositol becomes a limiting growth factor only when other nutrilites are added. Inositol is present in both plant and

FIG. 131 —Nicotinic acid crystals (*Courtesy Merck & Company, Inc*)

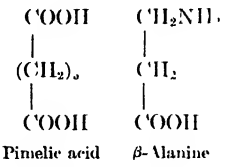
animal cells, but its exact physiological function is not clearly understood. It has the following structural formula




Inositol

**Pimelic Acid and β-Alanine.**—Mueller (1935) and Mueller and Kapnick (1935) showed that the diphtheria bacillus *Corynebacterium diphtheriae* produced a luxuriant growth in a medium composed entirely of amino acids to which were added a purified liver extract and a carbon source, such as glycerol and lactic acid, and suitable inorganic salts Mueller (1937*a*, *b*) identified two of the constituents of the liver extract, essential for growth of the diphtheria bacillus, as pimelic acid and nicotinic acid. In a later report, Mueller and Cohen (1937) found β-alanine to be a third-growth accessory substance present in liver extract responsible for the

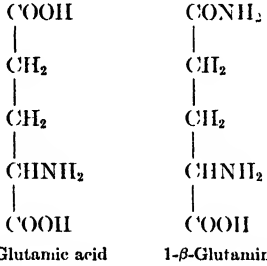
luxuriant growth of the diphtheria bacillus in a synthetic medium. The structural formulas for pimelic acid and β-alanine are as follows:




Pimelic acid β-Alanine

**Glutamic Acid and Glutamine.**—McIlwain, Fildes, Gladstone, and Knight (1939) found that glutamine was an essential nutrient for the growth of some strains of *Streptococcus pyogenes* but not for others. Glutamine is present in beef extract and is probably widely distributed in the animal body. Later, Fildes and Gladstone (1939) and McIlwain (1939) reported that glutamine was indispensable for the growth of many strains of *S. pyogenes* and other bacterial species. Pollack and Lindner (1942) stated that nine species of lactic acid-producing bacteria required either glutamine or glutamic acid for growth. They concluded that bacteria required glutamine or glutamic acid for the construction of cell proteins because the requirements of these amino acids are of the order of magnitude that would be expected for this function. Lankford and Snell (1943) found that certain strains of *Neisseria gonorrhoeae* required glutamine for growth.

The structural formulas for glutamic acid and glutamine are as follows:




1-Glutamic acid 1-β-Glutamine

**Folic Acid.**—Snell and Peterson (1939, 1940) found that *Lactobacillus casei* required a hitherto unrecognized growth substance, which they called the *L. casei* eluate factor. They noted that the factor possessed many of the properties of a purine. Stokstad (1941) found that a mixture of guanine and thymine possessed some of the growth-promoting properties of the eluate factor. Mitchell, Snell, and Williams (1941) concentrated the factor to a state approaching purity and named it "folic acid." The chemical identity of folic acid is not known.

For additional information, see Wright and Skeggs (1944*b*) and Stokes (1944).

**Purines and Pyrimidines.**—Richardson (1936) found that uracil was necessary for the growth of *Micrococcus pyogenes* var. *aureus*. Snell and

Mitchell (1941) reported that uracil was necessary for the growth of *Lactobacillus arabinosus* and *L. mesenteroides*, thymine for *Streptococcus lactis*, adenine for *L. arabinosus* and *L. pentosus*. Shull, Hutchings, and Peterson (1942) and Pollack and Lindner (1942) found adenine, guanine, uracil, and xanthine to be necessary for the growth of *L. casei*. Hutner (1944) reported the necessity of uracil for the nutrition of *Shigella paradysenteriae*. Rogers (1944) found uracil to be a growth factor for *Streptococcus pyogenes*. Chattaway (1944) reported that orotic acid (uracil-4-carboxylic acid) stimulated the growth of *L. casei*, and Hitchings, Falco, and Sherwood (1945) found that thymine could replace folic acid in the nutrition of the same organism.

The structural formulas of the various purines and pyrimidines may be found on pages 586 to 588.

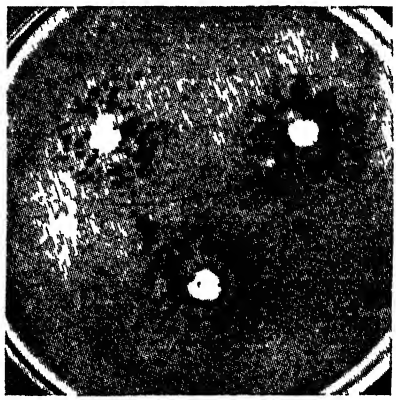


Fig. 132.—Satellite colonies of *Neisseria gonorrhoeae*. The plate was streaked with *N. gonorrhoeae*, then spotted with a *Micrococcus*. The colonies of *N. gonorrhoeae* grow as satellites at some distance from the colonies of the *Micrococcus*. The colonies of *N. gonorrhoeae* were developed with dimethyl-paraphenylene-diamine. (*After Lankford, Scott, Cox, and Cooke.*)

**V and X Factors.** Pfeiffer (1893) reported that the organism *Hemophilus influenzae* would not grow in a broth medium unless blood was added. Thjotta (1921), Thjotta and Avery (1921*a*, *b*), and Fildes (1921, 1922) found that the same organism required the presence of two factors which they named the *V* and *X* factors, both of which are present in blood. The *V* factor is also present in many plant extracts and in a large number of bacteria. It is thermolabile, being destroyed in 15 min. at 90°C., is very sensitive to alkali but not to acid, diffuses through parchment membranes, and is not readily destroyed by atmospheric oxygen. The *X* factor is found in potatoes and in some bacteria. It is thermostable, resisting a temperature of 120°C. for 45 min.

*Hemophilus influenzae* is unable to grow on media containing only the *X* factor. However, it will grow on media in association with an organism, such as *Micrococcus pyogenes* var. *aureus*, which is capable of producing the *V* factor. The characteristic arrangement of colonies of *H. influenzae* in such an association is sometimes referred to as the satellite phenomenon. The hemophilic organisms grow as satellites in isolated colonies at some distance from the colonies of *M. pyogenes* var. *aureus*.

Lankford, Scott, Cox, and Cooke (1943) observed the same phenomenon on agar plates streaked with a mixture of a nonpigmented *Micrococcus* and *Neisseria gonorrhoeae* (Fig. 132).

The organism *H. parainfluenzae* requires only the *V* factor for growth. Lwoff and Lwoff (1937*a*, *b*) isolated the *V* factor from yeast and added the extract to a culture of the organism. They noted that *V* activity paralleled the codehydrogenase I concentration of the yeast extract. This substance was found to replace the *V* factor in extremely low concentration. Codehydrogenases I and II are very similar chemically but are not, as a rule, interchangeable. As growth factors, however, codehydrogenase II can replace codehydrogenase I. Since one or the other factor must be supplied before growth can occur, they are considered to be true vitamins. Chemically, codehydrogenase I is diphosphopyridine nucleotide and codehydrogenase II is triphosphopyridine nucleotide (see page 321).

The *X* factor requirement is supplied largely by the addition of hemin. Since the addition of a small amount of blood enhances activity of the organism, it is quite likely that other factors are also involved.

For further reading, see Gingrich and Schlenk (1944), Hoagland, Ward, Gilder, and Shank (1942), and Gregory (1944).

**Glutathione.**—Gould (1944) reported that glutathione was necessary for the growth of *Neisseria gonorrhoeae*. The factor was found to be present in meat infusion, yeast infusion, and red blood cell extract. Freshly isolated strains of the organism were found not to require glutathione, but they showed a tendency to develop dependence on glutathione after some weeks of subculturing on a medium containing meat infusion.

The structural formula of glutathione is given on page 325.

**Effect of pH on Growth-factor Requirements.**—Doede (1945) found that the pH of the medium had a marked influence upon the growth-factor requirements of a number of bacterial species. The amount of nicotinic acid required by *Micrococcus pyogenes* var. *aureus* for maximum growth at pH8 was approximately fifteen times that required at pH6. *Shigella paradysenteriae* required nicotinic acid in the medium at pH7 and pH8 but not at pH6. At pH6, the organisms were capable of synthesizing the growth factor. *Lactobacillus casei* grew in a medium without pyridoxine at pH5 but failed to grow at pH6 and pH7 unless the factor was added. Folic acid, riboflavin, biotin, nicotinic acid, and pantothenic acid were found to be most effective at pH6.

**Bacterial Destruction of Vitamins.**—A number of bacterial species have been shown to be capable of destroying growth factors. Young and Rettger (1943) found that vitamin C (ascorbic acid) was easily destroyed by the enteric bacteria, including the intestinal streptococci. In the presence of an easily fermented carbohydrate, like glucose, the vitamin is protected from decomposition, whereas in the absence of the competitive agents the ascorbic acid content of the medium becomes rapidly depleted. The ascorbic acid was utilized as a carbon food when the medium contained a suitable source of organic nitrogen. Foster (1944*a*, *b*) showed that

the organism *Pseudomonas riboflavinus* was capable of oxidizing riboflavin to lumichrome, according to the reaction:

$$\underset{\text{Riboflavin}}{C_{17}H_{20}O_6N_4} + 5\tfrac{1}{2}O_2 \rightarrow \underset{\text{Lumichrome}}{C_{12}H_{10}O_2N_4} + 5CO_2 + 5H_2O$$

The reaction is essentially an oxidation of the ribityl side chain of the riboflavin molecule, leaving the ring structure intact (see page 246). Koser and Baird (1944) reported the destruction of nicotinic acid by *P. fluorescens* and allied types, and also by *Serratia marcescens* and related species. Destruction occurred during periods of active cell multiplication.

**Vitamin Content of Ingredients of Culture Media.**—Information concerning the vitamin content of ingredients of culture media should prove of interest and value in the cultivation of microorganisms. Such information could be used to decide whether a particular culture medium satisfies the growth factor requirements of an organism.

Stokes, Gunness, and Foster (1944) assayed a number of culture media for their content of eight members of the B complex: thiamine, riboflavin, pantothenic acid, nicotinic acid, biotin, pyridoxine, folic acid, and *p*-aminobenzoic acid, and compared the results with the vitamin requirements of various microorganisms that were unable to synthesize these factors. They arranged the various culture media ingredients in order of descending value on the basis of their content of vitamins of the B complex.

The classification is as follows:

1. Yeast extract.
2. Meat extract, brain infusion, heart infusion.
3. Various peptones.

With the possible exception of thiamine, yeast extract is an excellent source of all vitamins of the B complex. This explains why yeast extract is held in such high favor as an ingredient of culture media.

Stokes, Gunness, and Foster concluded that if peptone, meat extract, etc., were used singly or in combinations of 1 or 2 per cent, the resultant media were likely to be deficient in thiamine, riboflavin, pantothenic acid, pyridoxine, and *p*-aminobenzoic acid but not nicotinic acid, biotin, or folic acid. They suggested that such growth-factor deficiencies in culture media could be remedied by proper combinations of ingredients or by the addition of yeast concentrates or synthetic vitamins.

For more information on growth factors, see Mueller (1940), Williams (1941*b*), and the excellent review by Peterson and Peterson (1945).

## GROWTH PHASES IN A CULTURE

It is well known that the smaller an object, the greater will be the ratio of the area to its weight. For example, an organism of average size, such as *Escherichia coli*, has a volume of $2 \times 10^{-9}$ cu. mm. and a surface area

of $10^{-5}$ sq. mm. If the specific gravity is taken as 1 (bacteria have a specific gravity slightly greater than 1), the weight of a single cell of *E. coli* will be $2 \times 10^{-9}$ mg. The ratio

$$\frac{\text{Area } (10^{-5} \text{ sq. mm.})}{\text{Weight } (2 \times 10^{-9} \text{ mg.})} = 5000$$

A similar calculation for an average man becomes

$$\frac{\text{Area } (2.4 \text{ sq. meters})}{\text{Weight } (100 \text{ kg.})} = 0.024$$

Comparing the two results, it is seen that the ratio area/weight for a bacterial cell is about 200,000 times greater than for a man.

Bacterial organisms are believed to absorb the necessary nutrients through their cell walls. The greater the ratio of surface area to weight, the greater will be the amount of food absorbed in proportion to size. This explains why bacteria are able to multiply at such a rapid rate and produce pronounced changes in culture media in a short period of time. Under favorable conditions, a single cell of *E. coli* divides into two about every 20 min. If this same rate is maintained, a single organism will give one billion new cells after a period of about 10 hr. However, this rate of multiplication is not maintained indefinitely, owing to the exhaustion of the nutrients, to the accumulation of toxic metabolic waste products, and to the fact that many of the cells die. The rate of death increases as the culture ages. The more vulnerable cells die first, leaving the resistant forms in the culture at the end of the incubation period.

When an organism is inoculated into a tube of medium such as nutrient broth, multiplication does not take place in a regular manner. On the contrary, various growth phases may be recognized which are known as the life phases of a culture. Buchanan (1918) recognized seven distinct cultural phases which he designated as follows:

1. *Initial Stationary Phase.*—During this phase, the number of bacteria remains constant. Plotting the results on graph paper gives a straight horizontal line (1*a*) in Fig. 133.

2. *Lag Phase or Phase of Positive Growth Acceleration.*—During this phase, the rate of multiplication increases with time (*ab*).

3. *Logarithmic Growth Phase.*—During this phase, the rate of multiplication remains constant (*bc*). This means that the generation time is the same throughout.

4. *Phase of Negative Growth Acceleration.*—During this phase, the rate of multiplication decreases (*cd*). The average generation time increases. The organisms continue to increase in numbers but at a slower rate than during the logarithmic growth phase.

5. *Maximum Stationary Phase.*—During this phase, the number of living organisms remains constant, *i.e.*, the death rate equals the rate of increase (*de*).

6. *Phase of Accelerated Death.*—During this phase, the numbers fall off with increasing rapidity (*ef*). The average rate of death per organism increases to a maximum.

7. *Logarithmic Death Phase.*—During this phase, the rate of death is constant (*fg*).

The reasons for the existence of a number of growth phases are not fully understood. Kojima (1923) believed that the phases in a bacterial culture were due to changes in the environment, such as alteration in pH, exhaustion of food supply, and accumulation of waste products. A glucose broth medium was inoculated with *Escherichia coli*. After an incubation period of 48 hr., sodium hydroxide was added to the culture to neutralize the high acidity. This caused the viable count to rise from 12,000 to 192,000,000 cells at the end of 60 hr., showing that the decrease in multiplication was due to the high acidity. However, the 60-hr. count did not

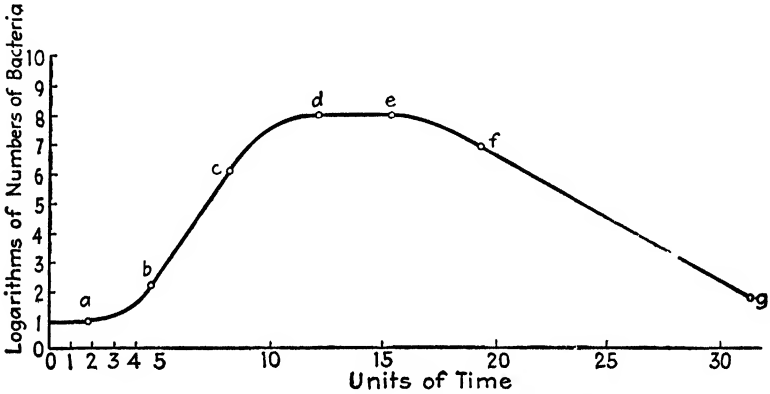



FIG. 133.—Growth phases in a culture. (*After Buchanan.*)

reach the height attained in a parallel tube of the same medium without glucose. This might indicate either an exhaustion of the nutrients or the presence of inhibitory waste products. To test the first hypothesis, fresh broth was added with the same amount of sodium hydroxide. The viable count rose to 380,000,000 at the end of 60 hr. When glucose was added to the nutrient broth instead of alkali, approximately the same count was obtained, but the falling off occurred at a more rapid rate.

Lodge and Hinshelwood (1939*a*, *b*) also believed that exhaustion of the nutrients, toxic metabolic products, and adverse pH might all be the cause of the cessation of growth. Topley and Wilson (1946) suggested that in the case of the obligate and facultative aerobic forms, a lack of oxygen might be the cause of the retardation of growth, since aeration resulted in a considerably increased maximum population of viable cells.

**Lag Period.**—When bacteria are transferred from an old culture to a new medium, they exhibit a period of delayed multiplication or lag. Müller (1895) was probably the first to make this observation. He recognized three distinct phases in a culture, which he designated the lag, the logarithmic increase, and the slackened growth phases. Müller found that, when cultures of differing ages were used for the inoculation of new medium,

the generation times in the new cultures showed considerable differences. Transfers from a typhoid culture 2½ to 3 hr. old gave a generation time of 40 min. in the new medium; a culture 6¼ hr. old gave a generation time of 80 to 85 min.; and a culture 14 to 16 hr. old gave a generation time of 160 min.

Another characteristic of organisms is that they show considerable variation to harmful influences in the different growth phases. Reichenbach (1911) found that cultures in the lag and early logarithmic phases exhibited greater sensitivity to heat than those in the older phases of growth. Schultz and Ritz (1910) reported that a 20-min. culture of *Escherichia coli* was more resistant to heat than a 4-hr. culture. Then the resistance showed a steady rise as the culture aged. Sherman and Albus (1923) exposed *E. coli* to various unfavorable conditions and found that old cells were considerably more resistant than very young cells. Hegarty and Weeks (1940) found that young cells of a culture of *E. coli* were more susceptible to cold shock than old cells. Mature cells were not affected by either an initial cold shock or a prolonged holding at 0°C. They concluded that the sensitivity of young cells to cold appeared to be related in some manner to cell division and to changes within the individual cell.

If a tube of fresh medium is inoculated from a culture in the logarithmic growth phase (*bc*, Fig. 133), the lag phase will be greatly reduced and in many cases completely eliminated. The organisms in the logarithmic growth phase are multiplying at the maximum rate and continue to do so when transferred to fresh medium. Buchanan (1928) concluded that transfer from any phase of a bacterial culture cycle to a new medium is followed by a continuance of the phase of the parent culture.

**Factors Affecting Cell Size.**—Henrici (1928) showed that organisms increased very markedly in size during the lag phase. He found that the average length of *Bacillus megatherium* was six times longer than the inoculated organisms taken from an old culture. This increase generally manifested itself after 2 hr., and the maximum size was usually noted between 4 and 6 hr. During the lag phase, the cells showed considerable fluctuation in form. On passing from the lag phase to the logarithmic death phase, the organisms gradually decreased in size and exhibited a more constant cell form.

It is well known that, when a parent culture is inoculated into a new medium, an initially slow rate of increase in bacterial numbers occurs. Hershey (1939) showed quite conclusively that this slow rate of multiplication cannot be interpreted as indicating a period of lag in the sense of decreased viability and activity. What actually happens is that the rate of multiplication decreases but the individual cells become larger giving a rapid increase in bacterial mass. Hershey inoculated new medium from 3- and 24-hr. parent cultures of *Escherichia coli* and measured the increase

in bacterial mass by means of a photelometer. During the first 2 or 3 hr., new cultures inoculated from 3-hr.-old parent cultures showed a slower multiplication rate than new cultures inoculated from 24-hr. parent cultures, but the increase in protoplasmic growth remained the same. The cells from a young parent culture showed the same increase in cell mass as the cells from an old parent culture, even though they multiplied at a slower rate. The rate of increase in cell mass is nearly constant from the time growth first begins until the maximum population is reached.

The results suggest that conditions in fresh medium favor an increase in cell size but inhibit cell division, with the result that a majority of the cells attain an abnormal size before fission occurs. Inoculation of a large number of cells into new medium tends to produce the reverse effect, *i.e.*, the average size is smaller and attained much sooner than with small inoculations. If cells are removed from a culture before their average maximum size is reached and transferred to fresh medium, the organisms attain a larger size and the critical point takes place later than in the case of the original culture. This would indicate that the size of the organisms is dependent upon the density of the bacterial culture. The concentration of the nutrients in the medium is another factor. The maximum size of the organisms in a dilute medium is smaller, and the critical point is reached earlier. A dilute medium produces a poorer growth than a more concentrated preparation. This means that a more concentrated medium showing a heavy, crowded growth produces the same effect on cell size as a more dilute medium showing a light growth.

**Effect of Carbon Dioxide on Growth.**—Valley and Rettger (1927) showed that many bacteria grew better in the presence of an increased concentration of carbon dioxide. Walker (1932) found that the length of the lag phase could be controlled by the concentration of carbon dioxide present. He noted that the multiplication of *Escherichia coli* could be indefinitely delayed by aeration of a culture with $CO_2$-free air. Reintroduction of $CO_2$ into the medium caused a rapid increase in bacterial numbers. He concluded that "the phenomenon of lag may be due largely, if not entirely, to the time it takes the culture to build up the $CO_2$ content of the medium or of the cells themselves to a value essential for growth." Others have come to a similar conclusion. There appears to be no doubt that the amount of carbon dioxide present in a new medium is an important factor in controlling the length of the lag phase, but it is probably not the only factor involved.

**Factors Affecting Rate of Reproduction.**—The rate of multiplication of bacteria is increased by a rise in temperature. This continues until a certain maximum is reached after which the rate decreases until death finally occurs. The generation times of *E. coli* at different temperatures of incubation are as follows:

| °C. | Time | °C. | Time |
|---|---|---|---|
| 10 | 14 hr., 20 min. | 35 | 22 min. |
| 15 | 120 min. | 40 | 17½ min. |
| 20 | 90 min. | 45 | 20 min. |
| 25 | 40 min | 47½ | 77 min. |
| 30 | 29 min. | | |

Penfold and Norris (1912) found that the generation time of *Salmonella typhosa* in 1 per cent peptone solution at 37°C. was about 40 min. If the amount of peptone in the medium was less than 0.2 per cent, the generation time was almost inversely proportional to the concentration of peptone; if above 0.4 per cent, an increase in the amount was practically without effect on the growth rate of the organisms. The addition of 0.175 per cent of glucose to a medium containing only 0.1 per cent peptone lowered the generation time from 111 to 50 min. The addition of the same amount of glucose to a 1 per cent peptone solution reduced the generation time only from 39 to 34 min.

**Effect of Age on Cell Morphology.**—Under some environmental conditions, bacteria show the presence of granules; under other conditions, they do not. It has been observed that when cells are largest (2 to 4 hr. old) intracellular granules disappear and the protoplasm becomes more hyaline and stains more deeply. As the cells age and decrease in size, they become increasingly more granular. Old cells are, in general, very granular whereas young cells do not exhibit the presence of granules. The development of granules in cells of *Bacillus mycoides* with age is shown in Fig. 134. An exception to this rule is the organism *Corynebacterium diphtheriae*, the causative agent of diphtheria. This organism appears to be smaller in young than in old cultures and to exhibit the presence of granules in both young and old cells.

**Effect of Constant Environment on Cell Numbers.**—Jordan and Jacobs (1944) cultivated *Escherichia coli* in an apparatus that permitted rigid control of temperature, pH, aeration, and culture volume, and allowed food to be supplied at a constant rate by means of an automatic syringe mechanism. Determinations of total and viable cell populations were made. Results showed an initial period in which the total and viable counts both increased, followed by a steady phase in which the viable counts remained constant or decreased slightly while the total cell counts steadily increased. When during the steady phase the food supply was suddenly stopped, the total cell population remained constant but the viable cells decreased to a constant low level.

For further reading, consult Rahn (1943), Stephenson (1939), and Winslow and Walker (1939).

## HYDROGEN-ION CONCENTRATION OF CULTURE MEDIA

Culture media are adjusted to different degrees of acidity or alkalinity, depending upon the pH requirements of the organisms to be cultivated. Some organisms grow best in acid media; others grow best in alkaline media; still others prefer media that are approximately neutral in reaction. This last group includes the majority of bacteria that have been studied. It is necessary, therefore, to adjust the reaction of media to the pH requirements of the organisms to be studied.

Two methods are employed for adjusting the reaction of culture media: (1) the determination of the actual numbers of free hydrogen ions and (2) the determination of the net amount of acid- or base-binding groups present. The former is spoken of as the hydrogen-ion ($H^+$) concentration; the latter as the titratable acidity or alkalinity.

The hydrogen-ion concentration can be determined either colorimetrically or electrometrically. The titratable acidity is determined by titration, finding the difference between the titer of the solution and whatever is accepted as the neutral point or initial acidity. Both methods serve very useful purposes in bacteriological technique. The adjustment of media is more accurately carried out by means of the hydrogen-ion method. The titratable acidity determination is of great value in learning the buffer content of the medium, *i.e.*, its ability to resist changes in reaction on the addition of acid or alkali.

**Measuring the Concentration of Hydrogen ($H^+$) Ions.**—Pure water is neutral in reaction because it ionizes into equal numbers of hydrogen ($H^+$) and hydroxyl ($OH^-$) ions. One liter of a normal solution of hydrochloric acid contains approximately 1 gm. of hydrogen ($H^+$) ions. One liter of pure water contains approximately 0.0000001 gm. of hydrogen ions. This may be written $10^{-7}$ gm. per liter. For each $H^+$ ion there is a corresponding and neutralizing $OH^-$ ion.

According to the law of mass action,

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

or

$$\frac{(H^+)(OH^-)}{HOH} = K$$

Since the concentration of the undissociated water is very great, it can be taken as a constant. The equation can be expressed as follows:

$$(H^+)(OH^-) = K$$

The numbers of $H^+$ and $OH^-$ ions being equal, each must have a concentration of $1 \times 10^{-7}$. The product of the concentration of the hydrogen and hydroxyl ions is equal to $1 \times 10^{-14}$. The equation now becomes

$$(H^+) \times (OH^-) = 1 \times 10^{-14}$$

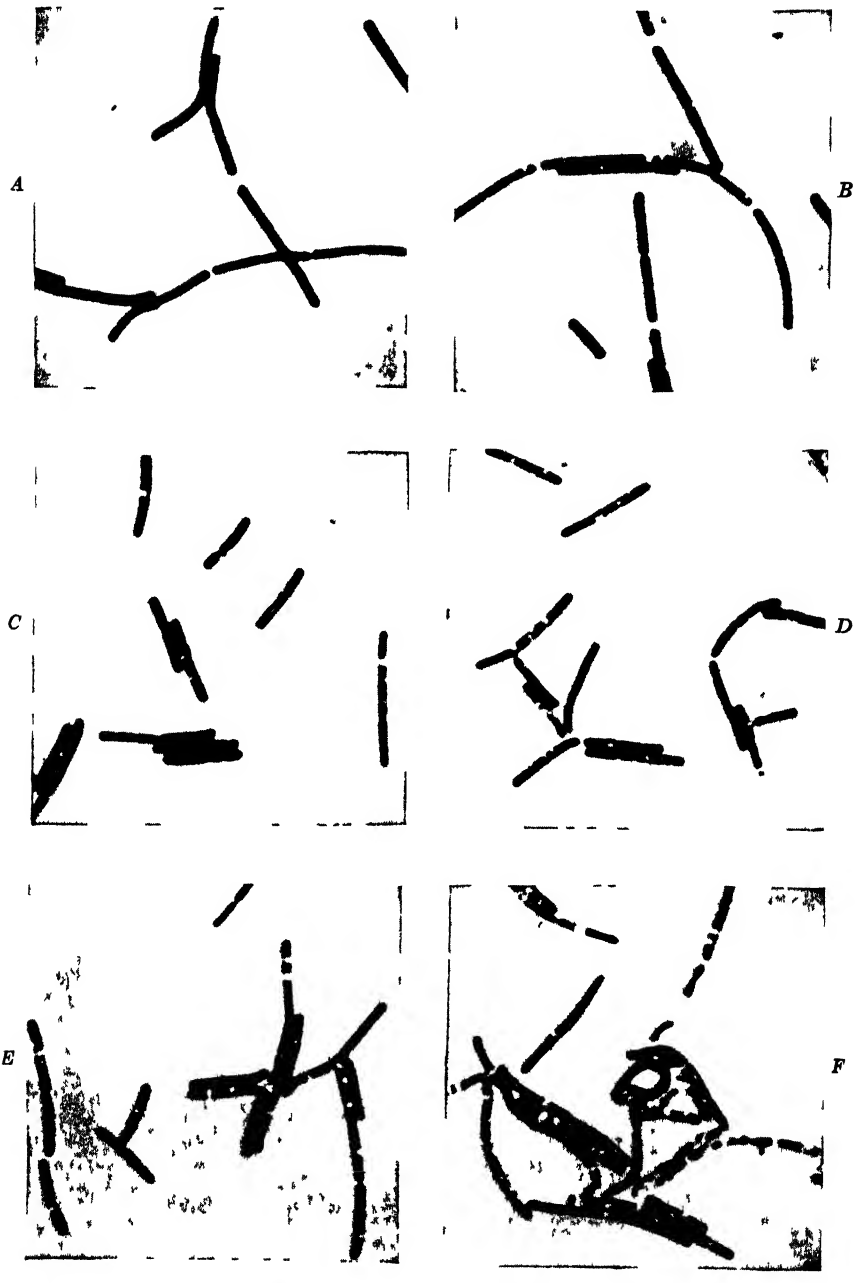



FIG. 134.—*Bacillus mycoides*. *A*, 6-hr. culture; *B*, 9-hr. culture; *C*, 18-hr. culture; *D*, 21-hr. culture; *E*, 24-hr. culture, *F*, 48-hr. culture. Note the development of granules with age.

Pure water, which has a hydrogen-ion concentration of $1 \times 10^{-7}$, is neutral in reaction. If the hydrogen-ion concentration of a solution is smaller than $1 \times 10^{-7}$, it will have an alkaline reaction; if greater than $1 \times 10^{-7}$, it will have an acid reaction.

The term "pH" is defined as the logarithm of the reciprocal of the hydrogen-ion concentration. For convenience, only the exponent is used in expressing pH. If a solution has a pH less than 7, it is acid in reaction; if greater than 7, it is alkaline. Most organisms grow best in culture media adjusted to a pH of about 7.0.

The relation of pH to the strength of hydrochloric acid and sodium hydroxide is given in Table 27. Since the acids and alkalies are not 100 per cent dissociated, the various pH values are only approximate.

*Colorimetric Method.*—The determination of the hydrogen-ion concentration by the colorimetric method depends upon the color changes produced in certain weakly acid or basic dyes by varying the reaction of the medium. Such dyes are called "indicators." All indicators dissociate on the addition of acid or alkali. An indicator changes in color within a short distance each side of that point in the pH scale at which it is 50 per cent dissociated. At this point, one-half the dye is undissociated and the other half is dissociated in the form of free ions. The pH at which this occurs is denoted by the symbol pK.

A short distance each side of the pK point gives a zone which is referred to as the sensitive range of the indicator. Every shade of color of the indicator in this sensitive range corresponds to a definite pH value so that, by comparing the shade of the indicator with standards of known reaction, the hydrogen-ion concentration of a solution can be determined. Indicators can be selected showing a certain amount of overlapping in their sensitive ranges so that the scale from pH1.0 to pH10.0 can be covered.

A list of the most important pH indicators used in bacteriology and some of their characteristics are given in Table 28.

**Buffers.**—The salts of weak acids have the power of preventing pronounced changes in the reactions of solutions on the addition of relatively large amounts of strong acids or alkalies. Substances that possess the power of resisting changes in acidity or alkalinity are spoken of as buffers.

The addition of 1 cc. of $N/10$ hydrochloric acid to 1 liter of neutral distilled water (pH7.0) gives a solution having a pH of about 4.0. The addition of 1 cc. of $N/10$ NaOH to 1 liter of neutral distilled water gives a solution having a pH of about 10.0. The addition of the same amount of acid or alkali to 1 liter of distilled water, in which is dissolved a few grams of sodium phosphate, produces very little, if any, change in the reaction. Sodium phosphate is classed as a buffer. This may be shown in the following reactions:

The addition of a strong acid:

$$Na_2HPO_4 + HCl \rightarrow NaH_2PO_4 + NaCl$$

The strong acid (HCl) reacts with the weak alkali ($Na_2HPO_4$) to give the weak acid ($NaH_2PO_4$) and sodium chloride. In other words, the strong

TABLE 27.—STRENGTHS OF SOLUTIONS OF HCl AND NaOH AND THEIR APPROXIMATE pH VALUES

| Acid or basic | Strength of solution | Grams hydrogen per liter | Expressed in logarithms | pH |
|---|---|---|---|---|
| Acid | $\frac{N}{1}$ HCl | 1.0 | $10^{-0}$ | 0.0 |
| Acid | $\frac{N}{10}$ HCl | 0.1 | $10^{-1}$ | 1.0 |
| Acid | $\frac{N}{100}$ HCl | 0.01 | $10^{-2}$ | 2.0 |
| Acid | $\frac{N}{1000}$ HCl | 0.001 | $10^{-3}$ | 3.0 |
| Acid | $\frac{N}{10,000}$ HCl | 0.0001 | $10^{-4}$ | 4.0 |
| Acid | $\frac{N}{100,000}$ HCl | 0.00001 | $10^{-5}$ | 5.0 |
| Acid | $\frac{N}{1,000,000}$ HCl | 0.000001 | $10^{-6}$ | 6.0 |
| Neutral | Pure water | 0.0000001 | $10^{-7}$ | 7.0 |
| Basic | $\frac{N}{1,000,000}$ NaOH | 0.00000001 | $10^{-8}$ | 8.0 |
| Basic | $\frac{N}{100,000}$ NaOH | 0.000000001 | $10^{-9}$ | 9.0 |
| Basic | $\frac{N}{10,000}$ NaOH | 0.0000000001 | $10^{-10}$ | 10.0 |
| Basic | $\frac{N}{1000}$ NaOH | 0.00000000001 | $10^{-11}$ | 11.0 |
| Basic | $\frac{N}{100}$ NaOH | 0.000000000001 | $10^{-12}$ | 12.0 |
| Basic | $\frac{N}{10}$ NaOH | 0.0000000000001 | $10^{-13}$ | 13.0 |
| Basic | $\frac{N}{1}$ NaOH | 0.00000000000001 | $10^{-14}$ | 14.0 |

HCl is replaced by the weak acid phosphate, resulting in a relatively small change in the final hydrogen-ion concentration.

The addition of a strong alkali:

$$NaH_2PO_4 + NaOH \rightarrow Na_2HPO_4 + H_2O$$

The strong alkali (NaOH) reacts with the weak acid ($NaH_2PO_4$) to give the weak alkali ($Na_2HPO_4$) and water. The strong NaOH is replaced by the weak basic phosphate, resulting in a relatively small change in the final hydrogen-ion concentration.

The important salts commonly added to nutrient media for their buffer-

ing action include phosphates and carbonates. These compounds are particularly valuable because they are relatively nontoxic.

Bacteriological peptones contain such substances as proteoses, peptones, peptids, and amino acids, all of which are buffers. These possess both acidic and basic properties, *i.e.*, have the power of uniting with both bases and acids. Therefore, all culture media containing peptone are well buffered, the degree of buffering being dependent upon the amount of peptone added.

TABLE 28.—COLOR CHANGES OF THE IMPORTANT BACTERIOLOGICAL INDICATORS

| Indicator | Concentration recommended* | Full acid color | Full alkaline color | Sensitive range (pH) | pK† |
|---|---|---|---|---|---|
| *m*-Cresol purple (acid range) | 0.04 | Red | Yellow | 1.2– 2.8 | 1.5 |
| Thymol blue (acid range) | 0.04 | Red | Yellow | 1.2– 2.8 | 1.5 |
| Bromophenol blue | 0.04 | Yellow | Blue | 3.0– 4.6 | 4.1 |
| Bromochlorophenol blue | 0.04 | Yellow | Blue | 3.0– 4.6 | 4.0 |
| Bromocresol green | 0.04 | Yellow | Blue | 3.8– 5.4 | 4.7 |
| Chlorocresol green | 0.04 | Yellow | Blue | 4.0– 5.6 | 4.8 |
| Methyl red | 0.02 | Red | Yellow | 4.4– 6.0 | 5.1 |
| Chlorophenol red | 0.04 | Yellow | Red | 4.8– 6.4 | 6.0 |
| Bromophenol red | 0.04 | Yellow | Red | 5.2– 6.8 | 6.2 |
| Bromocresol purple | 0.04 | Yellow | Purple | 5.2– 6.8 | 6.3 |
| Bromothymol blue | 0.04 | Yellow | Blue | 6.0– 7.6 | 7.0 |
| Phenol red | 0.02 | Yellow | Red | 6.8– 8.4 | 7.9 |
| Cresol red | 0.02 | Yellow | Red | 7.2– 8.8 | 8.3 |
| *m*-Cresol purple (alkaline range) | 0.04 | Yellow | Purple | 7.4– 9.0 | 8.3 |
| Thymol blue (alkaline range) | 0.04 | Yellow | Blue | 8.0– 9.6 | 8.9 |
| Cresolphthalein | 0.04 | Colorless | Red | 8.2– 9.8 | 9.4 |
| Phenolphthalein | 0.04 | Colorless | Red | 8.3–10.0 | 9.7 |

* Per cent of dye dissolved in 50 per cent ethyl alcohol.
† The pH at which the dye is 50 per cent dissociated.

Buffers are of special importance in carbohydrate media that are vigorously fermented. In the various fermentations, organic acids are produced. As the concentration of hydrogen ions increases, the pH decreases. Finally a pH is reached where the organisms no longer continue to multiply. Sufficient acid has accumulated to stop the activity of the organisms. In the presence of a buffer, this takes place usually in from 24 to 48 hr. In the absence of a buffer, the activity of the organisms may cease after a few hours. A good culture medium, besides containing the required nutrients, is also well buffered.

**Buffer Standards.**—Clark and Lubs (1917) proposed a series of buffer solutions covering the range from pH1.2 to pH10 at intervals of 0.2 pH (Table 29). By selection of the proper indicators (Table 28), these buffer solutions may be used as standards for the adjustment of the reaction of

culture media. For this purpose, comparable concentrations of indicator must be used in both the buffer standards and the medium under adjustment.

For additional information on the theory and measurement of the hydrogen-ion concentration and on buffers, consult Britton (1929), Clark (1928), Committee on Bacteriological Technic (1945), Grant (1930), Michaelis (1926), and Van Slyke (1922).

TABLE 29.—COMPOSITION OF BUFFERS GIVING pH VALUES AT 20°C.

KCl-HCl mixtures

| pH | *M*/5 KCl, ml. | *M*/5 HCl, ml. | Dilute to, ml. |
|---|---|---|---|
| 1.2 | 50 | 64.5 | 200 |
| 1.4 | 50 | 41.5 | 200 |
| 1.6 | 50 | 26.3 | 200 |
| 1.8 | 50 | 16.6 | 200 |
| 2.0 | 50 | 10.6 | 200 |
| 2.2 | 50 | 6.7 | 200 |

Phthalate-HCl mixtures

| pH | *M*/5 KH phthalate, ml. | *M*/5 HCl, ml. | Dilute to, ml. |
|---|---|---|---|
| 2.2 | 50 | 46.70 | 200 |
| 2.4 | 50 | 39.60 | 200 |
| 2.6 | 50 | 32.95 | 200 |
| 2.8 | 50 | 26.42 | 200 |
| 3.0 | 50 | 20.32 | 200 |
| 3.2 | 50 | 14.70 | 200 |
| 3.4 | 50 | 9.90 | 200 |
| 3.6 | 50 | 5.97 | 200 |
| 3.8 | 50 | 2.63 | 200 |

Phthalate-NaOH mixtures

| pH | *M*/5 KH phthalate, ml. | *M*/5 NaOH, ml. | Dilute to, ml. |
|---|---|---|---|
| 4.0 | 50 | 0.40 | 200 |
| 4.2 | 50 | 3.70 | 200 |
| 4.4 | 50 | 7.50 | 200 |
| 4.6 | 50 | 12.15 | 200 |
| 4.8 | 50 | 17.70 | 200 |
| 5.0 | 50 | 23.85 | 200 |
| 5.2 | 50 | 29.95 | 200 |
| 5.4 | 50 | 35.45 | 200 |
| 5.6 | 50 | 39.85 | 200 |
| 5.8 | 50 | 43.00 | 200 |
| 6.0 | 50 | 45.54 | 200 |
| 6.2 | 50 | 47.00 | 200 |

TABLE 29.—(*Continued*)

$KH_2PO_4$-NaOH mixtures

| pH | *M*/5 $KH_2PO_4$, ml. | *M*/5 NaOH, ml. | Dilute to, ml. |
|---|---|---|---|
| 5.8 | 50 | 3.72 | 200 |
| 6.0 | 50 | 5.70 | 200 |
| 6.2 | 50 | 8.60 | 200 |
| 6.4 | 50 | 12.60 | 200 |
| 6.6 | 50 | 17.80 | 200 |
| 6.8 | 50 | 23.65 | 200 |
| 7.0 | 50 | 29.63 | 200 |
| 7.2 | 50 | 35.00 | 200 |
| 7.4 | 50 | 39.50 | 200 |
| 7.6 | 50 | 42.80 | 200 |
| 7.8 | 50 | 45.20 | 200 |
| 8.0 | 50 | 46.80 | 200 |

Boric acid, KCl, NaOH mixtures

| pH | *M*/5 $H_3BO_3$ *M*/5 KCl, each, ml. | *M*/5 NaOH, ml. | Dilute to, ml. |
|---|---|---|---|
| 7.8 | 50 | 2.61 | 200 |
| 8.0 | 50 | 3.97 | 200 |
| 8.2 | 50 | 5.90 | 200 |
| 8.4 | 50 | 8.50 | 200 |
| 8.6 | 50 | 12.00 | 200 |
| 8.8 | 50 | 16.30 | 200 |
| 9.0 | 50 | 21.30 | 200 |
| 9.2 | 50 | 26.70 | 200 |
| 9.4 | 50 | 32.00 | 200 |
| 9.6 | 50 | 36.85 | 200 |
| 9.8 | 50 | 40.80 | 200 |
| 10.0 | 50 | 43.90 | 200 |

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## CHAPTER XII

## ENZYMES OF BACTERIA

Catalysis may be defined as the acceleration of a reaction produced by the presence of a substance known as a catalyst. The term is compounded from the two Greek words, κατά, down, and λύειν, to loose. It means literally to destroy or to dissolve but has now come to mean the hastening of a chemical reaction.

A catalyst is an agent that accelerates a chemical reaction without itself being destroyed or used up. It may be recovered at the end of the reaction.

An enzyme or ferment may be defined as an organic catalyst, thermolabile and elaborated by a living cell, and capable of functioning independently of the cell.

An enzyme acts by catalysis; *i.e.*, it increases the velocity of a chemical reaction without itself being permanently changed. The enzyme may be recovered in an active condition after the completion of the reaction. There is a close analogy between inorganic catalysts and enzymes. For example, hydrogen peroxide slowly decomposes into water and oxygen, according to the equation:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

In the presence of an inorganic catalyst, such as platinum, or the enzyme catalase, the decomposition of the peroxide is greatly accelerated and ceases only when the destruction of the compound is complete.

Other inorganic catalysts and the industrial processes for which each is used are given in Table 30.

It is usually stated that a catalyst does not initiate a new reaction but merely speeds up one already in progress. There are probably exceptions to this statement. Newer work indicates that a catalyst may initiate a new reaction rather than just cause an acceleration of one already in progress.

TABLE 30.—SOME INDUSTRIAL PROCESSES AND THEIR CATALYSTS

| Catalyst | Process |
|---|---|
| Hydrochloric acid | Hydrolysis of starch to glucose |
| Lime—magnesia mixture | Preparation of acetone from acetic acid |
| Magnetic iron oxide with potassium aluminate | Manufacture of ammonia from hydrogen and nitrogen |
| Nitric oxide | Lead chamber process for manufacture of $H_2SO_4$ |
| Spongy platinum | Contact process for manufacture of $H_2SO_4$ |

## CLASSIFICATION OF ENZYMES

Enzymes form colloidal solutions, dialyze through membranes either very slowly or not at all, and have high molecular weights. Proteins in solution have similar properties.

The great majority of enzymes have not been purified by crystallization but by precipitation and absorption methods. Since the same procedures are also used for the purification of proteins, it is never known when enzymes are free from such compounds. For this reason, enzymes are classified on the basis of what they do rather than of what they are. For example, lactase hydrolyzes lactose to glucose and galactose; arginase hydrolyzes arginine to urea and ornithine; adenase converts adenine to hypoxanthine and ammonia; etc. There are also general names for groups of enzymes, such as esterases, carbohydrases, proteases, dehydrogenases, and oxidases. It is customary to name the enzyme after the substance acted upon and to add the suffix –ase. The first discovered enzymes, such as pepsin, trypsin, and rennin, were not named according to this terminology, but these are exceptions.

The classification of enzymes is a difficult task, owing to the fact that it is never known when a certain preparation under examination contains only a single enzyme. Many preparations that were at one time believed to be composed of a single enzyme have been shown later to consist of more than one enzyme. Evidence that has accumulated during the past few years seems to point to the fact that enzymes now placed into different groups are really the same enzyme acting under slightly different conditions.

As has already been seen, enzymes behave as colloids and have large molecular weights. The water content of bacteria ranges from 73 to 98 per cent. If it is assumed that an average bacterial cell is a cube 1.25 $\mu$ on each side and the diameter of a colloid particle of the order of 100 m$\mu$ (0.1 $\mu$), it is difficult to picture how a cell so small as that of a bacterium could accommodate upwards of 100 different enzymes. The work on bacterial dehydrogenases indicates that it is better to assume the presence of an active group located at or near the cell surface, together with some other factor, such as specific absorption, rather than to the presence of many different enzymes.

Regardless of whether vital reactions are produced by one or many enzymes, a classification is believed to be essential for a clearer understanding of the subject. Table 31 is not complete, but it shows the method followed in naming enzymes. The chemical reactions are included as far as it is possible to do so.

## CRYSTALLINE ENZYMES

About 20 enzymes have been isolated and prepared in pure crystalline form. These pure enzymes give positive tests for proteins, even after re-

TABLE 31.—CLASSIFICATION OF ENZYMES

A. Esterases

The esterases catalyze the reversible reaction

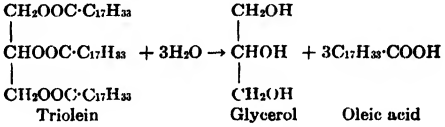
$$R{\cdot}COOR' + H_2O \rightleftharpoons R{\cdot}COOH + R'OH$$

The acid may be a higher or lower fatty acid and R'OH may be glycerol or a simple aliphatic or aromatic alcohol or a carbohydrate.

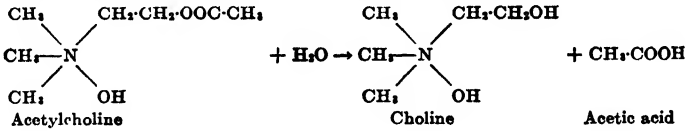
1. Butyrases + lower esters → alcohols + lower fatty acids

$$\underset{\text{Ethyl acetate}}{CH_3{\cdot}COO{\cdot}C_2H_5} + H_2O \rightarrow \underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + \underset{\text{Alcohol}}{C_2H_5OH}$$

2. Glyceridases + glycerides → glycerol + higher fatty acids




3. Cholinesterase + acetylcholine → choline + acetic acid




4. Cholesterol esterase + cholesterol esters → cholesterol + R·COOH

5. Chlorophyllase + chlorophyll A + alcohol → ethyl chlorophyllide + phytol

$$\underset{\text{Chlorophyll A}}{C_{32}H_{30}N_4MgO(COOCH_3){\cdot}COO{\cdot}C_{20}H_{39}} + \underset{\text{Alcohol}}{C_2H_5OH} \longrightarrow$$

$$\underset{\text{Ethyl chlorophyllide}}{C_{32}H_{30}N_4MgO(COOCH_3){\cdot}COOC_2H_5} + \underset{\text{Phytol}}{C_{20}H_{39}OH}$$

6. Lecithinases

*a.* Lecithinase A + lecithin → lysolecithin + oleic acid

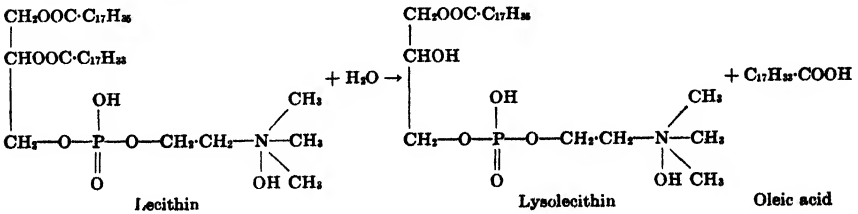
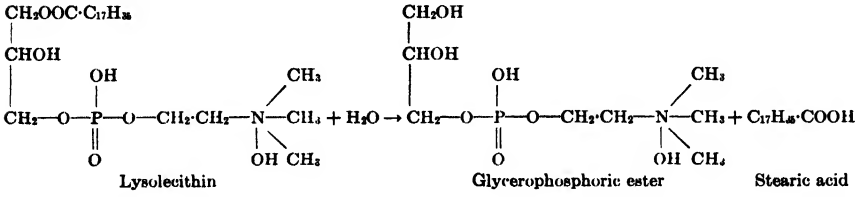
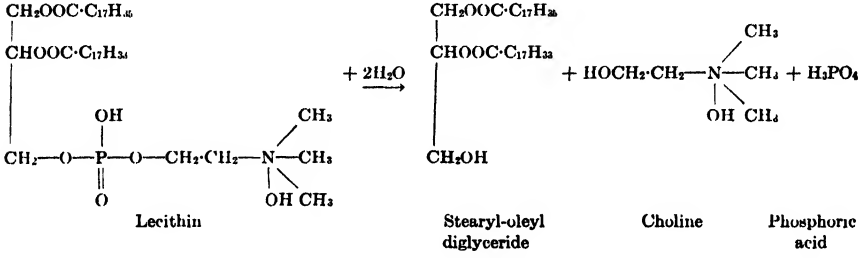


TABLE 31.—(*Continued*)

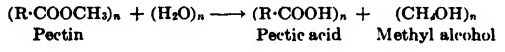
*b.* Lecithinase B + lysolecithin → glycerophosphoric ester + stearic acid




*c.* Lecithinase + lecithin → fat + choline + phosphoric acid



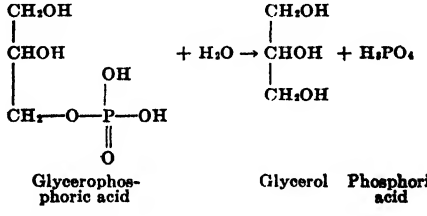

7. Pectase + pectin → pectic acid + methyl alcohol




8. Phosphatases

*a.* The phosphomonoesterases consist of a cophosphatase (coenzyme) and an apophosphatase (carrier). The coenzyme of one enzyme may be combined with the carrier of another to give two new enzymes.

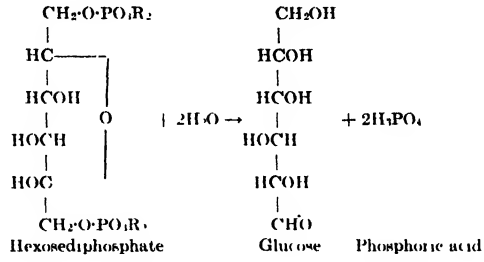
Phosphomono- esterases + monoesters of *o*-phosphoric acid → alcohol + phosphoric acid



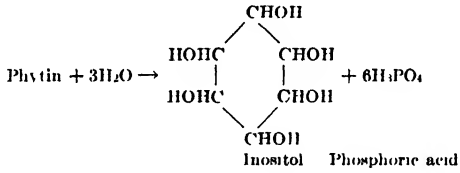

*b.* Phosphodiesterase + diesters of phosphoric acid → alcohol + monoester

TABLE 31.—(*Continued*)

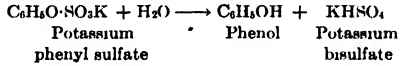
*c.* Hexosediphos- + hexosediphosphoric → hexose + phosphoric acid
phatase acid



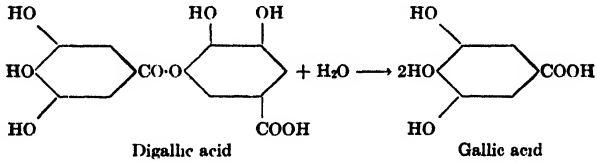

*d.* Phytase + phytin → inositol + phosphoric acid




9. Sulfatases + ethereal sulfates → phenols + potassium bisulfate




10. Tannase + tannins (esters of polyhydroxy phenols → gallic acid
with aromatic acids)

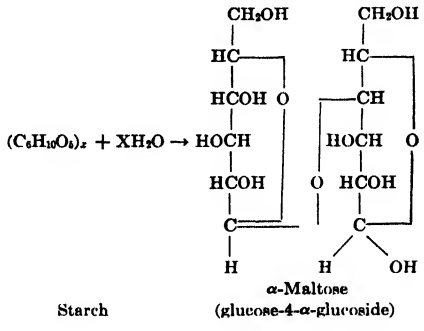



B. Carbohydrases

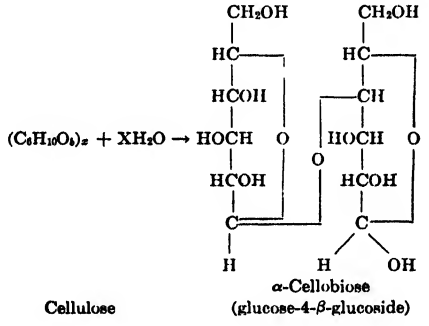
The carbohydrases catalyze the hydrolysis of carbohydrates to compounds that are simpler and generally more soluble.

TABLE 31.–(*Continued*)

1. $\alpha$-Amylase ($\alpha$-diastase) + starch → $\alpha$-maltose




2. Cellulase + cellulose → cellobiose




3. Cytase + hemicelluloses → simple sugars having formula $C_6H_{12}O_6$

4. Lichenase + lichenin → glucose

5. Inulase + inulin → *d*-fructose (levulose)

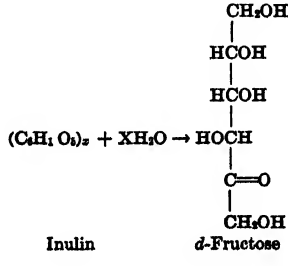
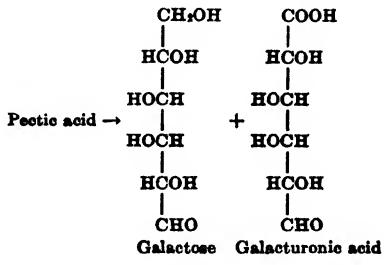
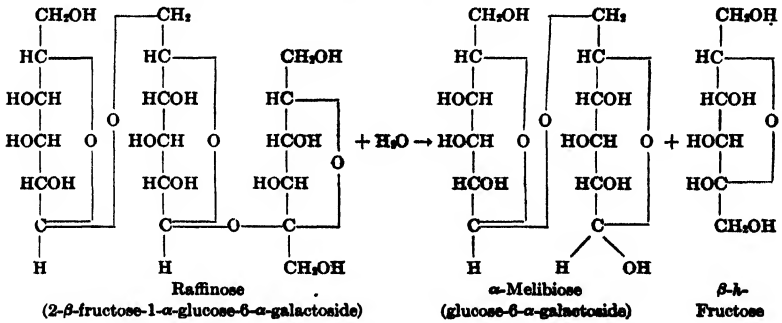


TABLE 31.—(*Continued*)

6. Pectinase + pectic acid → galactose + galacturonic acid




7. Raffinase + raffinose → $\alpha$-melibiose + $\beta$-*h*-fructose




8. Gentianase + gentianose → $\alpha$-gentiobiose + $\beta$-*h*-fructose

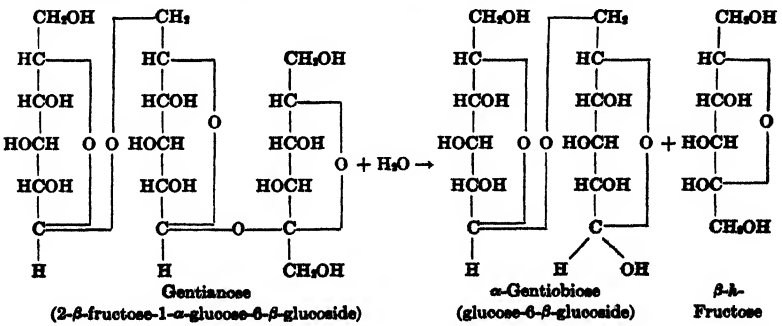
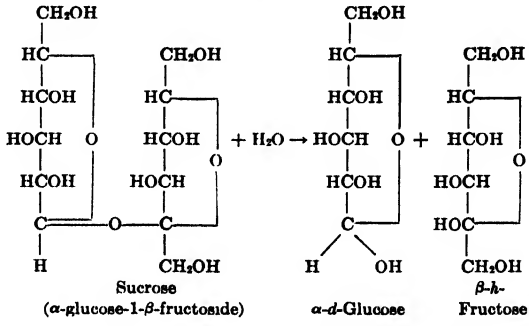
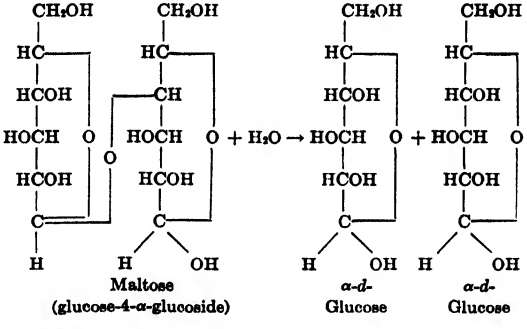


TABLE 31.—(*Continued*)

9. *β-h*-Fructosidase (sucrase) + sucrose → *α-d*-glucose + *β-h*-fructose




10. Maltase + α-maltose → *α-d*-glucose + *α-d*-glucose




11. Trehalase + trehalose → *α-d*-glucose + *α-d*-glucose

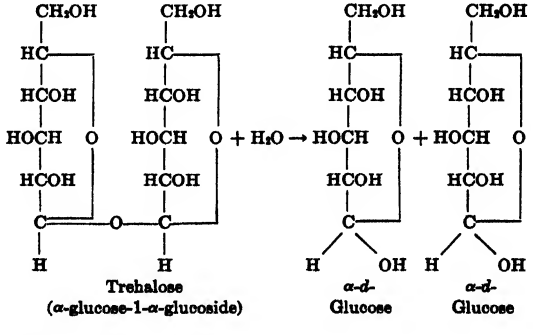
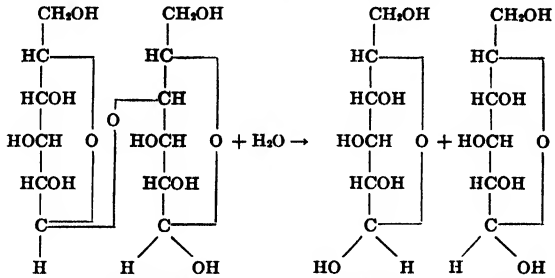
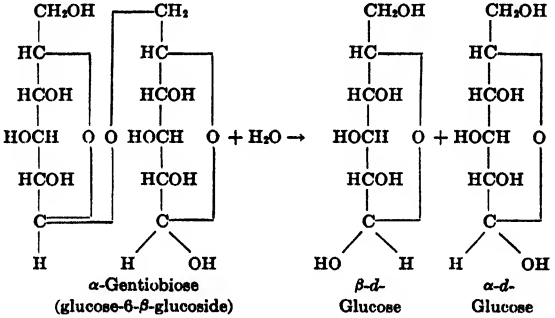


TABLE 31.—(*Continued*)

12. Cellobiase + α-cellobiose → β-*d*-glucose + α-*d*-glucose




13. Gentiobiase + α-gentiobiose → β-*d*-glucose + α-*d*-glucose




14. β-Galactosidase (lactase) + α-lactose → β-*d*-galactose + α-*d*-glucose

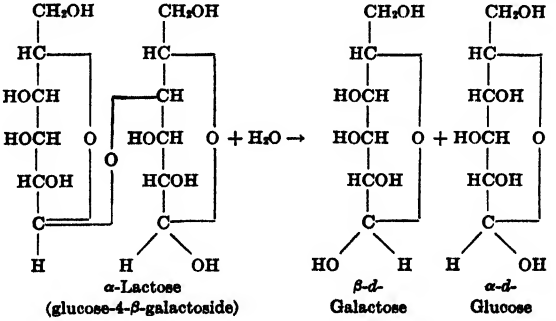
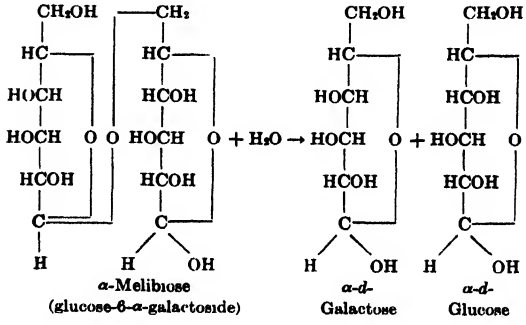
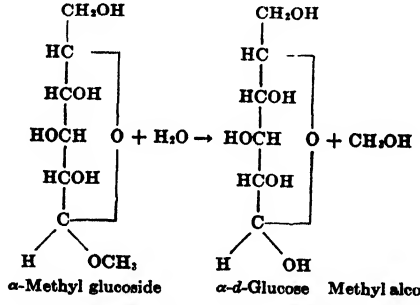


TABLE 31.—(*Continued*)

15. Melibiase + α-melibiose → α-*d*-galactose + α-*d*-glucose




16. α-Glucosidase + α-glucosides → α-*d*-glucose + alcohol or phenol residue




17. β-Glucosidase + β-glucosides → β-*d*-glucose + alcohol or phenol residue

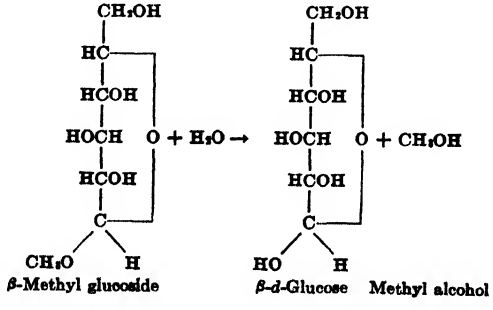
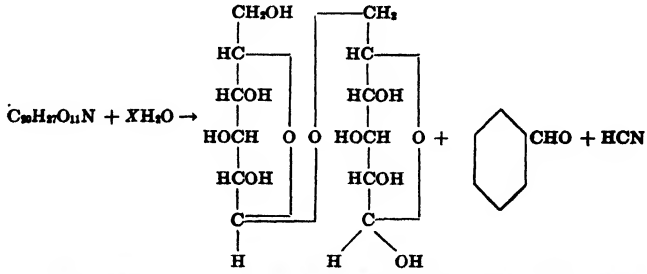
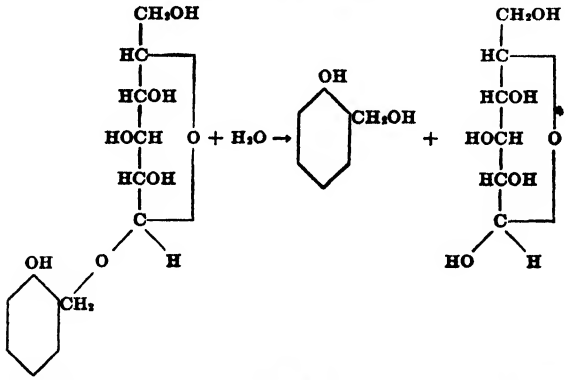


TABLE 31.—(*Continued*)

18. Amygdalase + amygdalin → $\alpha$-gentiobiose + benzaldehyde + hydrocyanic acid




19. Emulsin + salicin → saligenin + $\beta$-*d*-glucose




C. Enzymes of carbohydrate metabolism

1. Phosphorylase + glycogen + $H_3PO_4 \rightleftharpoons$ glucose-1-phosphate (Cori ester)

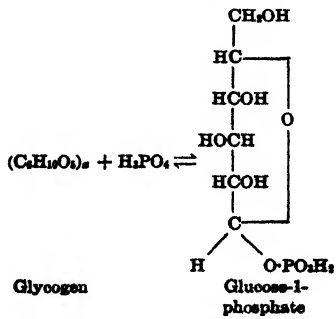
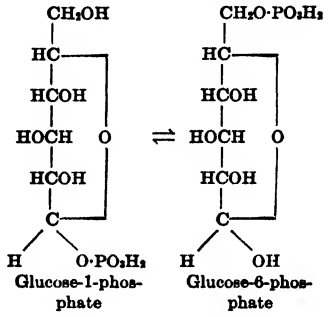


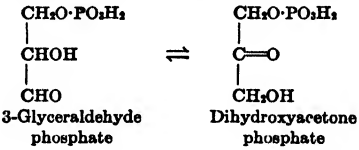
TABLE 31.—(*Continued*)

2. Phosphoglucomutase + glucose-1-phosphate ⇌ glucose-6-phosphate

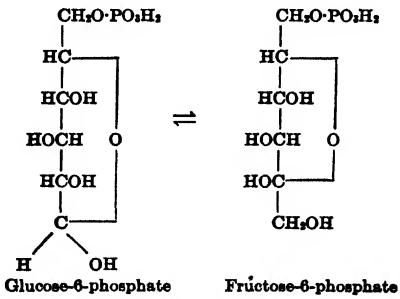



3. Hexokinase + adenosine triphosphate + 2 glucose → adenylic acid + 2 glucose-6-phosphate
(see page 370) (see page 285)

4. Isomerase + 3-glyceraldehyde phosphate ⇌ dihydroxyacetone phosphate




5. Phosphohexoisomerase + glucose-6-phosphate ⇌ fructose-6-phosphate




6. Phosphoglyceromutase + 3-phosphoglyceric acid ⇌ 2-phosphoglyceric acid

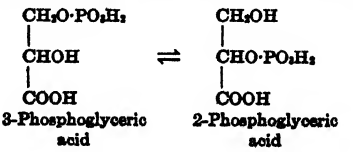
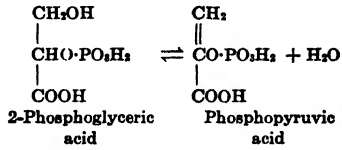


TABLE 31.—(*Continued*)

7. Enolase + 2-phosphoglyceric acid ⇌ phosphopyruvic acid



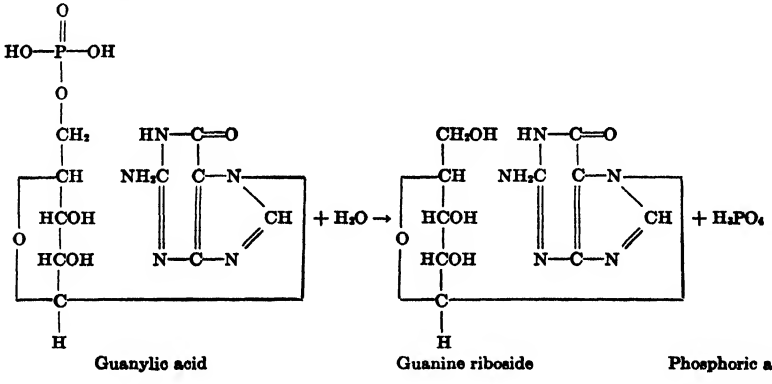

D. Nucleases

The nucleases attack nucleic acids or their decomposition products.

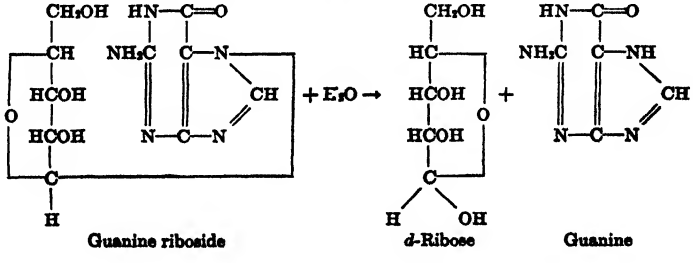
1. Ribonuclease + ribonucleic acid → mononucleotides (see page 587)

2. Desoxyribonuclease + desoxyribonucleic acid → mononucleotides (see page 588)

3. Nucleotidases + nucleotides → nucleosides + phosphoric acid




4. Nucleosidases + nucleosides → sugars + purine bases

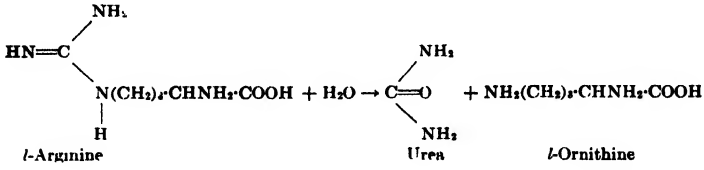



E. Enzymes hydrolyzing nitrogen compounds

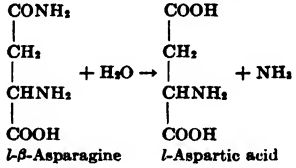
TABLE 31.—(*Continued*)

1. Amidases

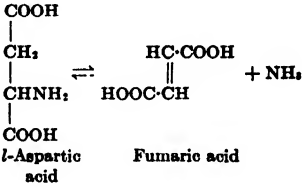
*a.* Arginase + *l*-arginine → urea + *l*-ornithine



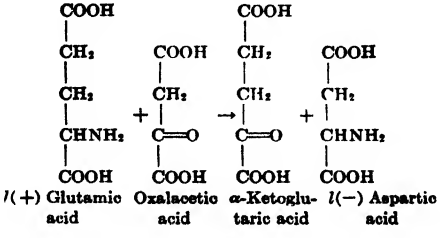

*b.* Asparaginase + *l*-β-asparagine → *l*-aspartic acid + $NH_3$




*c.* Aspartase + *l*-aspartic acid ⇌ fumaric acid + $NH_3$




*d.* Transaminase + glutamic acid → amino group transferred to some reactive carbohydrate residue




*e.* Urease + urea → $NH_3 + CO_2$

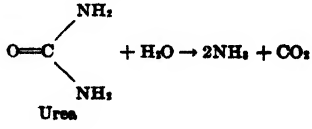
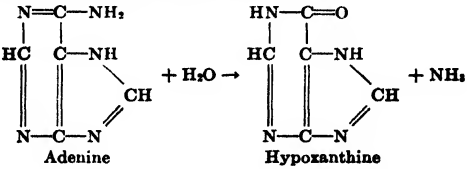


TABLE 31.—(*Continued*)

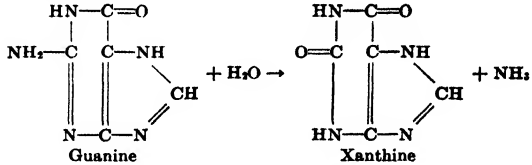
2. Nuclein desaminases

The nuclein desaminases produce a deamination of purines and pyrimidines, either free or combined.

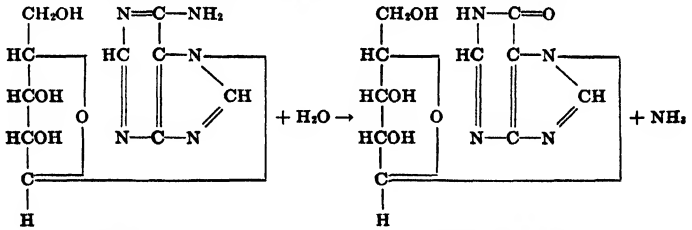
*a.* Adenase + adenine → hypoxanthine + $NH_3$




*b.* Guanase + guanine → xanthine + $NH_3$




*c.* Adenosine desaminase + adenosine → hypoxanthosine + $NH_3$




*d.* Adenylic acid desaminase + adenylic acid → hypoxanthylic acid + $NH_3$

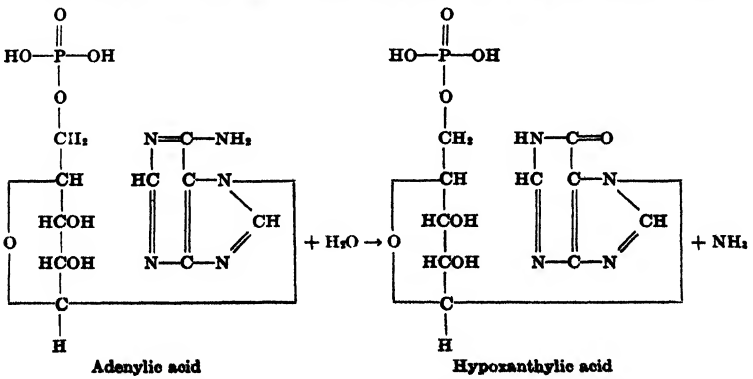
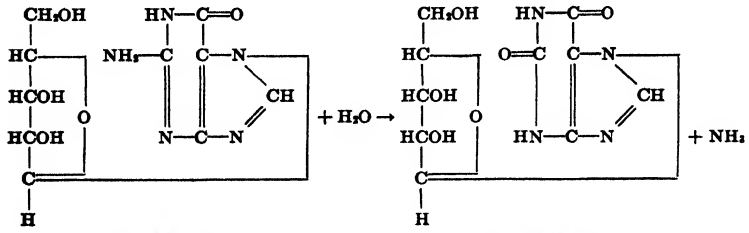


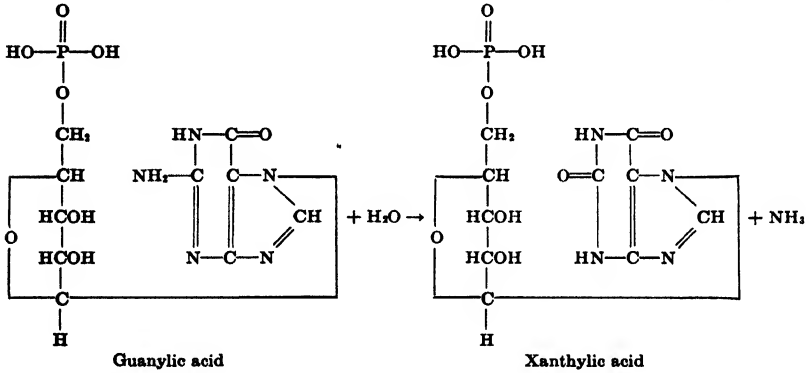
TABLE 31.—(*Continued*)

*e.* Guanosine desaminase + guanosine → xanthosine + $NH_3$



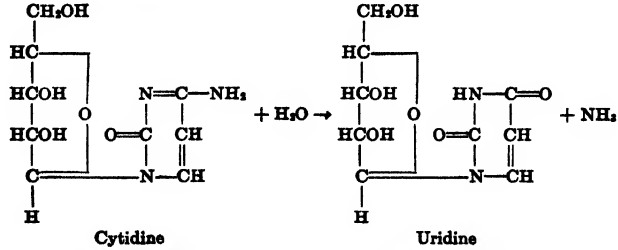

Guanosine (guanine riboside) — Xanthosine (xanthine riboside)

*f.* Guanylic acid desaminase + guanylic acid → xanthylic acid + $NH_3$




Guanylic acid — Xanthylic acid

*g.* Cytidine desaminase + cytidine → uridine + $NH_3$




Cytidine (cytosine riboside) — Uridine (uracil riboside)

3. Proteinases

The proteinases attack the true proteins.

*a.* Pepsin + native proteins → proteoses + peptones

TABLE 31.—(*Continued*)

*b.* Rennin + casein → paracasein

*c.* Trypsin + native proteins, proteoses, peptones, and peptids → polypeptids and amino acids

*d.* Erepsin + proteoses, peptones, and polypeptids → amino acids

*e.* Papain + native proteins → polypeptids and dipeptids

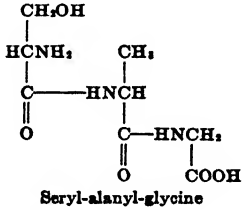
*f.* Bromelin + native proteins → polypeptids and dipeptids

*g.* Protaminase + protamines with terminal arginine group → arginine + residue

4. Peptidases

The peptidases hydrolyze polypeptids and dipeptids to the stage of amino acids.

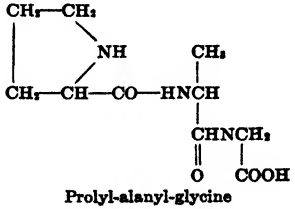
*a.* Aminopolypeptidase attacks amino acid on the amino end of a polypeptid chain.



Seryl-alanyl-glycine

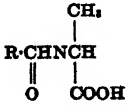
The amino acid serine is liberated on hydrolysis.

*b.* Prolinase attacks proline on the imino end of peptid chain.



Prolyl-alanyl-glycine

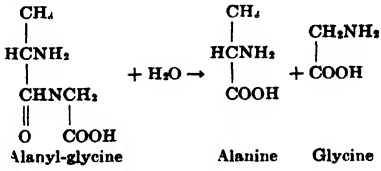
*c.* Carboxypeptidase attacks polypeptids having free COOH groups.



The amino acid alanine is liberated on hydrolysis.

TABLE 31.—(*Continued*)

*d.* Dipeptidase + dipeptids → amino acids




Alanyl-glycine Alanine Glycine

F. Enzymes involved in oxidation-reduction reactions

1. Oxidases

The true oxidases function by activating oxygen so that it will quickly oxidize slowly autooxidizable compounds. They are metalloproteins being inhibited by hydrogen sulfide and potassium cyanide. They do not form hydrogen peroxide as a result of the reduction of oxygen. Oxidases are active only under aerobic conditions.

The iron oxidases

These enzymes are combined with prosthetic groups containing iron. The prosthetic groups are either hematin, or substances closely related to hematin. Catalase is not a true oxidase but is included here for convenience.

*a.* Catalase + hydrogen peroxide → water + molecular oxygen

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Hydrogen peroxide

*b.* Peroxidase + hydrogen peroxide → water + atomic oxygen

$$\text{Peroxidase} + H_2O_2 \rightarrow \text{peroxidase}\cdot H_2O_2$$
$$\text{Peroxidase}\cdot H_2O_2 + RH_2 \rightarrow \text{peroxidase} + R + 2H_2O$$

Enzyme-peroxide compound — Oxidizable substance — Oxidized substance

*c.* Cytochrome oxidase + cytochrome c·H + $\frac{1}{4}O_2$ → cytochrome c + $\frac{1}{2}H_2O$

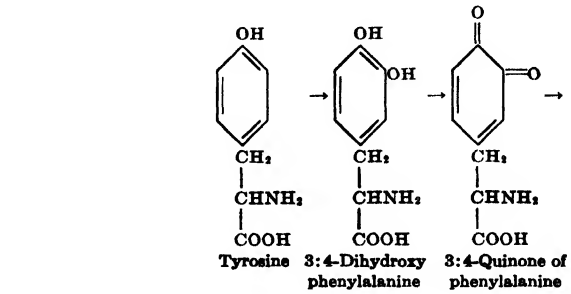
(1) Cytochrome c·H + $\frac{1}{4}O_2$ → cytochrome c + $\frac{1}{2}H_2O$
(2) Cytochrome c + H → cytochrome c·H

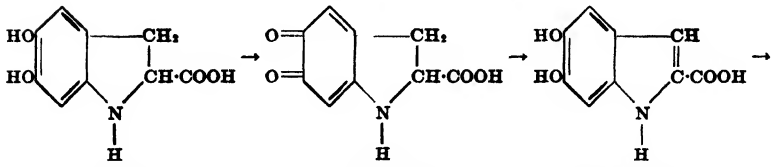
The copper oxidases

These enzymes contain copper instead of iron in their molecules.

*a.* Tyrosinase (polyphenol oxidase) + tyrosine → melanin (black pigment)

TABLE 31.—(*Continued*)

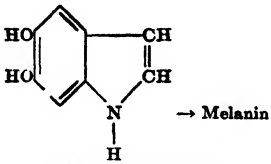



5:6-Dihydroxy-dihydroindole-2-carboxylic acid

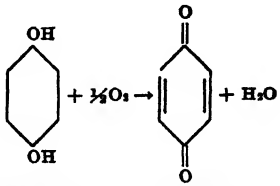
Hallochrome

5:6-Dihydroxyindole carboxylic acid




5:6-Dihydroxyindole

*b.* Laccase + phenols → ortho- and para-quinones

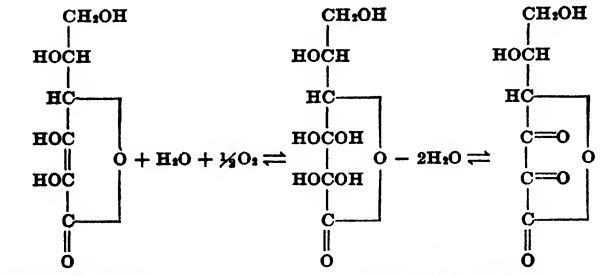



*p*-Dihydroxy benzene

Quinone

TABLE 31.—(*Continued*)

*c.* Ascorbic acid oxidase + *l*-ascorbic acid → oxidized ascorbic acid




Miscellaneous oxidases

*a.* Luciferase + luciferin + $O_2$ → oxyluciferin + light

(1) Oxyluciferin + 2H (from some donator) → luciferin·2H
(2) Luciferin·2H + luciferase → oxyluciferin + luciferase·2H
(3) Luciferase·2H + $O_2$ → luciferase + $H_2O_2$ + light

*b.* Dopa oxidase + *l*-3, 4-dihydroxyphenylalanine → melanin (see under Tyrosine)

2. Dehydrogenases

The dehydrogenases are responsible for the activation of the hydrogen of metabolites so that they can be oxidized in the presence of oxygen or a suitable reducible substance. They convert nonreducing substances into reducing substances. The aerobic dehydrogenases require neither coenzyme nor cytochrome systems. The anaerobic dehydrogenases catalyze reactions between metabolites and carriers.

The aerobic dehydrogenases

*a.* Tyramine dehydrogenase + tyramine + $O_2$ → parahydroxyphenyl acetaldehyde + $NH_3$ + $H_2O_2$

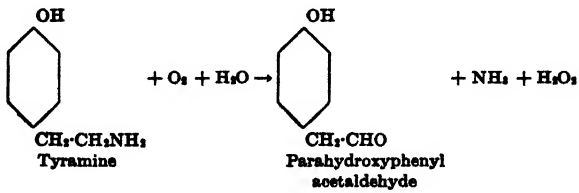
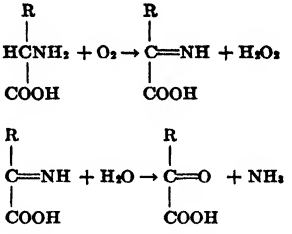
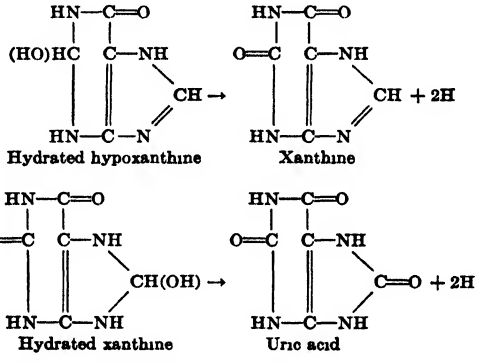


TABLE 31.—(*Continued*)

*b.* *d*-Amino acid dehydrogenase + *d*-amino acids → α-keto acids



*c.* Xanthine dehydrogenase + xanthine and hypoxanthine → uric acid




The anaerobic dehydrogenases

Coenzyme 1-linked

*a.* Alcohol dehydrogenase + alcohols → aldehydes

$$R\cdot CH_2OH \rightarrow R\cdot CHO + 2H$$

Alcohol — Aldehyde

*b.* β-Hydroxybutyric + *l*-β-hydroxybutyric → acetoacetic
dehydrogenase — acid — acid

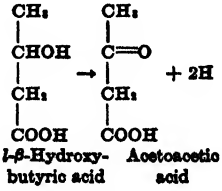
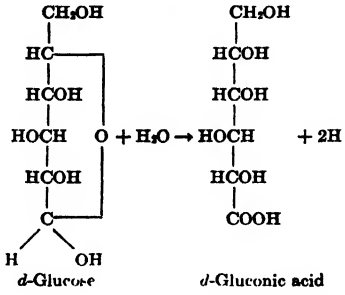


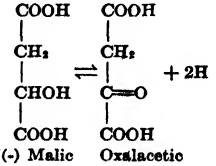
TABLE 31.—(*Continued*)

*c.* Glucose dehydrogenase + *d*-glucose → *d*-gluconic acid



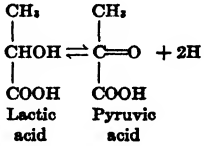

*d*-Glucose — *d*-Gluconic acid

*d.* Malic dehydrogenase + *l*(-)malic acid ⇌ oxalacetic acid



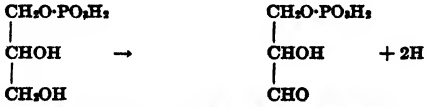

*l*(-) Malic acid — Oxalacetic acid

*e.* Lactic dehydrogenase + lactic acid ⇌ pyruvic acid




Lactic acid — Pyruvic acid

*f.* α-Glycerophosphoric dehydrogenase + α-glycerophosphoric acid → 3-phosphoglyceric aldehyde




α-Glycerophosphoric acid — 3-Phosphoglyceric aldehyde

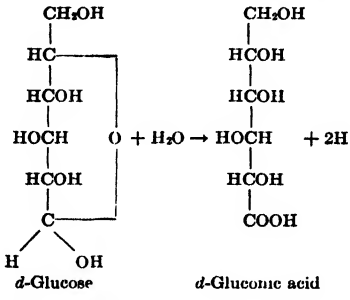
*g.* 1, 3-Diphosphoglyceric aldehyde dehydrogenase + 3-phosphoglyceric aldehyde → 3-phosphoglyceric acid

(1) 3-Phosphoglyceric aldehyde + $H_3PO_4$ ⇌ 1,3-diphosphoglyceric aldehyde
(2) 1,3-Diphosphoglyceric aldehyde + coenzyme 1 ⇌ 1,3-diphosphoglyceric acid + reduced coenzyme 1
(3) 1,3-Diphosphoglyceric acid + adenosine diphosphate ⇌ 3-phosphoglyceric acid + adenosine triphosphate

TABLE 31.—(*Continued*)

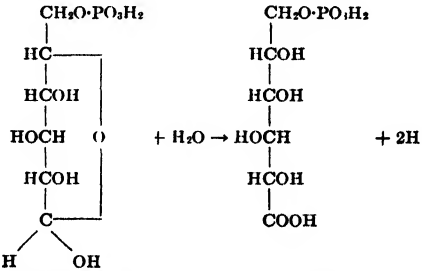
Coenzyme 2-linked

*a.* Glucose dehydrogenase + *d*-glucose → *d*-gluconic acid




*d*-Glucose *d*-Gluconic acid

*b.* Robison ester + hexose-6-phosphate → phosphohexonic acid dehydrogenase

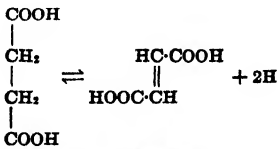



α-*d*-Glucose-6-phosphate *d*-Phospho-gluconic acid

Cytochrome c-linked

*a.* Succinic dehydrogenase + succinic acid → fumaric acid

(1)



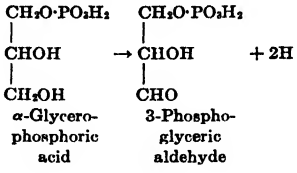

Succinic acid Fumaric acid

(2) 2 Cytochrome c + 2H ⇌ 2 cytochrome c·H (reduced)

(3) 2 Cytochrome c·H + ½$O_2$ + cytochrome oxidase → 2 cytochrome c + $H_2O$

TABLE 31.—(*Continued*)

*b.* α-Glycerophosphoric dehydrogenase + α-glycerophosphoric acid → 3-phosphoglyceric aldehyde + 2H




3. The yellow enzymes

These are carriers capable of being reversibly oxidized and reduced.

They are composed of specific proteins (apoenzymes) united with flavins (coenzymes).

*a.* The old yellow enzyme

(riboflavin phosphate protein) + coenzyme 2·2H + $O_2$ → $H_2O_2$ + coenzyme 2

(1) Hexosemonophosphate + coenzyme 2 → phosphohexonic acid + coenzyme 2·2H
(2) Coenzyme 2·2H + yellow enzyme → coenzyme 2 + yellow enzyme·2H
(3) Yellow enzyme·2H + $O_2$ → yellow enzyme + $H_2O_2$

*b.* Diaphorases + coenzyme 1·2H or coenzyme 2·2H + suitable carriers + $O_2$ → coenzyme 1 or coenzyme 2 + $H_2O_2$

(1) Coenzyme 1·2H + diaphorase → coenzyme 1 + diaphorase·2H
(2) Diaphorase·2H + methylene blue → diaphorase + leuco-methylene blue
(3) Leuco-methylene blue + $O_2$ → methylene blue + $H_2O_2$

*c.* Cytochrome c reductase + cytochrome c + coenzyme 2·2H → cytochrome c·2H + coenzyme 2

(1) Coenzyme 2·2H + cytochrome c reductase → coenzyme 2 + cytochrome c reductase·2H
(2) Cytochrome c reductase·2H + 2 cytochrome c → cytochrome c reductase + 2 cytochrome c·H

G. Desmolases

The desmolases break or form carbon chains.

1. Aldolase + fructose-1, 6-diphosphate → dihydroxyacetone phosphate + phosphoglyceric aldehyde

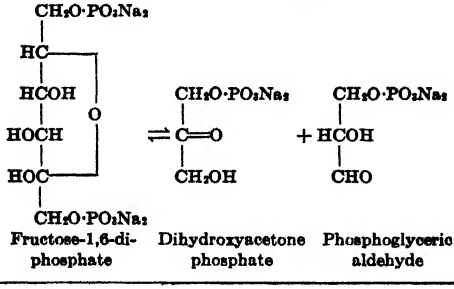
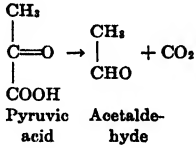
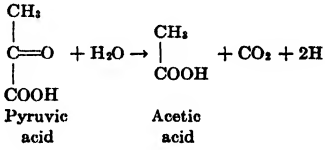


TABLE 31.—(*Continued*)

2. Carboxylase + cocarboxylase + pyruvic acid → acetaldehyde + $CO_2$



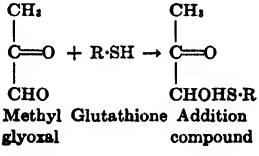

3. Pyruvic dehydrogenase + cocarboxylase + pyruvic acid → acetic acid + $CO_2$

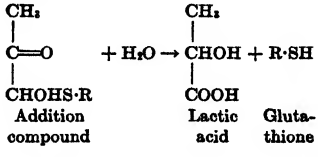



H. Hydrases

The hydrases add water to organic compounds without causing a hydrolysis.

1. Glyoxalase + coglyoxalase + methyl glyoxal → lactic acid (glutathione)




I. Mutases

The mutases bring about the Cannizzaro reaction. One molecule of an aldehyde is oxidized to an acid, and another molecule is reduced to an alcohol.

1. Aldehyde mutase + aldehyde → acetic acid + alcohol

$$\underset{\text{Acetaldehyde}}{2CH_3{\cdot}CHO} + H_2O \rightarrow \underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + \underset{\text{Ethyl alcohol}}{CH_3{\cdot}CH_2OH}$$

peated recrystallizations, indicating that they are pure preparations free from the presence of any foreign protein.

The crystalline enzymes, arranged in chronological order, include urease (1926), pepsin (1930), trypsin (1931), chymotrypsin (1933), yellow enzyme (1934), carboxypeptidase (1935), beef liver catalase (1937), alcohol dehydrogenase (1937), papain (1937), lysozyme (1937), tyrosinase (1938), lecithinase (1938), glyceric aldehyde diphosphate dehydrogenase (1939), ribonuclease (1939), ascorbic acid oxidase (1939), lactic dehydrogenase (1940), beef erythrocyte catalase (1941), chymopapain (1941), carbonic anhydrase (1941), and animal phosphorylase (1942).

Photomicrographs of crystalline preparations of trypsin, pepsin, and chymotrypsin are shown in Figs. 135 to 137.

## FACTORS THAT INFLUENCE ENZYME ACTIONS

Enzyme actions are greatly influenced by a number of factors including temperature, pH, ultraviolet light, activators, inhibitors, concentration of enzyme, and concentration of substrate. The same factors that influence the growth of microorganisms also affect the action of enzymes. In general, enzymes are more resistant to unfavorable environmental conditions than the cells producing them. For example, if dried yeast is heated at 100°C. for 6 hr. it loses the power of growth but still retains the power of fermenting sugar to alcohol and carbon dioxide.

**Effect of Temperature.**—The velocity of the reaction of an enzyme on its substrate is accelerated by an increase in temperature. This continues until a maximum is reached after which the speed gradually decreases, resulting finally in the destruction of the enzyme. In general, the velocity of enzyme reactions is doubled for each 10°C. rise in temperature.

Each enzyme has its own characteristic optimum temperature. That temperature above which an enzyme no longer shows any activity is known as the maximum temperature; that temperature below which it cannot function is known as the minimum temperature. The optimum, maximum, and minimum temperatures are influenced by several factors such as concentration of enzyme, nature and concentration of substrate upon which the enzyme acts, the hydrogen-ion concentration of the medium, and the presence of activating and paralyzing substances.

Most enzymes in solution are more or less stable at temperatures below 45°C., but above 50°C. inactivation rapidly increases with a rise in temperature. The majority of enzymes are destroyed at temperatures of 70 to 80°C. On the other hand, enzymes in the dried state are more resistant to high temperatures than the same enzymes in solution. For example, dried rennin is only slowly destroyed at 158°C.

**Effect of pH.**—Enzymes are greatly influenced by the hydrogen-ion concentration of the solution. Some enzymes act best in a neutral solution;

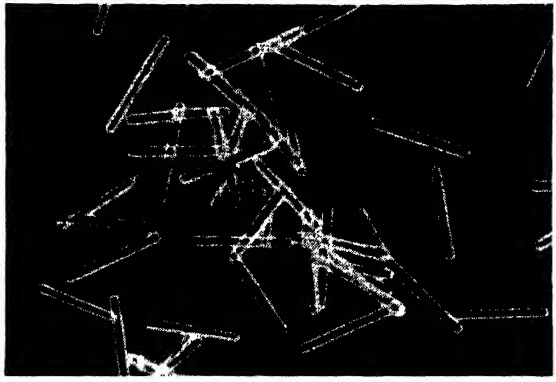


FIG. 135.—Trypsin crystals. (*From Northrop, "Crystalline Enzymes," Columbia University Press.*)

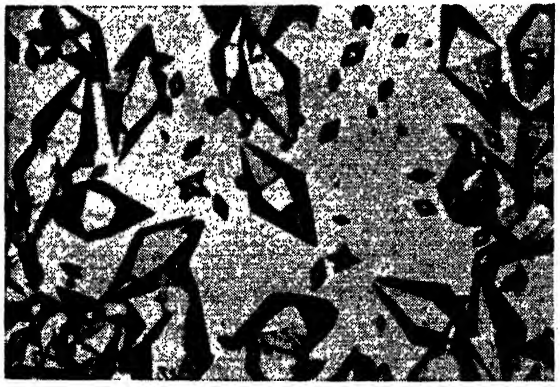


FIG. 136.—Pepsin crystals. (*From Northrop, "Crystalline Enzymes," Columbia University Press.*)

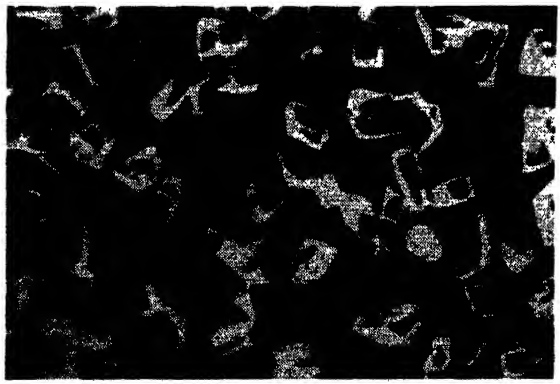


FIG. 137.—Chymotrypsin crystals. (*From Northrop, "Crystalline Enzymes," Columbia University Press.*)

others prefer alkaline solutions; still others do not function unless their solutions are acid in reaction. There exists for every enzyme a maximum, an optimum, and a minimum hydrogen-ion concentration under certain specified conditions. The optimum pH of an enzyme varies with the substrate, the source of the enzyme, and the type of buffer used.

**Effect of Ultraviolet Light.**—Ultraviolet light either destroys or modifies the action of enzymes. A destructive action usually occurs only in the presence of oxygen, although some exceptions have been reported. The rate of destruction is practically independent of temperature but is affected by pH and other environmental conditions.

Purified enzymes are more easily destroyed by light than the same enzymes in less purified preparations. Impurities from the culture media, especially proteins, may afford considerable protection. In general, the greater the degree of purification of an enzyme, the more susceptible it becomes to the toxic light rays.

The addition of small amounts of fluorescent substances, such as eosin, to enzyme solutions increases the toxic effect of the light rays. This is discussed in greater detail on page 218.

**Activators and Inhibitors.**—Some substances increase the activity of enzymes whereas others produce the reverse effect. The former are spoken of as "activators"; the latter are called "inhibitors." These substances may be either specific or nonspecific. The specific activators may be more appropriately referred to as coenzymes and are discussed on page 321.

*Pepsins.*—These enzymes are active only in acid solutions. The inactive form is known as pepsinogen or propepsin and becomes activated on the addition of hydrogen ions. The greatest activity occurs at a pH of about 1.6.

An active pepsin may be inhibited by the addition of hydroxyl ions (alkalies). If the solution is adjusted to a pH higher than 6.8, the enzyme is destroyed.

*Trypsins.*—The trypsins have an optimum pH range of 8 to 9. Inactive trypsin or trypsinogen is usually activated by a substance known as enterokinase. Northrop (1932*a*, *b*) and Kleiner and Tauber (1934) reported the isolation of trypsin from pancreatic tissue which does not require enterokinase for activity. Kunitz and Northrop (1933) isolated a crystalline trypsin from beef pancreas which was inactive both in the presence or absence of enterokinase.

*Papainases.*—The two best known representatives are cathepsin and papain. The former is found in animal cells; the latter is present in the cells of many plants, especially in papaya, the fruit of the melon tree. The optimum pH range of these proteinases is from 4 to 7.

A sulfhydryl group is believed to be an essential part of the active enzymes. Oxidation renders the enzymes "inactive" and reduction re-

stores their activity. Activation can be effected by hydrogen sulfide, cysteine, glutathione, or HCN.

This may be represented as follows:

$$\underset{\text{Inactive papain}}{PSSP} + \underset{\text{Reducing agent}}{2GSH} \rightarrow \underset{\text{Active papain}}{2PSH} + GSSG$$

*Catalase.*—This enzyme decomposes hydrogen peroxide into water and oxygen, and the oxygen may act as an acceptor of hydrogen in dehydrogenation reactions. Catalase is unstable in acid solutions being destroyed at pH3.0. It is inactivated by hydrogen sulfide, hydroxylamine, sodium azide, and hydrocyanic acid, but not affected by carbon monoxide, carbon dioxide, cysteine, and glutathione.

The enzyme is readily digested and destroyed by tryptic digestion.

*Urease.*—This enzyme hydrolyzes urea to ammonia and carbon dioxide. In the absence of buffers, these unite to form ammonium carbamate; in the presence of buffers, the products are ammonium salts and carbonic acid.

Jacoby (1933) found the enzyme to be inactivated by cupric chloride, mercuric chloride, and silver nitrate. Sumner and Poland (1933) believed that the destructive action of metal salts was due to the presence of sulfhydryl groups (–SH) in the urease molecule. Urease inactivated by heavy metals may be reactivated by the addition of hydrogen sulfide. Urease is also inactivated by fluorides, halogens, borates, quinones, formaldehyde, and hydrogen peroxide. Quastel (1932) found basic dyes to inhibit the action of urease. On the other hand, acid dyes were without effect. Urea, amino acids, methylamine, and hydroxylamine protected the enzyme against the inhibiting action of basic dyes.

The enzyme acts best at a pH of about 7.0.

*Inactivation of Enzymes by Proteases.*—In general, proteolytic enzymes produce an inhibitory or destructive action on other enzymes. This would indicate that those enzymes which are destroyed are proteins or are associated with colloidal carriers that are proteins. Sumner, Kirk, and Howell (1932) reported that urease was rapidly digested and inactivated by pepsin and by papain—$H_2S$ at pH4.3. Tauber and Kleiner (1933) found maltase to be readily destroyed by trypsin. The same workers (1932) stated that rennin was rapidly digested by both pepsin and trypsin. Tauber and Kleiner (1934) and Northrop and Kunitz (1932) found that trypsin was digested by pepsin at pH2.0, but at pH5.5 pepsin was digested by trypsin.

These are a few of the many observations that have been reported. In general, it may be concluded that those enzymes which are digested by proteases are probably proteins.

For more information, see Northrop (1937), Sumner and Somers (1947), and Tauber (1935).

**Effect of Concentration of Substrate and Enzyme.**—An increase in the concentration of substrate may increase or decrease the velocity of an enzymatic reaction. If the substrate concentration is small in proportion to the amount of enzyme, an increase in the substrate content will increase the velocity of the reaction. For example, sucrose is hydrolyzed to glucose and levulose by the enzyme sucrase. The velocity of hydrolysis is increased by an increase in the sucrose concentration up to 4 or 5 per cent. Above this concentration, the relative amount of sugar hydrolyzed becomes progressively less as the sugar concentration is increased. It may be concluded that a strong solution of cane sugar diluted to a concentration of about 5 per cent will be more efficiently hydrolyzed by sucrase than a more concentrated solution.

The time required for sucrase to hydrolyze cane sugar is proportional to the concentration of enzyme. In general, this is true for all enzymes when the amount of enzyme is much smaller than the concentration of substrate so that all of the enzyme can combine with the latter. When an excess of enzyme is used, however, the velocity appears to be proportional to the square root of the enzyme concentration.

## SYNTHETIC ACTIVITIES OF ENZYMES

A great number of chemical reactions are termed reversible reactions. The characteristics of a reaction of this type are that it progresses in one direction or the other until an equilibrium specific to the reaction and to the concentration of the reacting materials is established. Any change in concentration of the reacting components is immediately followed by a change to a new equilibrium.

Many enzymatic reactions are known to be of the above type. In a series of investigations, Doudoroff, Kaplan, and Hassid (1943), Doudoroff (1943, 1945), Hassid, Doudoroff, and Barker (1944), and Doudoroff, Hassid, and Barker (1944) found that a dry preparation of the organism *Pseudomonas saccharophila* was capable of phosphorolyzing sucrose to glucose-1-phosphate and fructose. With glucose-1-phosphate and fructose as substrates, the formation of sucrose was demonstrated by the reversal of the reaction. The competing hydrolytic and phosphorolytic properties of the enzyme were summarized as follows:

$$\begin{array}{l} \text{Sucrose} + H_3PO_4 \rightleftharpoons \text{glucose-1-phosphate} + \text{fructose} \\ \quad + \\ \quad H_2O \\ \quad \downarrow \\ \text{Glucose} + \text{fructose} \end{array}$$

In a later report Hassid, Doudoroff, Barker, and Dore (1945) showed that the enzyme that synthesized sucrose from glucose-1-phosphate also combined glucose-1-phosphate with 1-sorbose or *d*-ketoxylose to form the corresponding disaccharides.

Hehre (1945) prepared cell-free enzymes from *Streptococcus salivarius* and from a spore-forming bacillus capable of synthesizing polysaccharides from sucrose and raffinose. The polysaccharides formed by the action of these enzymes were found to possess properties similar to those isolated from sucrose broth cultures of the living bacteria.

Bergmann and Fruton (1944) selected pairs of suitable amino acid derivatives and found that proteolytic enzymes could synthesize single CO-NH linkages to produce dipeptids. The following example may be cited:

Enzyme: Papain

Benzoyl leucine + leucine anilide $\rightleftarrows$ benzoyl leucyl leucine anilide

For additional information, see Doudoroff and O'Neal (1945).

## SPECIFICITY OF ENZYMES

Inorganic catalysts such as nickel, platinum, and palladium, are able to catalyze many reactions. On the other hand, enzymes show a greater degree of specificity with respect to the substrates acted upon. Enzymes exhibit absolute specificity, stereochemical specificity, and relative specificity. Examples of absolute specificity: Carbohydrases do not act on fats or proteins but only on carbohydrates; lipases do not attack carbohydrates or proteins but only fats; and proteases act on proteins but have no effect on carbohydrates and fats. An example of stereochemical specificity: Maltase catalyzes the hydrolysis of many $\alpha$-glucosides but has no effect on $\beta$-glucosides. An example of relative specificity: $\beta$-glucosidase hydrolyzes some $\beta$-glucosides at much faster rates than other glucosides.

## ADAPTIVE AND CONSTITUTIVE ENZYMES

Most bacterial enzymes are elaborated irrespective of the composition of the medium in which the organisms are grown. These are known as constitutive enzymes because they are always produced. They appear to be essential enzymes carried by the cell.

The constitutive enzymes may be divided into two groups. The enzymes in one group always appear in approximately the same amount regardless of the composition of the medium. The enzymes in the other group vary in the amounts produced, depending upon the composition of the medium. The presence of certain nutrients causes an increased elaboration of the specific enzymes. For example, an organism capable of producing diastase (amylase) will produce more of the enzyme in the presence of starch than in its absence.

In some instances, an organism can be made to elaborate an enzyme that it normally does not secrete. The continued action of an organism on a specific substrate will finally result in the utilization of the compound. These are known as adaptive enzymes. For example, arabinose is not

attacked by *Salmonella enteritidis* but several serial transfers to the same medium finally results in the fermentation of the sugar. Since the enzyme appears only when the specific substrate is present, it is not considered to be an essential enzyme. Such action is usually a transient one, the acquired characteristic being quickly lost on transferring the organism to a medium lacking in the specific substrate.

Rahn (1938) stated that the adaptive enzymes are elaborated by most cells under the following conditions: (1) The reaction does not take place unless it is needed. (2) The mechanism necessary to produce this reaction is not preformed in the cell, but is formed only when needed. (3) The mechanism is not inherited, but the ability to set up this mechanism in case of need is inherited. (4) All reactions involved are highly specific, chemically speaking. Rahn, Hegarty, and Deuel (1938) grew *Streptococcus lactis* in glucose broth, separated the cells by centrifugation, and resuspended them in phosphate buffer (pH7.0) with the addition of glucose or galactose. The suspensions were so concentrated that growth failed to take place. A parallel set of suspensions was prepared with cells grown in galactose broth. The results are shown in Table 32. The organisms grown in glucose broth for 12 hr. cannot ferment galactose, but those grown in galactose broth ferment both sugars. Glucose is always fermented even when the bacteria are grown without any sugar. This indicates that the enzyme that attacks glucose is constitutive. On the other hand, the enzyme that attacks galactose is adaptive.

TABLE 32.—MILLIGRAMS OF LACTIC ACID PRODUCED BY *Streptococcus lactis* GROWN UNDER DIFFERENT CONDITIONS

| Time, hr. | Cells grown in glucose broth, then suspended in | | Cells grown in galactose broth, then suspended in | |
|---|---|---|---|---|
| | Glucose | Galactose | Glucose | Galactose |
| 1 | 240 | 0 | 160 | 180 |
| 2 | 360 | 0 | 300 | 270 |
| 3 | 420 | 0 | 370 | 400 |
| 4 | 470 | 0 | 420 | 410 |

The most important factor in adaptation appears to be the age of the cells when they are placed in contact with the new substrate. The older the cells, the slower will be the adaptation. The delay is necessary for the cells to build the mechanism that is responsible for the elaboration of the enzyme acting on galactose.

Karström (1938) grew the organism *Leuconostoc mesenteroides* in a medium containing only one of a series of sugars. After growth, the organisms were separated from the medium, washed, and then inoculated

into a series of tubes, each containing one of the carbohydrates. The results are given in Table 33. It may be seen that glucose, levulose, and mannose are fermented, indicating that the enzymes for these sugars are constitutive. On the other hand, the enzymes that ferment galactose, arabinose, and lactose are developed only in the presence of the appropriate substrate, indicating that the enzymes for these sugars are adaptive. Maltose is an exception in that the enzyme appeared in the absence of any sugar.

TABLE 33.—FORMATION OF CONSTITUTIVE AND ADAPTIVE ENZYMES

| Carbohydrate | Fermentation reaction | | | | |
|---|---|---|---|---|---|
| | Glucose, levulose, mannose | Galactose | Arabinose | Maltose | Lactose |
| Glucose | + | − | − | − | − |
| Galactose | + | + | − | − | − |
| Arabinose | + | − | + | − | − |
| Sucrose | + | − | − | − | − |
| Maltose | + | − | − | + | − |
| Lactose | + | + | − | − | + |
| No sugar | + | − | − | + | − |

The production of adaptive enzymes is not always the result of a specific response to the presence of the homologous substrate in the culture medium. For instance, calcium bears a definite relation to the formation of gelatinase by some bacterial species. Yet this stimulatory element is unrelated to the substrate attacked by the enzyme.

For additional information on adaptive and constitutive enzymes, see Burris, Phelps, and Wilson (1942), Dubos (1940), Karström (1938), and Rahn (1938).

## EXTRACELLULAR ENZYMES

Enzymes of bacteria may be divided into two groups depending upon whether they are secreted into the surrounding culture medium or remain confined within the cell. The enzymes that belong to the first group are known as the extracellular or exoenzymes; those which remain within the bacterial cell are known as the intracellular or endoenzymes.

It has been recognized from the time of Pasteur that the changes produced by organisms acting on carbohydrates (fermentation) and on proteins (protein decomposition and putrefaction) are brought about by the enzymes that they elaborate. Enzymes are capable of producing their specific actions in the complete absence of the living cell. This may be easily shown, in the case of the extracellular enzymes, by centrifugating a culture of organisms, passing the clear supernatant liquid through a

suitable filter, and demonstrating enzyme action by adding the filtrate to the appropriate substrate.

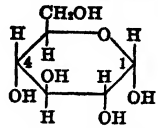
Extracellular enzymes may be demonstrated also by incorporating insoluble, indiffusible compounds into solid culture media. The presence of caseolytic, hemolytic, amylolytic (diastatic), and lipolytic enzymes may be shown by employing appropriate substrates.

**Hydrolysis of Starch.**—Starch is found widely distributed throughout the plant kingdom. It occurs in the form of granules having characteristic striations. The size, shape, and striations of the granules are typical of many plants and may be used for the microscopical identification of the origin of the starch.

When starch is heated with dilute mineral acids, it is hydrolyzed to dextrins, α-maltose and α-glucose. These products are all soluble in water. Concentrated mineral acids hydrolyze the starch molecule completely to α-glucose. Hydrolysis of starch is also effected by the enzyme amylase or diastase.

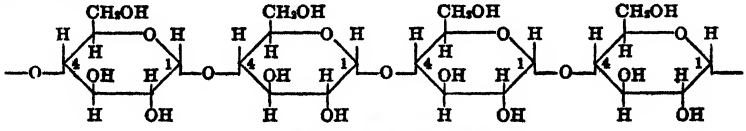
Starch can be separated into two fractions possessing different physical properties. These have been designated as β-amylose (amylose) and α-amylose (amylopectin). The β-amylose is soluble in water and is colored blue by iodine. The α-amylose is insoluble in water and gives no color with iodine. The β-amylose is believed to consist of long, unbranched chains of α-glucose units; α-amylose is believed to be made up of similar chains that show branching.

The structure of glucose may be represented in the conventional manner, as shown on page 278, or by the normal glucopyranose form, in which the first and fifth carbon atoms are joined through an oxygen to form an internal ring, as shown in the following perspective formula:




α-Glucopyranose (α-glucose)

The starch molecule consists of a chain structure made up of α-glucose units joined by glucosidic linkages through the first carbon atom and the fourth carbon atom of the next glucose unit through the sharing of an oxygen atom:




α-Linked glucose units in starch

The linkage between any pair of hexose units in starch is the same as in the disaccharide maltose.

Many organisms are capable of elaborating the enzyme amylase, which

is capable of hydrolyzing starch to maltose. Amylase is an extracellular enzyme secreted by organisms to convert the indiffusible compound into diffusible maltose. The maltose is then capable of entering the cell where it is utilized. Maltose is probably further hydrolyzed to glucose intracellularly by the enzyme maltase. The products of fermentation of starch are the result of the intracellular utilization of glucose.

The presence of amylase may be demonstrated by filtering a culture and mixing some of the filtrate with starch. The disappearance of the starch indicates the presence of amylase. This may be detected by the addition of a few drops of a dilute iodine solution (Fig. 138). A blue color indicates the presence of starch; a brown color indicates the complete hydrolysis of the starch to maltose. A simple procedure is to streak a loopful of a culture over the surface of a starch agar plate. After incubation, the plate is flooded with a dilute solution of iodine. The absence of a blue color at some distance from the bacterial growth indicates the extracellular hydrolysis of starch (Fig. 139).

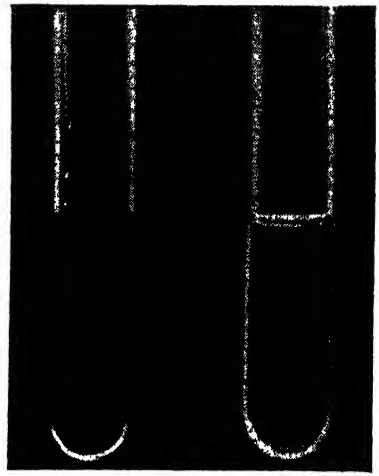



FIG. 138.—Hydrolysis of starch in the absence of bacteria. *A*, *Escherichia coli* filtrate. Blue color with iodine, indicating absence of starch hydrolysis; *B*, *Bacillus subtilis* filtrate. No color with iodine, indicating complete hydrolysis of starch.

For excellent discussions on the structure of the starch granule, see the reports by Alsberg (1938) and Hassid (1943, 1945).

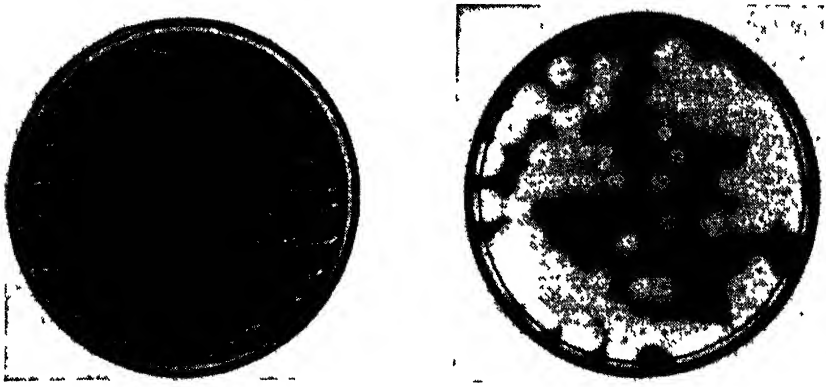
**Liquefaction of Gelatin.**—Gelatin possesses the property of forming a gel when dissolved in warm water. Since it is a protein, it can be attacked by some bacterial organisms, resulting in the loss of its property to gel. The hydrolysis of gelatin is an enzymatic reaction. The enzyme responsible for this change is known as a gelatinase. In the presence of carbohydrates which are rapidly fermented, gelatinase is usually not produced at all, or else in very minute amounts. In general, noncarbohydrate media should be used to demonstrate the ability of an organism to elaborate this enzyme.

Bacteria may be divided into two groups on the basis of their action on gelatin.

**Hydrolysis of Casein.**—Some organisms possess the power of attacking and hydrolyzing casein to smaller molecular units. This results in a conversion of the insoluble casein into soluble products. This transformation is generally spoken of as peptonization. The enzyme responsible for the

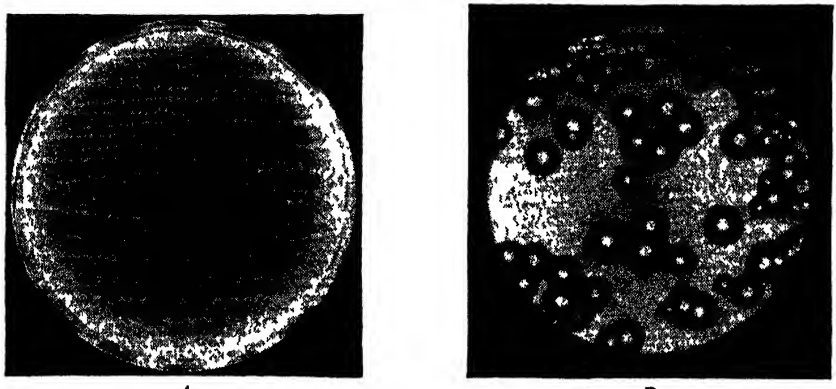
hydrolysis of casein is a proteinase. It is more specifically referred to as a casease.

The presence of a casease can be determined as follows: Sufficient milk



A B

FIG. 139.—Hydrolysis of starch in the presence of bacteria. *A*, *Escherichia coli*. Blue color with iodine, indicating absence of starch hydrolysis; *B*, *Bacillus subtilis*. No color with iodine around colonies, indicating hydrolysis of starch.



A B

FIG. 140.—Hydrolysis of casein. *A*, milk agar plate streaked with *Escherichia coli*. The casein is not hydrolyzed. *B*, same streaked with *Bacillus subtilis*. The casein is hydrolyzed resulting in the appearance of clear zones around each colony.

is incorporated in an agar medium to produce an opalescent appearance. The organism to be tested is then streaked over the surface of the milk agar. If a culture of *Bacillus subtilis* is used, clear zones appear around each colony on the plate. This indicates that the casein has been peptonized or digested to soluble compounds by the extracellular enzymes. On the other hand, *Escherichia coli* does not produce an extracellular caseolytic enzyme and is unable to attack the casein (Fig. 140). The digestion of

other compounds, such as blood (hemoglobin), starch, and inulin, may be demonstrated in a similar manner.

The tests are of value in identifying and classifying bacteria.

## INTRACELLULAR ENZYMES

The intracellular enzymes are concerned with the respiratory activities of the organism. They are not secreted outside of cells and cannot, therefore, be demonstrated in culture filtrates. Such enzymes may be detected by employing either living, disintegrated, or dissolved bacteria. Cells may be disintegrated by making use of various types of ball mills. Suspensions of pneumococci and other species, when mixed with bile, are dissolved. The disintegrated cells and lysates may be used for demonstrating the presence of various endoenzymes by incorporating them with appropriate substrates.

## OXIDIZING AND REDUCING ENZYMES

The term "oxidation" means the reverse of reduction. The oxidation of one compound is always accompanied by the reduction of another. An oxidation can be defined as (1) the addition of oxygen, or (2) the loss of hydrogen, or (3) the loss of electrons.

The reaction between ferric chloride and potassium iodide involves the oxidation of the iodine atom with simultaneous reduction of the iron atom by the transference of negative electrons from the former to the latter:

$$Fe^{+++} + 3Cl^- + K^+ + I^- \rightarrow Fe^{++} + 3Cl^- + K^+ + I$$

Many theories have been advanced to explain the mechanisms involved in intracellular oxidations and reductions. These will be discussed in Chap. XIII (page 310).

For further reading, consult the articles by Adams, Richtmyer, and Hudson (1943), DuBois and Potter (1943), Gale (1943), Hellerman, Chinard, and Deitz (1943), Lichstein and Cohen (1945), Moog (1946), Swenson and Humfeld (1942), Wagreich, Halpert, and Hirschman (1943), and Young and Hawkins (1944).

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# CHAPTER XIII

## RESPIRATION OF BACTERIA

The term "respiration" has undergone several changes in meaning since it was first coined. Originally, it was used to denote the passing of air into and out of the lungs. Following this, the term was extended to include the transference of oxygen to, and of carbon dioxide and water away from, the tissues. Then the term was employed to include various types of bacterial oxidations, which were referred to as aerobic and anaerobic respiration. In the discussion of bacterial respiration, the term can be used only in its broader meaning. Therefore, any cellular reaction, whether aerobic or anaerobic, capable of yielding energy to the organism, is called "bacterial respiration."

During the early years of bacteriology, it was believed that free oxygen was necessary for all organisms and that life was not possible in the complete absence of this element. Pasteur demonstrated very early in his studies that such a statement, which was then considered fundamental, had to be abandoned. He showed that there were bacteria that could not grow in the presence of free oxygen. He classified bacteria as aerobic, anaerobic, facultative aerobic, and facultative anaerobic, depending upon their action toward free oxygen.

Pasteur considered all fermentations, *i.e.*, the action of organisms on carbohydrates with the production of acid or of acid and gas, to be anaerobic. The escaping stream of carbon dioxide gas evolved during respiration was capable of driving the dissolved oxygen out of the media. Even though the media were exposed to the air, the conditions in the deeper portions were anaerobic. The organisms produced oxidations by the removal of hydrogen rather than by the addition of oxygen.

### OXIDIZING-REDUCING ACTIVITIES OF ORGANISMS

A compound may be oxidized by the transfer of hydrogen (or electrons) from a donator to an acceptor. The oxidation of one compound results in the reduction of another. Clark *et al.* (1923) defined biological oxidations as "the withdrawal of electrons from a substance with or without the addition of oxygen or elements analogous to oxygen; or as the withdrawal of electrons with or without the withdrawal of hydrogen or elements analagous to hydrogen." It is generally believed that oxygen acts as a hydrogen acceptor to form $H_2O$ and $H_2O_2$. Oxygen as such does not enter the

molecule of the substrate. Compounds that are active as hydrogen acceptors take the place of oxygen in various oxidations, being themselves reduced in the reactions. Bacterial oxidations and reductions are associated phenomena and must be studied together.

Wieland (1922) believed that almost all oxidations could be explained on the basis of the removal of hydrogen rather than of the addition of oxygen. Hydrogen acceptors were necessary for the reactions to take place. Oxidations that occurred in this manner were called "dehydrogenations." A few of the exceptions included the oxidation of aldehydes to acids, and purine bases to uric acid. In these instances, Wieland believed that a preliminary hydration occurred prior to the removal of hydrogen.

1. Oxidation without preliminary hydration:

$$\underset{\text{Alcohol}}{R{\cdot}CH_2OH} \rightarrow \underset{\text{Aldehyde}}{R{\cdot}CHO} + 2H + \text{hydrogen acceptor}$$

2. Oxidation with preliminary hydration:

$$R{\cdot}CHO + H_2O \rightarrow R{\cdot}C(H)(OH)(OH)$$

Aldehyde — Aldehyde hydrate

$$R{\cdot}C(H)(OH)(OH) \rightarrow R{\cdot}COOH + 2H + \text{hydrogen acceptor}$$

Theobald Smith was apparently the first to show that anaerobic, facultative, and aerobic organisms possessed the power to reduce methylene blue. Avery and Neill (1924*a*,*b*) cultivated the pneumococcus (*Diplococcus pneumoniae*) under anaerobic conditions and prepared an extract of the organisms by subjecting a suspension to repeated freezing and thawing. The suspension was then centrifugated and the clear supernatant liquid passed through a Berkefeld filter in an atmosphere of nitrogen. The extract prepared in this manner was capable of producing hydrogen peroxide in the presence of oxygen and of reducing methylene blue. Heating the extract to from 55 to 60°C. destroyed the power both to reduce and to form hydrogen peroxide.

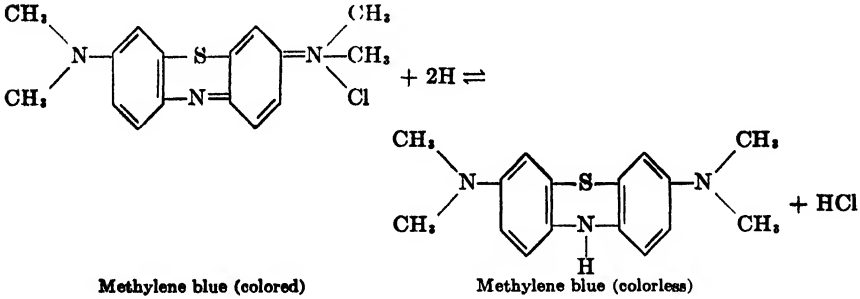
Other dyes that have been added to culture media for testing the reducing activities of bacteria include neutral red, litmus, indigo (indigo blue), and indigo carmine (sodium indigodisulfonate). Methylene blue has undoubtedly been used more than any of the other dyes for this purpose.

Probably all bacteria possess the power to reduce the foregoing dyes. There are, however, quantitative differences in their reducing powers. For example, *Escherichia coli* is capable of reducing methylene blue twice as fast as *Vibrio comma*, but the reverse is true for litmus.

Bacterial reductions normally occur intracellularly or at the cell surface. The evidence is not very striking to support the statement that bacterial-cell filtrates are capable of actively reducing dyes to their colorless forms. It is true that culture filtrates do possess some reducing power, which is probably due to the enzymes liberated after autolysis of a small number of dead cells. However, the action is very mild in comparison to the vigorous reduction that occurs when filtrates of disintegrated organisms are used.

The speed of decolorization of dyes is proportional to the number of organisms present. Methylene blue is added to milk to determine quickly its approximate bacterial count. The method is of value where speed is desired. A test based on this principle is known as the reductase test (see page 501).

**Reduction of Methylene Blue.**—Methylene blue acts as a hydrogen acceptor and loses its color in the reduced form. If air or oxygen is bubbled through the medium containing the reduced compound, the blue color is again restored. The reaction may be represented as follows:




Methylene blue (colored) Methylene blue (colorless)

One atom of hydrogen is taken up by the double-bonded nitrogen, converting the blue-colored compound into the colorless or leuco form. The reaction is easily reversible from one form to the other.

By means of the methylene blue technique, Thunberg (1929) demonstrated the presence of respiratory enzymes in animal tissues, which were capable of activating a number of organic compounds. The methylene blue acted as a hydrogen acceptor and became reduced to the colorless or leuco form. The speed of decolorization of the dye is an indication of the rate at which oxidation takes place. The reaction must be carried out in an anaerobic environment since the presence of air quickly reoxidizes the methylene blue to the colored form.

Thunberg found that methylene blue added to suspensions of minced animal tissue was quickly decolorized because the tissue contained many substances activated by certain enzymes known as "dehydrogenases." By first extracting minced tissue with water, he was able to remove these substances but not the dehydrogenases. The tissue had now lost its power

to reduce methylene blue. By adding various compounds to the washed tissue, Thunberg succeeded in demonstrating the presence of a large number of dehydrogenases. Such enzymes have been found to be widely distributed in higher plant and animal tissues, in yeast cells, bacteria, and other organisms.

Since the number of hydrogen donators is very large, it is not likely that each compound has its own specific dehydrogenase. According to Quastel (1930), the explanation that accounts most readily for the facts is that there are relatively few dehydrogenases but that each enzyme deals with a particular type of molecule rather than with a specific substrate.

**Dehydrogenases.**—A dehydrogenase is defined as an enzyme that is capable of activating the hydrogen of metabolites. With the possible exception of uricase, all dehydrogenases can act in either the presence or the absence of oxygen. Uricase appears to be an exception in that it acts only in the presence of oxygen.

The dehydrogenases that are linked with carriers are generally referred to as the anaerobic dehydrogenases. The hydrogen from metabolites may be transported over several intermediate compounds, eventually combining with oxygen to produce hydrogen peroxide. The anaerobic dehydrogenases are differentiated according to the first carrier that receives the hydrogen from the substrate-dehydrogenase reaction. Those enzymes which transfer hydrogen directly to oxygen without the necessity of one or more carriers are called the "aerobic" dehydrogenases.

*Aerobic Dehydrogenases.*—The characteristics of the aerobic dehydrogenases, according to Potter (1939) are as follows:

1. They reduce dyes.
2. They act in the absence of oxygen if suitable dyes are present.
3. They catalyze a direct reaction between metabolites and oxygen.
4. They produce peroxide in the presence of oxygen.
5. They may or may not be inhibited by cyanide.
6. They require neither coenzyme nor cytochrome systems.

Under the aerobic dehydrogenases are included:

1. Xanthine dehydrogenase.
2. *d*-Amino acid dehydrogenase.
3. Tyramine dehydrogenase.
4. Uricase.

*Anaerobic Dehydrogenases.*—The anaerobic dehydrogenases catalyze reactions between metabolites and carriers. They are differentiated according to the first carrier.

1. Coenzyme I—linked.
   *a.* Lactic dehydrogenase.
   *b.* β-Hydroxy butyric dehydrogenase.
   *c.* Malic dehydrogenase.

*d.* Citric dehydrogenase.
*e.* Alcohol dehydrogenase.
*f.* Glucose dehydrogenase.
*g.* Aldehyde dehydrogenase.
*h.* α-Glycerophosphoric dehydrogenase.
*i.* 1,3-Diphosphoglyceric aldehyde dehydrogenase.

2. Coenzyme II—linked.
   *a.* Glucose dehydrogenase.
   *b.* Robison ester dehydrogenase.
3. Cytochrome—linked.
   *a.* Succinic dehydrogenase.
   *b.* α-Glycerophosphoric dehydrogenase.

The reactions between the dehydrogenases and their specific substrates are given on page 290 ff.

Many of the dehydrogenases are differentiated by the presence of certain active groups probably attached to a common colloidal protein carrier of large molecular weight. It is difficult to believe that each dehydrogenase is a distinct enzyme with its own protein carrier. As Harrison (1935) stated,

> If each enzyme be considered distinct both as regards its active group and its colloidal carrier, it becomes difficult, in view of a large number of different substrates activated by bacteria, to imagine how so many individual enzymes, each with a very large molecule, can be accommodated in a space so small as that of the cells of bacteria.

For more information on dehydrogenases, see Ames and Elvehjem (1944, 1945), Greisen and Gunsalus (1944), Guggenheim (1944), Potter and Albaum (1943), and Potter and DuBois (1943).

**Oxidases.**—The oxidases differ from the dehydrogenases in that they appear to function by activating oxygen so that it will quickly oxidize slowly auto-oxidizable compounds (Wieland, 1932). Other characteristics of the oxidases that distinguish them from the dehydrogenases are

1. They are metalloproteins (copper or iron) and are inhibited by KCN, $H_2S$, and other substances that stabilize the metallic groups.
2. They do not produce hydrogen peroxide.
3. They do not act under anaerobic conditions.

The properties of the iron oxidases are due largely to the presence of this metal. The iron is located in prosthetic groups which are either hematin or a substance closely related to hematin. The hematin is united to various specific proteins, or apoenzymes, to give the complete enzymes.

The following enzymes are generally classified as iron oxidases:

1. Cytochrome.
2. Catalase.
3. Peroxidase.

*Cytochrome and Cytochrome Oxidase.*—Cytochrome is a respiratory pigment, which was discovered by MacMunn (1886) but was forgotten until

1925 when it was "rediscovered" by Keilin. He named the compound cytochrome which means "cellular pigment."

The pigment is widely distributed in plant and animal cells being found in baker's yeast, bacteria, muscles of mammals and birds, insects, molluscs and crustacea, etc. It is easily oxidized and reduced under suitable conditions. It is not a single compound but consists of three components designated as cytochrome *a*, *b*, and *c*. The cytochrome in a yeast suspension is completely reduced by the addition of a solution of KCN. The cytochrome remains reduced because the KCN acts as an inhibitor of oxidation.

Chemically cytochrome is a hemochromogen having a structure similar to that of hemoglobin. It acts as a carrier of hydrogen. Under normal conditions, it is present in cells in the oxidized or only partly reduced form. If a suspension of yeast cells is well aerated, the cytochrome becomes oxidized; if exposed to an atmosphere of nitrogen, it becomes reduced. During conditions of great activity, the cytochrome is partly oxidized, but during periods of inactivity it is again reduced.

Cytochrome oxidase is sometimes referred to as indophenolase and indophenol oxidase. The enzyme is widely distributed in nature, being found in yeasts, molds, algae, aerobic bacteria, higher plants and animals, etc.

Cytochrome oxidase is capable of oxidizing *p*-phenylenediamine to indophenol. The presence of the enzyme is easily detected by pouring a solution of *p*-phenylenediamine over the surface of an agar plate containing bacterial colonies. In the presence of the enzyme, the *p*-phenylenediamine is oxidized to indophenol, resulting in the appearance of a blue color. The oxidase is believed to oxidize phenols, amines, etc., not directly, but by oxidizing cytochrome *c*. The cytochrome *c* then oxidizes the phenols, or amines. The aerobic bacteria and a few of the facultative species give a strong positive reaction; the facultative anaerobes grown aerobically give a weak positive reaction; the obligate anaerobes give a negative reaction.

Keilin (1933) reported the following properties for cytochrome oxidase:

1. It oxidizes *p*-phenylenediamine rapidly.
2. It is thermostable, being destroyed at temperatures above 60°C.
3. It is destroyed on the addition of strong alcohol or acetone.
4. Oxygen uptake during the oxidation of *p*-phenylenediamine is inhibited by the addition of minute amounts of KCN, $H_2S$, $NaN_3$, or CO.
5. *p*-Phenylenediamine does not require activation by a dehydrogenase to be oxidized by cytochrome oxidase.

Keilin and Hartree (1938) stated that the only property that could be definitely ascribed to cytochrome oxidase was the oxidation of reduced cytochrome *c*. The reactions may be represented as follows:

$$\text{Metabolite}\cdot 2H + \text{dehydrogenase} + \text{cytochrome } c \rightarrow \text{reduced cytochrome } c$$
$$\text{Reduced cytochrome } c + \text{cytochrome oxidase} + O_2 \rightarrow \text{oxidized cytochrome } c$$

The reactions do not occur on the addition of KCN, $H_2S$, $NaN_3$, or CO.

For additional information on cytochrome and cytochrome oxidase, see Haas (1943), Keilin and Hartree (1939), Schultze (1939), and Stotz (1942).

*Catalase.*—Some bacteria produce hydrogen peroxide ($H_2O_2$) in the presence of free oxygen. Since hydrogen peroxide is toxic to living cells, its destruction is of considerable importance. The accumulation of peroxide in cultures is controlled by two factors: (1) bacterial catalase and (2) the degree of sensitiveness of the organisms to the compound.

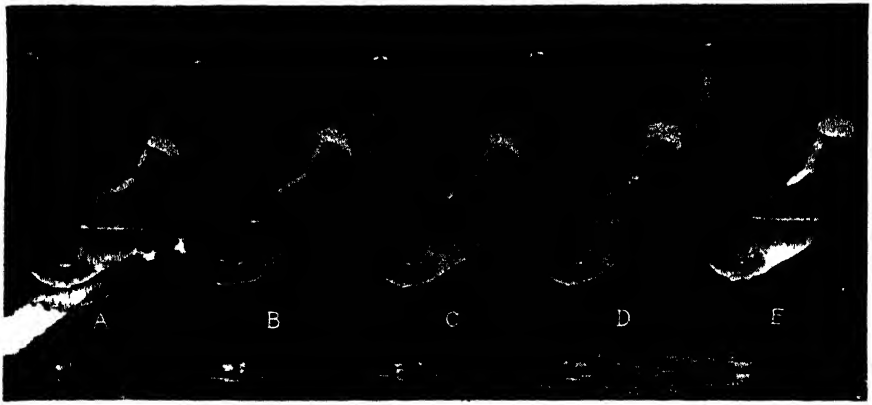



FIG. 141.—Reduction of hydrogen peroxide with the liberation of oxygen. *A, Bacillus subtilis; B, Micrococcus pyogenes* var. *aureus; C, Pseudomonas fluorescens; D, Escherichia coli; E,* control. *Bacillus subtilis* shows slight decomposition whereas *Pseudomonas fluorescens* shows vigorous decomposition. Note the absence of gas in the control.

Catalase is an enzyme capable of decomposing hydrogen peroxide into water and molecular oxygen, according to the equation:

$$2H_2O_2 + \text{catalase} \rightarrow 2H_2O + O_2$$

The presence of hydrogen peroxide was first detected in the pneumococcus, an organism incapable of producing catalase and only moderately sensitive to the toxic action of $H_2O_2$. Organisms that do not produce catalase may be protected by being cultivated with certain plant or animal tissues, or with other organisms capable of producing the enzyme.

Catalase is produced by many bacteria. Some produce more of it than others. It is present in largest amounts in the strictly aerobic bacteria. On the other hand, its presence has not been demonstrated in the obligately anaerobic bacteria.

The concentration of catalase can be determined by adding hydrogen peroxide and noting the amount decomposed. The test is easily performed by adding peroxide to bacterial cultures contained in Smith fermentation tubes and by measuring the volumes of oxygen evolved (Fig. 141).

*Peroxidase.*—The recognition of peroxidase as a specific enzyme was first reported by Linossier (1898). The enzyme is widely distributed in nature, being found in vegetables, many sprouts, sugar beet, wheat flour, bran, brewer's yeast, insects, birds, animals, aerobic bacteria, milk, potatoes, etc. Horseradish is one of the richest sources of peroxidase and is frequently employed for the preparation of the enzyme.

Peroxidase is very resistant to heat. It is destroyed at a temperature of 98°C. in 10 min. but, on standing, as much as 25 per cent of the original activity is restored.

The function of peroxidase is to transfer oxygen from peroxides to oxidizable substances. Peroxidase does not decompose hydrogen peroxide

TABLE 34.—OXIDATION OF NITRITE TO NITRATE

| Enzyme System | Oxidation of Nitrite |
|---|---|
| Xanthine dehydrogenase + xanthine | Negative |
| Xanthine dehydrogenase + peroxidase | Negative |
| Xanthine + peroxidase | Negative |
| Xanthine dehydrogenase + xanthine + peroxidase | Positive |

in the absence of an oxidizable substance. In this respect it differs from catalase, which decomposes hydrogen peroxide in the absence of an oxygen acceptor. The two enzymes differ also in the kind of oxygen released. Peroxidase releases atomic oxygen whereas catalase decomposes hydrogen peroxide with the liberation of molecular oxygen. A large number of compounds may function as oxygen acceptors, thereby permitting the peroxidase to act. Among these may be mentioned glutathione and cytochrome *c*. The action of the enzyme on an oxidizable substance ($RH_2$) in the presence of hydrogen peroxide may be represented as follows:

$$\text{Peroxidase} + H_2O_2 \rightarrow \text{peroxidase}\cdot H_2O_2$$
$$\text{Peroxidase}\cdot H_2O_2 + RH_2 \rightarrow \text{peroxidase} + R + 2H_2O$$

The addition of KCN completely inhibits the above reactions, whereas CO has no effect.

*Dehydrogenase-peroxidase Systems.*—Thurlow (1925) showed that, when xanthine was added to xanthine dehydrogenase in the presence of a plentiful supply of air, hydrogen peroxide soon appeared in the solution. The amount present rapidly increased until a maximum was reached after which the quantity gradually decreased owing to its reaction with the xanthine. The presence of hydrogen peroxide was detected by the addition of peroxidase and a suitable oxidizable substance such as sodium nitrite. Peroxidase does not decompose hydrogen peroxide in the absence of an oxidizable substance. In this instance, the hydrogen peroxide rapidly converted the nitrite to nitrate and the amount utilized could be determined from the unoxidized nitrite still remaining in the mixture. A typical set of results reported by Thurlow is given in Table 34. The oxidation of nitrite to nitrate occurred only in the last experiment.

McLeod and Gordon (1923*a*) and McLeod (1928) proposed a classification of bacteria on the basis of catalase and peroxide production. The classification is as follows:

Group I. The anaerobes, devoid of catalase, extremely sensitive to $H_2O_2$, and considered as potential peroxide producers; *Clostridium perfringens*, *C. tetani*, *C. sporogenes*, etc.

Group II. Peroxide producers devoid of catalase and only moderately sensitive to $H_2O_2$; the pneumococcus; many types of streptococci; the lactic acid bacteria; and some sarcinae.

Group III. Nonperoxide producers and devoid of catalase; certain streptococci, dysentery bacilli (Shiga type), and some hemoglobinophilic bacteria.

Group IV. Bacteria producing catalase. The great majority of the bacteria capable of growing aerobically or both aerobically and anaerobically.

A number of oxidases contain copper instead of iron. The most important of these are

1. Ascorbic acid oxidase.
2. Laccase.
3. Tyrosinase (polyphenol oxidase).

*Ascorbic Acid Oxidase.*—Tauber, Kleiner, and Mishkind (1935) were the first to isolate a specific enzyme capable of oxidizing ascorbic acid or vitamin C (Fig. 142) to dehydroascorbic acid. The enzyme is known as ascorbic acid oxidase.

Ascorbic acid is extremely unstable, being readily oxidized to dehydroascorbic acid (see page 290). The oxidized form still displays some antiscorbutic properties. Antiscorbutic activity appears to parallel to a remarkable degree its reducing capacity. Hopkins and Morgan (1936) believed that glutathione was the chief protective substance in cells for maintaining ascorbic acid in the reduced form. It guarded the vitamin against irreversible oxidation with a loss of antiscorbutic activity. Glutathione, added to a mixture of ascorbic acid and its oxidase, maintained the vitamin in the reduced or active form.

The exact function of ascorbic acid in cellular respiration is not clearly understood. Since it is capable of alternate oxidation and reduction, it is probable that its function is that of a respiratory carrier.

For information on the preparation and properties of ascorbic acid oxidase, see Powers and Dawson (1944) and Powers, Lewis, and Dawson (1944).

*Laccase.*—This enzyme is found in the latex of the lac tree. The oxidase is widely distributed in the plant kingdom.

Laccase oxidizes phenols to ortho- and para-quinones (see page 289). It requires oxygen for its action. It is inhibited by hydrocyanic acid, hydrogen sulfide, sodium azide, and sodium diethyldithiocarbamate.

*Tyrosinase.*—The development of a brown, orange, red, or black color around colonies of some bacteria and molds growing on agar is due to the

action of tyrosinase on the amino acid tyrosine and related compounds. The reactions on tyrosine, according to Raper (1932), are given on page 289.

Waksman (1932), Skinner (1938), and others showed that a large number of aerobic actinomycetes were capable of producing dark-colored compounds in nonsynthetic media, and in synthetic media to which tyrosine was added Clark and Smith (1939) noted the production of a black pigment by many strains of *Bacillus niger* (*mesentericus*) growing on several peptone agars. The addition of glucose or maltose inhibited the formation of pig-

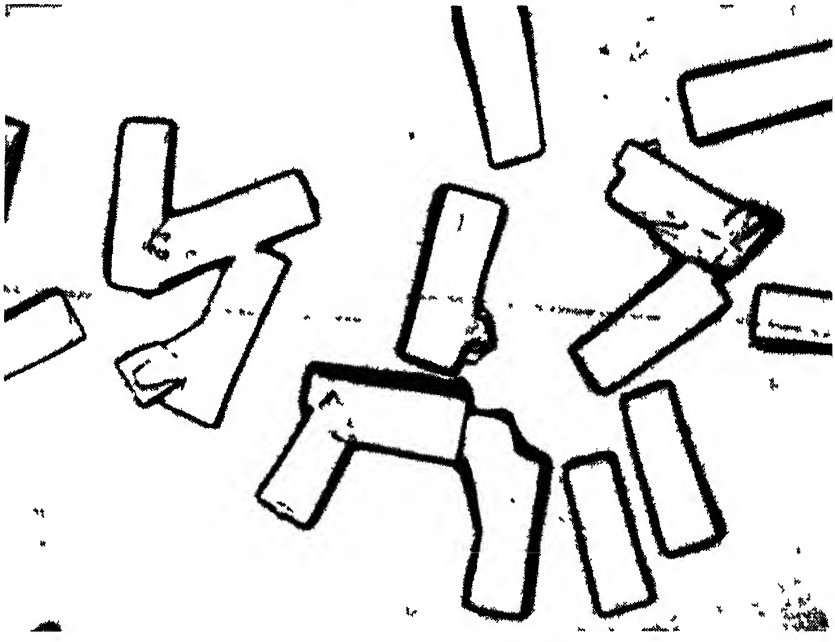


FIG. 142.—Ascorbic acid crystals (*Courtesy Merck & Co., Inc.*)

ment on those agars which were normally blackened by the organism. This suggested that pigment formation was due to the action of the organisms on the protein and that the addition of a fermentable carbohydrate produced a protein-sparing action. They concluded that *B. niger* produced a black pigment upon protein media, which contained free or metabolically available tyrosine. Some commercial peptones that did not contain readily available tyrosine were not blackened unless the amino acid was added. The addition of a fermentable carbohydrate to many protein media, normally blackened by *B. niger*, inhibited pigmentation unless free tyrosine was added

For more reading, see Behm and Nelson (1944*a*, *b*), Gould (1939), and Tenenbaum and Jensen (1943).

Miscellaneous oxidases:

1 Dopa oxidase.
2. Luciferase.

*Dopa Oxidase.*—This enzyme is so named because of its ability to oxidize 3,4-dihydroxyphenylalanine, or dopa, to melanin. Since it is unable to oxidize many phenolic derivatives, it is considered the most specific of the oxidases so far studied. The enzyme is active only in the presence

FIG 143.—Colonies of bacteria photographed with their own light. The bright colonies are the yellow variants (*After Giese*)

of oxygen. The addition of dopa to cells containing the enzyme results in the appearance of a black color (see page 290).

*Luciferase.*—A number of organisms are known that are capable of emitting light under appropriate conditions. These are referred to as bioluminescent organisms.

Most of the species are members of the genera *Achromobacter*, *Photobacterium*, and *Vibrio*. The majority of these are marine forms, having been isolated from dead fish. The halophilic forms are best cultivated on various media prepared with sea water (3 per cent sodium chloride). The nonhalophilic species are cultivated in media containing about 0.9 per cent sodium chloride.

The emission of light is due to the presence of an enzyme, luciferase, acting on its substrate, luciferin. The reactions are believed to be as follows: Oxidized luciferin is reduced in the presence of some hydrogen donor. The reduced luciferin reacts with luciferase to give oxidized luciferin and reduced luciferase. The reduced luciferase then reacts with oxygen to give light, hydrogen peroxide, and oxidized luciferase (see page 290).

Giese (1943) obtained a variant of *A. fisheri* when the medium was al-

lowed to become acid. The luminescence of this variant was four to five times more brilliant than that of the original strain (Fig. 143). The variant developed a yellow-brown pigment; the original strain was nonpigmented. The variant produced a more profuse growth than the original strain.

For more information, consult Doudoroff (1942*a*,*b*), Johnson, Zworykin, and Warren (1943), and McElroy and Ballentine (1944).

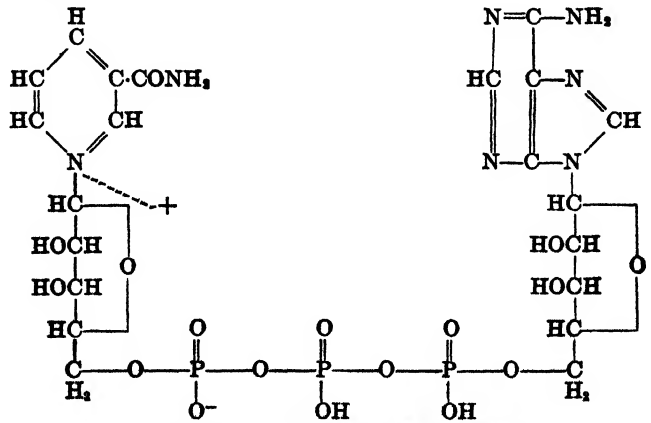
## COENZYMES

Enzymes may be defined as organic catalytic agents elaborated by living cells and capable of functioning independently of the cells. They are heat-labile, nondialyzable compounds of high molecular weight. Coenzymes are also catalytic agents produced by living cells and are necessary in enzymatic reactions, but they are heat-stable, dialyzable, and have smaller molecular weights.

**Codehydrogenases I and II.**—Warburg (1932) constructed an enzyme system for the oxidation of hexosemonophosphate (Robison ester) in vitro in the presence of molecular oxygen. The system was composed of

1. Hexosemonophosphate.
2. A dehydrogenase from yeast.
3. A coenzyme from red blood cells.
4. The yellow enzyme (flavoprotein).
5. Oxygen.

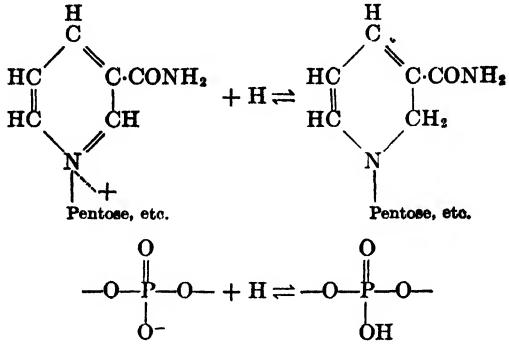
In a later communication, Warburg (1935) isolated the coenzyme and characterized it as a triphosphopyridine nucleotide (TPN). It consists of one molecule of $\beta$-nicotinic acid amide, one of adenine, two of pentose, and three of phosphoric acid. He showed that the compound was capable of oxidation and reduction. The structural formula of the coenzyme is believed to be as follows:



Triphosphopyridine nucleotide (codehydrogenase II)

Other names for the compound are coenzyme II and codehydrase II.

In the above system, the first reaction occurred between the substrate, codehydrogenase II, and the specific dehydrogenase. Two atoms of hydrogen were transferred from the hexosemonophosphate to the oxidized coenzyme to give phosphohexonic acid and dihydrocodehydrogenase II. When the coenzyme was unable to accept more hydrogen, the reaction stopped. The pyridine ring of the coenzyme accepted one atom of hydrogen and a molecule of phosphoric acid accepted the other:




This reaction occurred either in the presence or in the absence of oxygen. The second reaction was studied under anaerobic conditions. To the mixture of substrate, dehydrogenase, and coenzyme, was added the yellow enzyme or flavoprotein. The hydrogen was now transferred from the reduced coenzyme to the flavoprotein. When both coenzyme and flavoprotein were unable to accept more hydrogen, the reaction stopped. If air was now added to the system, hydrogen was transferred from the reduced flavoprotein to molecular oxygen, which resulted in the formation of hydrogen peroxide. The reactions may be represented as follows:

$$\underset{\text{(Robison ester)}}{\text{Hexosemonophosphate·2H}} + \text{coenzyme} + H_2O \rightarrow \underset{\text{acid}}{\text{phosphohexonic}} + \text{coenzyme·2H}$$

$$\text{Coenzyme·2H} + \text{flavoprotein} \rightarrow \text{coenzyme} + \text{flavoprotein·2H}$$

$$\text{Flavoprotein·2H} + O_2 \rightarrow \text{flavoprotein} + H_2O_2$$

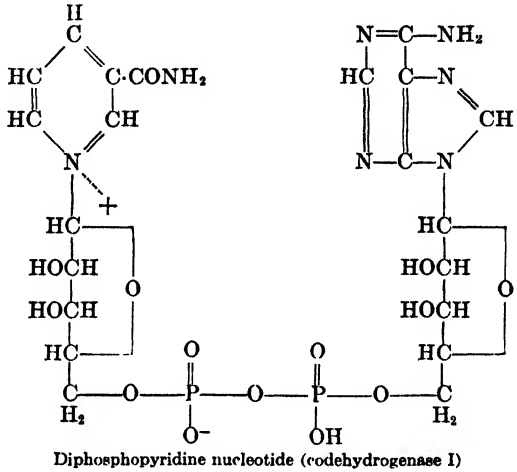
The over-all reaction becomes

$$\text{Hexosemonophosphate} + H_2O + O_2 \rightarrow \text{phosphohexonic acid} + H_2O_2$$

It may be seen that the hydrogen was transported over several compounds, eventually being united with oxygen to give hydrogen peroxide. Both the coenzyme and flavoprotein were reduced and regenerated many times without being used up in the reaction. Many compounds capable of accepting hydrogen may be substituted for the flavoprotein.

Warburg isolated another coenzyme from yeast which differed from codehydrogenase II in having one less molecule of phosphoric acid. This coenzyme is necessary in the fermentation of glucose to alcohol by the

yeast *Saccharomyces cerevisiae* and in other reactions. Chemically the coenzyme is diphosphopyridine nucleotide (DPN) and is generally known as codehydrogenase I. Other names for the coenzyme are cozymase, coenzyme I, codehydrase I, and yeast coenzyme. The active groups are also the pyridine ring and a molecule of phosphoric acid. The structural formula is as follows:



Diphosphopyridine nucleotide (codehydrogenase I)

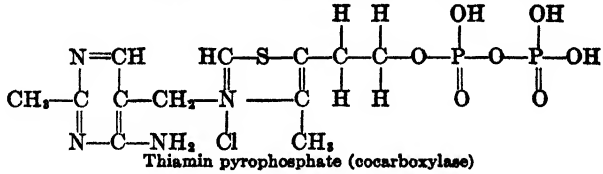
Diaphorases are enzymes that are capable of reoxidizing reduced coenzyme I or reduced coenzyme II in the presence of suitable carriers. The reactions may be written as follows:

Dihydrocoenzyme I + diaphorase → codehydrogenase I + reduced diaphorase
Reduced diaphorase + methylene blue → diaphorase + leuco methylene blue
Leuco methylene blue + $O_2$ → methylene blue + $H_2O_2$

The diaphorases are widely distributed in nature, being present in yeasts, bacteria, higher plants, animal tissues, and milk.

For more information, see Lockhart (1939).

**Cocarboxylase.**—This coenzyme was first isolated from yeast juice. It is composed of one molecule of thiamin (vitamin $B_1$) and two molecules of phosphoric acid, having the following structural formula:



Thiamin pyrophosphate (cocarboxylase)

The coenzyme is necessary for the decarboxylation of pyruvic acid to acetaldehyde by the enzyme carboxylase, according to the reaction:

$CH_3 \cdot CO \cdot COOH$ + carboxylase + cocarboxylase → $CH_3 \cdot CHO + CO_2$
Pyruvic acid — Acetaldehyde

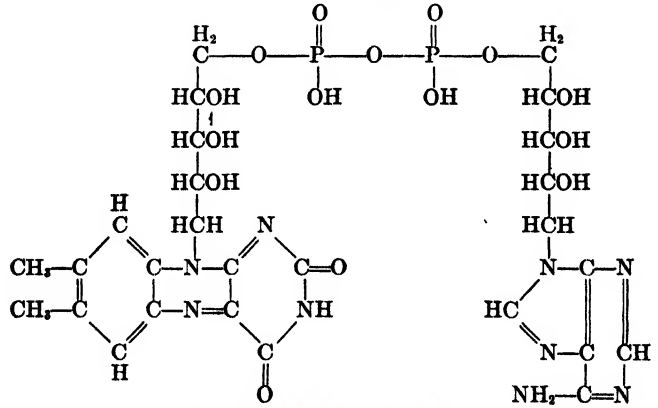
In animal tissues, the coenzyme appears to be intimately connected with thiamin (vitamin $B_1$) and the metabolism of pyruvic acid.

**Coenzyme of the *d*-Amino Acid Oxidase.**—This coenzyme is necessary for the action of *d*-amino acid oxidase, an enzyme which is capable of oxidizing many *d*-amino acids to the corresponding keto acids. The reaction is as follows:

$$\underset{\text{d-Amino acid}}{R\cdot CHNH_2\cdot COOH} + \text{d-amino acid oxidase} + \text{coenzyme} + O \rightarrow \underset{\text{Keto acid}}{R\cdot CO\cdot COOH} + NH_3$$

*d*-Amino acid oxidase has been shown to consist of protein (apoenzyme) united with a coenzyme (prosthetic group). Warburg showed the coenzyme to be isoalloxazine adenine dinucleotide. The isoalloxazine ring of the coenzyme accepts hydrogen from the *d*-amino acid, resulting in the formation of reduced coenzyme. The reduced compound may then be reoxidized by molecular oxygen.

The following formula has been suggested for the compound:



Isoalloxazine adenine dinucleotide

**Adenylic Acid.**—This important coenzyme is discussed on page 371 in connection with the fermentation of glucose to alcohol by brewer's yeast.

**Mode of Action of Coenzymes.**—Coenzymes function by accepting hydrogen and then transferring it to other compounds until it eventually unites with oxygen. Baumann and Stare (1939*a*) stated,

> Coenzymes are no longer looked upon as accelerators in any supplementary sense, but rather as essential, integral members of a complicated "bucket brigade," transferring hydrogen or phosphate from compound to compound; they empty their buckets and come back for more. They are "carriers" of both hydrogen and phosphate. But protein enzymes of the traditional type, the dehydrogenases, must be present to bring about hydrogen transfer.

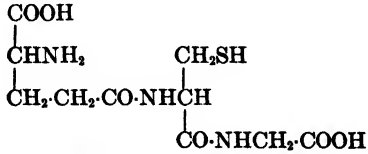
For additional information, see Baumann and Stare (1939*a*,*b*), Hogness (1942), and Schlenk (1945).

## GLUTATHIONE

Hopkins (1921) isolated a thermolabile substance from extracts of yeast and muscle, which functioned as an oxidation-reduction system. The compound was believed to be composed of one molecule of glutamic acid and one of cysteine united together by a peptid linkage. He named the compound glutathione.

In a later communication, Hopkins (1929) showed that the compound was not a dipeptid, as was at first believed, but a tripeptid composed of glycine, cysteine, and glutamic acid.

The structural formula is as follows:




Glutathione (glutamyl cysteinyl glycine)

Since it is the sulfhydryl group (—SH) which is of importance in reactions of oxidation-reduction, the above formula may be abbreviated to GSH. Two molecules of reduced glutathione readily give up the hydrogen of their sulfhydryl groups and become oxidized to a disulfide:

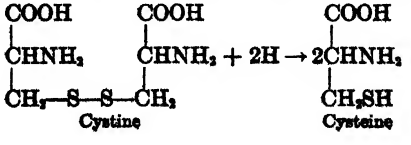
$$2GSH + B \rightleftharpoons GSSG + BH_2$$

Reduced glutathione — Oxidized glutathione

The disulfide form is readily reduced to the original compound by the addition of two atoms of hydrogen.

Glutathione has been shown to be of almost universal occurrence in living tissue where the concentration roughly parallels the metabolic activity of the cells. The concentration is higher in rapidly growing cells than in older cells. Blood is said to contain from 34 to 47 mg. of glutathione per 100 gm. Miller and Stone (1938) demonstrated the presence of determinable amounts of glutathione in many species of aerobic and anaerobic bacteria, yeasts, and molds. *Aerobacter aerogenes* contained about 27 mg. per 100 gm.; *Proteus vulgaris*, 29 to 31 mg.; *Chromobacterium violaceum*, 7 to 45 mg.; *Monilia sitophila* (mold), 20 to 38 mg.; *Saccharomyces cerevisiae* (yeast), 59 mg.

Since the oxidation-reduction mechanism of glutathione is due to the cystine-cysteine combination, it has been shown that cystine functions in a similar manner. This may be shown by the following equation:




Cystine — Cysteine

## OXIDATION-REDUCTION POTENTIALS

Oxidation-reduction potentials are of great importance in biology. Reactions that occur intracellularly and release energy to the organism involve a study of oxidation-reduction potentials. An oxidation occurs either by the addition of oxygen or by the removal of hydrogen. The oxidation of one compound involves the reduction of another.

An atom consists of a nucleus of positive electricity surrounded by a shell of electrons possessing negative electrical charges. The sum total of the negative charge must be equal to the positive charge of the nucleus. Some elements easily lose electrons whereas others add electrons. An oxidation involves the loss of one or more electrons; and a reduction, a gain. This may be shown in the following equations:

Oxidation:

$$Cu^{+} - \text{one electron } (-1e) \rightarrow Cu^{++}$$

Reduction:

$$Cu^{++} + 1e \rightarrow Cu^{+}$$

A substance that readily gives up electrons is a good reducing agent; conversely, a substance that readily takes up electrons is a good oxidizing agent.

A typical oxidation by the addition of oxygen involves the formation of cupric oxide in the reaction:

$$2Cu + O_2 \rightarrow 2CuO$$

The conversion of alcohol to aldehyde is an example of an oxidation by the removal of hydrogen:

$$CH_3{\cdot}CH_2OH \rightarrow CH_3{\cdot}CHO + 2H$$

Still other oxidations occur in which oxygen or hydrogen is not involved. Iron may have a valence of three or two. The former is regarded as more highly oxidized than the latter. The oxidation of ferrous chloride to ferric chloride may be represented by the equation:

$$FeCl_2 + Cl \rightleftharpoons FeCl_3$$

This reaction involves the transfer of an electron from the iron to the chlorine atom. The same is true of all oxidation reactions. The oxidized atom loses one or more electrons to some other atom.

The transfer of electrons from one compound to another sets up a potential difference between the reactants, which may be measured by an appropriate instrument. The magnitude of this potential difference depends upon the ease with which the electrons are lost or gained. The greater the oxidizing or reducing power of a substance, the greater will be the electrical potential on one side or the other of a zero point. The more highly oxidized a substance, the more positive will be its electrical potential and, the more highly reduced a substance, the more negative

will be the electrical potential. The direction in which a reaction proceeds is dependent upon the free electrons in the system. If the number of electrons is increased, the system will produce more of the reductant; if the number is reduced, the system will produce more of the oxidant. The electronic state of the system is a measure of its oxidizing or reducing power.

The oxidation-reduction potential of a system is expressed by the symbol Eh. The greater the proportion of reduced substance present, the lower will be the Eh value; conversely, the greater the proportion of oxidized substance, the higher will be the Eh value. When the concentration of the oxidant is equal to that of the reductant, the term becomes zero and the observed potential is equal to Eo.

## ANAEROBIOSIS

Anaerobes are usually defined as organisms that can live and multiply only in the complete absence of oxygen. This statement is not strictly correct as it has been shown that all obligately anaerobic bacteria can tolerate some free oxygen. However, they show considerable variation in the amount of free oxygen that they are able to tolerate. *Clostridium tetani* can grow in a liquid medium exposed to a gaseous environment containing from 10 to 20 mm. of air pressure, and *C. chauvoei* can tolerate 40 mm. On a blood agar plate, *C. tetani* was found to grow in 5 to 15 mm. of air pressure. *C. perfringens* was able to produce good growth in an atmosphere containing 200 mm. of air pressure and produced a slight growth at 380 mm. of air pressure.

**Mechanism of Oxygen Inhibition.**—The mechanism involved in the inhibition of growth of anaerobes in the presence of molecular oxygen has been the subject of many investigations. The most important theories appear to be the following:

1. Oxygen is directly toxic to the cell.
2. Hydrogen peroxide is produced and, since the organisms do not elaborate the enzyme catalase, the compound is toxic to the cells.
3. The growth of anaerobes is dependent upon a low oxidation-reduction potential, which is not possible in the presence of free oxygen.

1. If it is true that oxygen is toxic to bacteria, growth should not take place when the organisms are transferred to a favorable environment. Such is not the case, however. Growth is reestablished when anaerobic cultures in contact with air are again exposed to a reduced oxygen environment.

2. McLeod and Gordon (1923*b*) suggested that anaerobes produced small amounts of hydrogen peroxide when exposed to air and, since they are extremely sensitive to the compound, its presence prevented the organ-

isms from growing. This statement was based on certain observations made in connection with the growth of anaerobes in a blood medium. They observed that anaerobic organisms produced a zone of greenish discoloration about ⅛ in. below the surface of tubes of heated blood (chocolate) agar. The growth was very similar to that produced by the pneumococcus on the same medium, which is known to be a peroxide producer.

Avery and Morgan (1924), working with several strains of pneumococcus, *Streptococcus pyogenes*, and *Diplococcus mucosus*, arrived at the same conclusions. They found that conditions favoring the formation and accumulation of peroxide in broth cultures of the above organisms were free access of air and the absence of catalase, peroxidase, and other catalysts capable of decomposing the compound. In the absence of free oxygen, peroxide was not formed. In the presence of a suitable catalyst, peroxide did not accumulate when the organisms were cultivated under aerobic conditions.

It is known that the function of catalase is to decompose hydrogen peroxide into water and molecular oxygen. Although catalase was unable to promote the growth of anaerobic organisms in contact with air, the addition of an extract rich in catalase raised the level of growth in deep tubes of agar almost to the surface. Also, the appearance of a green-colored ring in chocolate agar cultures of anaerobes was greatly delayed and decreased by the addition of catalase.

It is exceedingly difficult to demonstrate peroxide production by anaerobes since their active life is inhibited by exposure to air before sufficient peroxide has accumulated in cultures to give a positive test. It was shown that colonies of *Clostridium botulinum* appearing on the surface of blood agar plates, previously treated with benzidine, developed dark halos within an hour after exposure to air. This test indicated the production of peroxide in the presence of oxygen. Since obligate anaerobes did not produce catalase, the organisms were unable to destroy the toxic compound.

Bacteria that produce peroxide have active reducing mechanisms. A reducing mechanism is necessary for bringing active hydrogen in contact with oxygen, resulting in the formation of hydrogen peroxide. All bacteria that are active reducers and devoid of catalase produce peroxide or the greenish discoloration in chocolate agar. The amount of catalase produced by different species varies, depending upon the sensitiveness of the organisms to the compound.

Broh-Kahn and Mirsky (1938), in their studies on anaerobiosis, did not agree with the findings and conclusions of McLeod and Gordon. They destroyed the aerobic mechanism of *Escherichia coli* by placing the organisms in a medium containing cyanide. The respiratory system of the cyanide-poisoned organisms was replaced by the addition of a reversible dye system of suitable potential. Under these conditions, the organism

could no longer tolerate free oxygen and behaved as an obligate anaerobe. To quote from their work,

> . . . it is demonstrated that *E. coli*, in the absence of oxygen consumption, is unable to form peroxide. It may, therefore, be assumed that oxygen consumption must precede peroxide formation. . . . To produce the amounts of peroxide found necessary to inhibit growth, amounts of oxygen more than sufficient to be detected manometrically must be consumed. Yet obligate anaerobes have never been found to consume any amount of this gas [Fujita and Kodama (1934); Stickland (1935)]. In the case of such organisms as the pneumococcus and of *E. coli* growing in the presence of cyanide and dye, large amounts of oxygen consumption and peroxide formation may be detected and inhibition may properly be attributed to this factor. In the former organism, the relative tolerance towards $H_2O_2$ allows growth to proceed until amounts of peroxide sufficient to inhibit have accumulated.

3. Quastel and Stephenson (1926) believed that anaerobic growth was dependent upon a low oxidation-reduction potential, which was not possible in the presence of free oxygen. Reed and Orr (1943) found that some 15 species of pathogenic clostridia grew luxuriantly from small inocula in a simple, slightly alkaline, peptone solution, provided it was poised at a favorable oxidation-reduction potential. The optimum Eh was in the region −0.2 volt. A low concentration of glucose produced an oxidation-reduction potential that approximated the optimum for the species. Sodium thioglycollate, cysteine, ascorbic acid, and sodium formaldehyde sulfoxylate produced better poised oxidation-reduction potentials than glucose.

A proper oxidation-reduction potential is generally believed to be the most important reason why anaerobes fail to grow in the presence of free oxygen. However, since cyanide-treated *E. coli* multiplied readily in the presence of air, it is questionable whether the growth of anaerobes depends entirely upon a suitable oxidation-reduction potential.

It may be concluded that no single theory explains all the facts why anaerobes fail to grow in the presence of free oxygen.

## METHODS EMPLOYED FOR THE CULTIVATION OF ANAEROBIC BACTERIA

**Reduction by Heat.**—Tubes of deep broth may be effective for the cultivation of anaerobes. The medium is first heated in an Arnold sterilizer to 100°C. and maintained at this temperature for 10 min. to drive out as much of the dissolved oxygen as possible. The broth is allowed to cool, without disturbing, to a temperature of 50°C. and then inoculated before there is opportunity for redissolution of oxygen from the atmosphere.

**Increasing the pH of the Medium.**—An increase in pH results in an increase in the reducing intensity of the medium. The anaerobic con-

ditions are poor but may permit growth of those anaerobes which are less exacting in their requirements.

**Addition of Reducing Compounds.**—The addition of reducing compounds such as glucose, cysteine, sodium formaldehyde sulfoxylate, and sodium thioglycollate to liquid media usually permits growth of anaerobes under aerobic conditions. Heavy inocula are more effective than light inocula because of the transfer of reducing substances to the new media.

Brewer (1942) introduced a Petri dish cover, together with an agar medium containing sodium thioglycollate and methylene blue, for the surface cultivation of anaerobic bacteria (Fig. 144). The anaerobic agar is melted, cooled to 50°C., poured into a Petri dish, and allowed to harden. The surface of the agar is streaked with the organism. Then the Petri dish cover is replaced by the Brewer anaerobic lid which is designed to touch the agar at the periphery and trap a small amount of air (less than 1 mm. in thickness) over the surface of the agar. The sodium thioglycollate in the medium uses up the oxygen in this small amount of air to create anaerobic conditions. The glass rim on the lid forms a seal with the moist agar preventing the entrance of more atmospheric oxygen. The methylene blue in the agar acts as an indicator, and the center of the dish, which is anaerobic, becomes colorless; the oxygenated edge of the plate, about 5 mm. in diameter, remains blue.

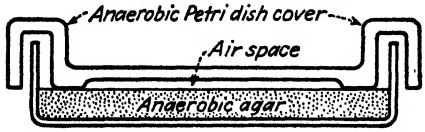



FIG. 144.—Technique for using the Brewer Petri dish cover and anaerobic agar.

**Use of Aerobic Bacteria.**—A tube of deep agar, containing an appropriate carbohydrate, is melted and poured into a Petri dish. When the agar has solidified, the plate is placed in an incubator until the surface is free from droplets of moisture. The plate is divided into two parts by making a heavy line on the bottom of the dish with a china-marking pencil. One-half of the plate is streaked with a culture of an obligate anaerobe; the other half is streaked with a culture of a facultative aerobic organism. The cover is replaced, and the edges of the two halves are carefully sealed with modeling clay. The plate is incubated in an inverted position.

The facultative aerobic organism utilizes the free oxygen and eliminates carbon dioxide. The oxygen tension is soon reduced to a level that permits growth of the anaerobe. Colonies of the anaerobic organism should appear in from 24 to 48 hr.

**Addition of Living or Dead Tissue.**—Pieces of kidney, liver, etc., removed aseptically from an animal, or minced and heat-sterilized beef heart or brain tissue, have been used for the cultivation of obligate anaerobes.

Minced and sterilized brain medium has been used probably more than any other tissue preparation for the cultivation of anaerobes. It is pre-

pared by suspending cooked, minced sheep brains in glucose broth. The medium is heated for about 10 min. in an Arnold sterilizer just before inoculation to drive out as much of the dissolved oxygen as possible. The tubes are allowed to cool to a temperature of about 50°C., then inoculated by loop or pipette plunged deep. A surface seal is not necessary because the minced brain tissue is very efficient in increasing the reducing

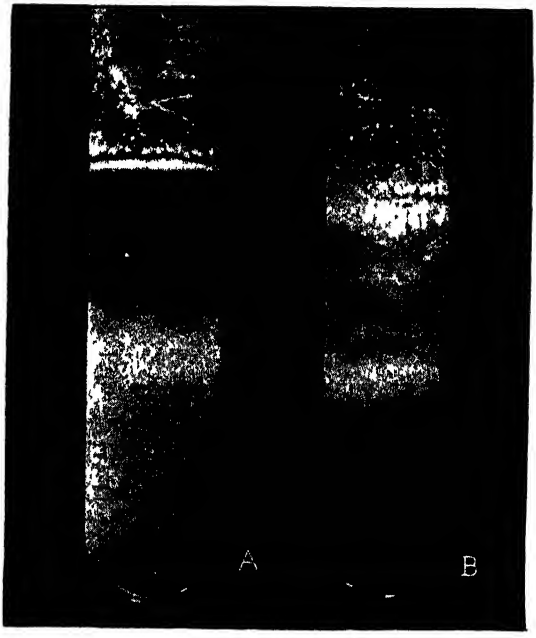



FIG. 145.—Anaerobic cultures of *Clostridium sporogenes*. *A*, minced brain medium; *B*, beef heart medium.

power of the medium. Aerobes grow throughout the medium and anaerobes multiply in the deeper portions (Fig. 145).

**Exclusion of Atmospheric Oxygen.**—A tube of deep nutrient broth or an appropriate carbohydrate broth is inoculated and then covered with a ½-in. layer of melted sterile vaspar (a mixture of equal parts of vaseline and paraffin). It does not prevent entirely the entrance of atmospheric oxygen, but it is usually sufficient in establishing initial growth. After growth has once set in, the elimination of carbon dioxide by the organisms creates optimum conditions for multiplication. It is advisable to heat the media in an Arnold sterilizer for 10 min. previous to inoculation in order to drive out as much of the dissolved oxygen as possible.

Agar may be substituted for the broth. A tube of the medium is melted and cooled to about 50°C. The agar is inoculated and thoroughly mixed by gently shaking and rotating the tube. The medium is allowed to

set in a vertical position after which the surface is covered with a ½-in. layer of melted vaspar

The value of this method is that it affords a simple means of grading the oxygen tension in the medium. On the surface, the pressure is atmospheric; at the bottom, the conditions are anaerobic. The agar cylinder is removed by cutting the tube in the center, pulling the two halves apart, and collecting the agar in a sterile Petri dish. The colonies can then be fished from the agar and examined

FIG. 146.—Bacilli and spores of *Clostridium sporogenes*

**Semisolid Agar Medium.**—An appropriate medium containing from 0.05 to 0 2 per cent of agar is sometimes sufficient in establishing growth of anaerobic organisms. The medium is either liquid or semisolid, depending upon the concentration of agar employed The agar lessens convection currents, thereby prolonging anaerobic conditions in the medium.

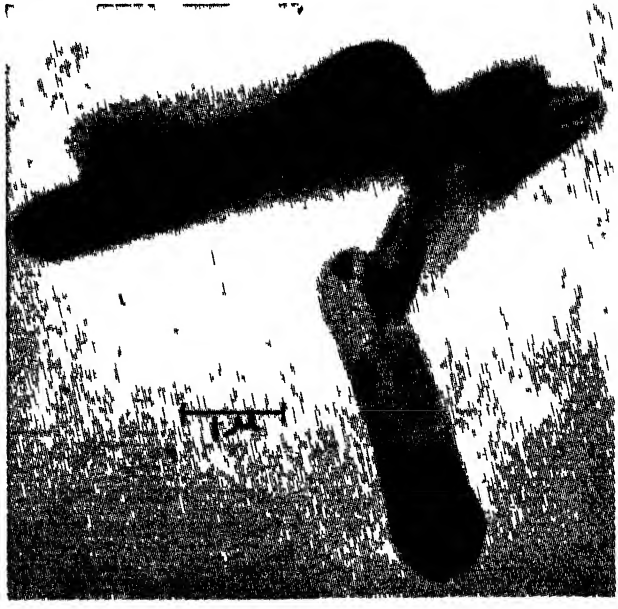


FIG. 147.—Electron micrograph of *Clostridium tetani* cells from a 3-day culture. The terminal "drumstick" spores are formed within the bacterial cells, × 14,000. (*After Mudd and Anderson*)

**Absorption of Atmospheric Oxygen.**—An agar slant is inoculated with the anaerobic organism. The cotton stopper is cut off flush with the neck

of the tube and pushed down about ½ in. from the opening. The surface of the cotton stopper is covered with a layer of pyrogallol crystals and moistened with a few drops of sodium hydroxide solution. A tight-fitting rubber stopper is inserted in the neck of the tube to prevent entrance of atmospheric oxygen. The alkaline pyrogallic acid absorbs oxygen from the environment, creating conditions compatible with the growth of the anaerobe. This procedure is usually satisfactory for those organisms which are not too exacting in their requirements.

A more convenient arrangement is to employ a Bray or a Spray anaerobic culture dish (Fig. 148). The dish is separated at the bottom by a raised center ridge. In one compartment is placed a solution of pyrogallic acid and in the other a solution of sodium hydroxide. Melted agar is poured into a Petri dish and allowed to harden. The surface is streaked with the anaerobic organism. The agar plate is then inverted over the top of the culture dish, and the edges are sealed with a suitable material to prevent entrance of atmospheric oxygen. Finally, the solutions in the bottom are mixed by gently tilting the dish.

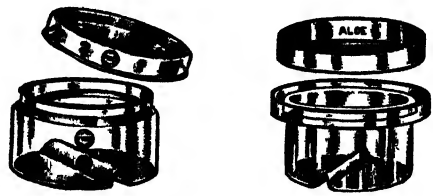


FIG. 148.—Left, Bray anaerobic culture dish; right, Spray anaerobic culture dish.

**Replacement of Atmospheric Oxygen with Hydrogen.**—In this procedure, the inoculated tubes are placed in a closed jar. The air is evacuated and replaced with hydrogen gas. The jar is then placed in an incubator.

This procedure does not always prove satisfactory, owing to the fact that sufficient oxygen usually remains in the medium to prevent growth. The method becomes considerably more efficient if an alkaline solution of pyrogallol is added to the jar just before the air is removed. This is best performed by placing some pyrogallol crystals on the bottom of the jar, followed by the addition of sufficient solution of sodium hydroxide to dissolve the compound. The lid is replaced immediately and the air removed as quickly as possible. The pyrogallol usually removes any oxygen still remaining in the medium, permitting growth of the strict anaerobes.

**Combustion of Oxygen with Hydrogen.**—Surface colonies are most essential for purifying and identifying anaerobes. The methods already described are not entirely satisfactory for the growth of the strict anaerobes.

The removal of oxygen by combustion with hydrogen is probably the most efficient method for obtaining surface colonies of anaerobic organisms. For a description of the apparatus and method of operation, see the report of Fildes (1931).

For additional information on anaerobiosis, see Commoner (1940), McClung (1944), Morton (1943), Vennesland and Hanke (1940).

## REDUCTION OF NITRATES

Many bacterial species are able to reduce nitrates to nitrites and finally to ammonia (Fig. 149). Some species are capable of reducing nitrates to nitrites but cannot reduce nitrites to ammonia. Still others are unable to attack nitrates but can reduce nitrites to ammonia. The nitrates support anaerobic growth by acting as hydrogen acceptors. An example of such a

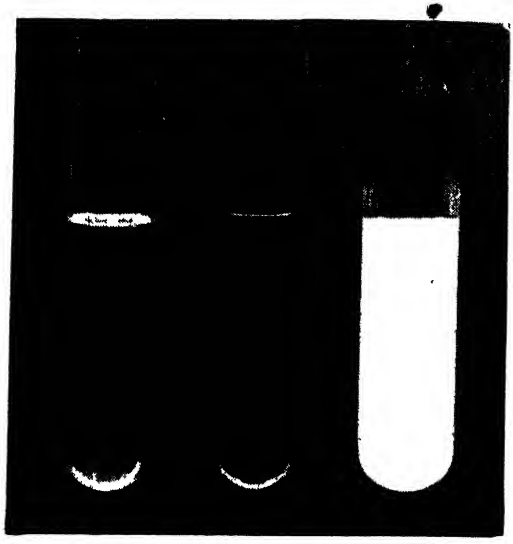


FIG. 149.—Reduction of nitrate to nitrite. Left, control, not inoculated; center, medium inoculated with *Proteus vulgaris*, nitrate reduced to nitrite (red color with sulfanilic acid + α-naphthylamine test solutions); right, medium inoculated with *Pseudomonas fluorescens*, nitrate not reduced.

reaction is the oxidation of lactic acid to pyruvic acid under anaerobic conditions in the presence of potassium nitrate:

$$\underset{\text{Lactic acid}}{CH_3{\cdot}CHOH{\cdot}COOH} + KNO_3 \rightarrow \underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} + H_2O + KNO_2$$

The potassium nitrate accepts hydrogen, being itself reduced to potassium nitrite.

The reduction of nitrates proceeds more rapidly in the presence of an anaerobic or partial anaerobic environment. Organisms capable of reducing nitrates and nitrites are well distributed in nature. It has been shown that many aerobic forms can live under anaerobic conditions in the presence of nitrates. If the culture is well aerated, nitrate reduction does not occur. The reaction of the medium is of importance in determining the extent of reduction. In an alkaline medium, the nitrates are reduced to nitrites, whereas in an acid environment the reaction may proceed to the ammonia stage.

A few species can produce nitrogen gas from nitrates. The organisms are capable of growing in the presence of oxygen but reduce nitrates only under anaerobic conditions. An increase in the oxygen supply causes a corresponding decrease in nitrate reduction.

In making tests for nitrate reduction, observe for (1) the reduction of nitrate to nitrite, (2) the disappearance of nitrite to ammonia, and (3) the presence of nitrogen gas. It is highly important that tests be made for these three products.

The presence of nitrite indicates that the nitrate has been reduced. The presence of gas shows that the nitrite has been reduced to ammonia and finally to nitrogen. Negative results may mean that (1) the organism in question is unable to reduce nitrate or (2) the medium is not satisfactory for growth. The medium may be improved by (1) increasing or decreasing the amount of peptone, (2) increasing the concentration of nitrate, (3) adjusting the reaction of the medium to a more favorable pH, (4) adding a readily available carbohydrate to stimulate growth, or (5) incorporating a small amount of agar to increase the viscosity of the medium.

The nitrite test may be negative even though good growth has occurred. This may indicate no action on the nitrate or complete reduction of the compound beyond the nitrite stage. Therefore, tests for nitrate should be made in every case where a negative nitrite test is obtained. Diphenylamine and concentrated sulfuric acid should be used for the test. A blue color indicates the presence of nitrate. Since nitrite gives the same reaction, the test should not be performed in the presence of this compound. If these tests do not show nitrate reduction, it is probable that the organism is not capable of attacking nitrate.

## REDUCTION OF SULFATES

As early as 1864, it was suggested that the presence of hydrogen sulfide in mud and water was the result of the reduction of sulfates by organisms. Beijerinck (1895) is generally believed to be the first to cultivate an organism from ditch mud capable of reducing sulfates. He named the organism *Spirillum desulfuricans*. It is now known as *Desulfovibrio desulfuricans*.

van Delden (1904) employed the following medium for the successful isolation of the organism:

| | |
|---|---|
| Gelatin | 10 gm. |
| Sodium lactate | 0.5 gm. |
| Asparagine | 0.1 gm. |
| $MgSO_4 \cdot 7H_2O$ | 0.1 gm. |
| $K_2HPO_4$ | 0.05 gm. |
| Ferrous ammonium sulfate | trace |
| Tap water | 1000 cc. |

The organisms obtained their energy from the anaerobic reduction of the sulfate, accompanied by a simultaneous oxidation of the lactate. The proportion of carbonic acid and hydrogen sulfide produced was in the ratio of 2:1. From this observation, van Delden considered the reaction to proceed as follows:

$$2CH_3{\cdot}CHOH{\cdot}COONa + 3MgSO_4 \rightarrow 3MgCO_3 + Na_2CO_3 + 2CO_2 + 2H_2O + 3H_2S$$

In the presence of sodium malate instead of lactate, the following reaction occurred:

$$2COONa{\cdot}CH_2{\cdot}CHOH{\cdot}COONa + 3MgSO_4 \rightarrow 3MgCO_3 + 2Na_2CO_3 + 3CO_2 + H_2O + 3H_2S$$

In this reaction, the molecular proportion between carbonic acid and hydrogen sulfide was about 8:3.

A general equation for the reduction of sulfates may be represented as follows:

$$2C + MeSO_4 + H_2O \rightarrow MeCO_3 + CO_2 + H_2S$$

in which C represents the organic substrate and Me a metal.

Baars (1930) showed that a large number of organic compounds could be substituted for the lactate or malate, all of which were oxidized in an appropriate sulfate medium inoculated with *D. desulfuricans*. The organic compounds were oxidized by the removal of hydrogen, *i.e.*, they acted as hydrogen donators. A few typical examples are the following:

$$\underset{\text{Formic acid}}{H{\cdot}COOH} \rightarrow CO_2 + 2H$$

$$\underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + 2H_2O \rightarrow 2CO_2 + 8H$$

$$\underset{\text{Lactic acid}}{CH_3{\cdot}CHOH{\cdot}COOH} + 3H_2O \rightarrow 3CO_2 + 12H$$

$$\underset{\text{Glycerol}}{CH_2OH{\cdot}CHOH{\cdot}CH_2OH} + 3H_2O \rightarrow 3CO_2 + 14H$$

In the reduction of sulfate, each molecule required 8 atoms of hydrogen for its conversion into hydrogen sulfide. The dehydrogenation of the organic substrate proceeded by steps followed by a corresponding hydrogenation of the sulfate to produce hydrogen sulfide:

$$H_2SO_4 + 8H \rightarrow H_2S + 4H_2O$$

It was shown that, unless the organic compound was present in large excess, complete oxidation did not occur. van Delden's medium contains 15 millimols of sulfate per liter. From this, it follows that 10 millimols of lactate should be sufficient to produce complete reduction of the sulfate. In actual experiment, however, it was found that 30 millimols were required for complete oxidation. The reaction for the incomplete oxidation was

$$2CH_3{\cdot}CHOH{\cdot}COONa + MgSO_4 \rightarrow 2CH_3{\cdot}COONa + MgCO_3 + CO_2 + H_2O + H_2S$$

*Organism.—Desulfovibrio desulfuricans* is an obligate anaerobe, which produces spores under natural conditions but fails to do so on laboratory media. The organisms can be isolated by inoculating soil or mud into synthetic media containing 0.5 per cent sodium sulfate and incubating the culture under anaerobic conditions. A loopful of the liquid culture is then streaked over the surface of a sulfate agar plate containing an iron salt as an indicator. The hydrogen sulfide produced by the organisms reacts with the iron to impart a black color to the colonies.

## CARBON DIOXIDE REQUIREMENT OF BACTERIA

It is now well known that bacteria do not grow in the complete absence of carbon dioxide. An accumulation of a definite amount of the gas is necessary to initiate growth. Many bacteria grow better in the presence of an increased concentration of carbon dioxide. Some organisms, when freshly isolated from disease processes, either do not grow or grow very poorly, unless the carbon dioxide concentration of the environment is increased to from 5 to 10 per cent. After growth on artificial media is once established, an increased concentration of the gas is no longer necessary. Since carbon dioxide is a product of the metabolism of organisms, it was formerly thought to be a useless, toxic, waste gas. Such a statement cannot now be considered true.

Certain bacteria contain a pigment known as bacteriochlorophyll, which enables them to manufacture their own carbohydrates from carbon dioxide and water in the presence of light. These are the photosynthetic bacteria. However, the great majority of bacteria do not contain bacteriochlorophyll and are not capable of synthesizing their own carbohydrates from carbon dioxide and water.

It is now generally accepted that carbon dioxide plays an important role in the metabolism of a variety of heterotrophic organisms. It is utilized by these organisms and becomes incorporated in the carboxyl groups of carboxylic acids (see also page 379).

Rahn (1941) was not able to detect the presence of carbon dioxide in cultures of *Lactobacillus acidophilus, L. casei,* and *Streptococcus lactis.* It is quite likely that the gas was utilized by the organisms as rapidly as it was formed.

Slade, Wood, Nier, Hemingway, and Werkman (1942) employed carbon dioxide containing heavy carbon ($C^{13}O_2$) as a tracer and found that the assimilation of the gas was a general phenomenon among the heterotrophic bacteria. The fixed carbon was located in the carboxyl groups of succinic, lactic, and acetic acids. Succinic acid contained fixed carbon in every case in which it was formed. Conversely, lactate and acetate did not contain fixed carbon in all cases. There were, therefore, fundamental dif-

ferences in the mechanism of formation of these compounds among the heterotrophic bacteria.

The following mechanism for the formation of succinic acid from pyruvic acid was suggested:

$$\underset{\text{Pyruvic acid}}{C^{13}O_2 + CH_3{\cdot}CO{\cdot}COOH} \rightleftharpoons \underset{\text{Oxalacetic acid}}{C^{13}OOH{\cdot}CH_2{\cdot}CO{\cdot}COOH}$$

$$C^{13}OOH{\cdot}CH_2{\cdot}CO{\cdot}COOH + 4H \rightleftharpoons \underset{\text{Succinic acid}}{C^{13}OOH{\cdot}CH_2{\cdot}CH_2{\cdot}COOH} + H_2O$$

Barker and Kamen (1945) showed that *Clostridium thermoaceticum* fermented carbohydrates, such as glucose and xylose, with the production of acetic acid without any accompanying carbon dioxide. They employed carbon dioxide containing heavy carbon ($C^{14}O_2$) as a tracer and concluded from their results that the acetic fermentation of glucose by *C. thermoaceticum* involved a partial oxidation of the substrate to two molecules each of acetic acid and carbon dioxide followed by a reduction and condensation of the carbon dioxide to a third molecule of acetic acid:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 8H$$
$$8H + 2CO_2 \rightarrow CH_3COOH + 2H_2O$$

The over-all reaction being,

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH$$

About 94 per cent of the $C^{14}$ from the added carbon dioxide disappeared during the fermentation and approximately 81 per cent was recovered in the acetic acid.

Barker, Kamen, and Haas (1945) cultivated *Butyribacterium rettgeri* in a medium containing lactate and $C^{14}O_2$ and found that the main products were carbon dioxide, acetic, and butyric acids. The fermentation was unusual because of the low yield of carbon dioxide and the high yield of fatty acids. Analyses showed that all the carbon positions of the fatty acids contained the labeled $C^{14}$ isotope. They suggested the following scheme for the synthesis of the fatty acids from lactate and $C^{14}O_2$:

$$CH_3{\cdot}CHOH{\cdot}COOH + H_2O \rightarrow CH_3{\cdot}COOH + CO_2 + 4H$$
$$2CO_2 + 8H \rightarrow CH_3{\cdot}COOH + 2H_2O$$
$$2CH_3{\cdot}COOH + 4H \rightarrow CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}COOH + 2H_2O$$

**Variation in $CO_2$ Production with Age.**—Young cells show a much higher rate of metabolism than old cells. Huntington and Winslow (1937) reported that carbon dioxide production of 1-hr.-old bacteria was from 3 to 14 times higher than that of 24-hr.-old cells. The peak of $CO_2$ production per cell was reached in from 2 to 5 hr. after inoculation of old cells into fresh medium. Rahn (1941) reported similar results. The removal of $CO_2$, by absorption in a solution of KOH, did not prevent growth of young cells unless the number per drop was very small. When a drop

showed less than 10 organisms, growth did not occur in the absence of $CO_2$.

**Effect of Large and Small Inocula.**—Rahn showed that heavy cell concentrations produced $CO_2$ more rapidly than it could be removed, with the result that the bacteria developed even though potassium hydroxide was present to absorb the gas. Light cell concentrations, on the other hand, failed to produce sufficient gas in the presence of KOH to permit growth. The probability of growth was lessened as the number of cells per drop decreased. The fact that a large inoculum produces more carbon dioxide than a small one would seem to afford an explanation for the shorter lag phase with larger inocula (see page 257).

For more information, see Foster, Carson, Ruben, and Kamen (1941), Krampitz, Wood, and Werkman (1943), van Niel, Ruben, Carson, Kamen, and Foster (1942), van Niel, Thomas, Ruben, and Kamen (1942), and Wood (1946).

## OXYGEN REQUIREMENT OF BACTERIA

It may be stated that all living plants and animals consume oxygen and eliminate carbon dioxide. Bacteria also utilize oxygen (either free or combined) and eliminate carbon dioxide.

Rahn and Richardson (1940) reported that the oxygen supply of bacteria in the usual cultural methods was far from adequate. As an example, the tubercle bacillus requires 1.22 per cent oxygen for the complete oxidation of 1 per cent of glycerol. The medium, at 37°C., contains only 0.00065 per cent of oxygen. The disproportion between supply and demand is so great that a majority of the cells in a culture probably starve for lack of oxygen.

Rahn and Richardson measured the oxygen consumption of multiplying bacteria per cell per hour. The results are recorded in Table 35. The consumption of oxygen did not depend entirely on cell size. *Pseudomonas* required more oxygen than *E. coli* for the same amount of cell substance.

**Sensitiveness of Organisms to Oxygen.**—Organisms differ widely in their sensitiveness to free oxygen. Some are depressed in growth by slight increases in the concentration of oxygen present in air; others are stimulated in growth by concentrations up to 60 per cent.

Aerobic organisms inoculated into favorable culture media grow in diminished oxygen tensions, but the rate of multiplication is less than under ordinary atmospheric conditions. However, entirely different results are obtained when such organisms are cultivated under slight or extensive starvation. Cahn-Bronner (1940) cultivated *Serratia marcescens, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhosa, Salmonella schottmuelleri, Shigella dysenteriae, Shigella paradysenteriae, Corynebacterium diphtheriae, Micrococcus pyogenes* var. *aureus*, and *M. pyogenes* var. *albus* under partial starvation in deep tubes of agar and found that the organisms

grew in a fine, dense layer below the surface of the medium where the relation between the nutrient content and oxygen was at an optimum. Under increasing starvation (more dilute medium), the bacteria became pro-

TABLE 35.—OXYGEN CONSUMPTION FOR A NUMBER OF BACTERIAL SPECIES

| Organism | Oxygen Consumption per Cell per Hr., $\times 10^{-10}$ Mg. |
|---|---|
| *Streptococcus liquefaciens* | 7.22 |
| *Streptococcus faecalis* | 2.32 |
| *Streptococcus durans* | 1.07 |
| *Streptococcus lactis* 125 | 0.46 |
| *Streptococcus lactis* L21 | 2.10 |
| *Escherichia coli* | 3.9 |
| *Escherichia coli* var. *communis* | 3.0 |
| *Aerobacter cloacae* | 4.0 |
| *Aerobacter aerogenes* | 3.3 |
| *Proteus vulgaris* | 5.3 |
| *Pseudomonas fluorescens* | 9.3 |
| *Pseudomonas aeruginosa* | 8.4 |
| *Bacillus subtilis* | 20.6 |
| *Bacillus cereus* | 32.4 |
| *Bacillus mesentericus* | 9.5 |
| *Bacillus megatherium* | 65.0 |
| *Bacillus peptogenes* | 82.4 |

gressively more sensitive to oxygen. The lower the concentration of carbon compounds in the medium, which may act as oxygen acceptors, the more inhibitory was the action of oxygen on the organisms.

Potter (1942) restricted the oxygen supply of human, bovine, and avian tubercle bacilli, in the presence of moisture and warmth, and found that the organisms slowly lost their capacity for subsequent growth, both in culture and in animal tissues.

## RESPIRATORY QUOTIENTS OF BACTERIA

The ratio of the amount of carbon dioxide eliminated to the oxygen consumed is known as the respiratory quotient. This may be written

$$\frac{CO_2}{O_2} = \text{R.Q.}$$

On complete oxidation, a carbohydrate, such as glucose, yields $CO_2$ and $H_2O$ according to the equation:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$$

Since the volume of $CO_2$ eliminated is the same as the oxygen consumed, the theoretical respiratory quotient of a carbohydrate is

$$\frac{6CO_2}{6O_2} = 1.00$$

Fat combustion is usually represented by the oxidation of palmitin as follows:

$$2(C_{15}H_{31}COO)_3C_3H_5 + 145O_2 \rightarrow 102CO_2 + 98H_2O$$

The theoretical respiratory quotient of a fat is

$$\frac{102CO_2}{145O_2} = 0.70$$

In like manner, the theoretical respiratory quotient of a protein on complete combustion is 0.80.

The presence of an easily utilizable carbohydrate in a medium gives a quotient of approximately 1.00. In the absence of such carbohydrate the quotient approaches that of a protein. The respiratory quotient gives an insight as to the kinds of nutrients being utilized by an organism.

TABLE 36.—RESPIRATORY QUOTIENTS OF A NUMBER OF BACTERIA ON DIFFERENT MEDIA

| Organism | Nutrient agar | Glycerol agar | Glucose agar | Serum agar |
|---|---|---|---|---|
| *Mycobacterium tuberculosis* var. *hominis* | ..... | 0.856 | 0.992 | 0.904 |
| *Mycobacterium tuberculosis* var. *bovis* | 0.888 | 0.903 | 1.036 | 0.852 |
| *Bacillus subtilis* | 0.912 | 0.843 | 1.278 | 0.874 |
| *Malleomyces mallei* | 0.841 | 0.859 | 0.972 | |
| Theoretical value | 0.810 | 0.857 | 1.000 | 0.810 |

Soule (1928) reported the respiratory quotients of a number of organisms grown on various types of culture media. The results are recorded in Table 36. It may be seen that the addition of glucose to media resulted in an increase in the respiratory quotients. Since the combustion of pure glucose yields a quotient of 1.00, this figure was closely approximated in several cases. Figures less than 1.00 indicated that the organisms utilized amino acids as well as glucose. In the absence of glucose, the respiratory quotients approximated that of pure protein.

A factor that complicates the accuracy of the quotients is the decarboxylation of amino acids and organic acids, yielding more carbon dioxide without a corresponding utilization of oxygen. This may possibly account for those quotients greater than 1.00.

## ASSIMILATION AND RESPIRATION

Barker (1936) showed that the oxidation of carbohydrates by certain organisms, in the presence of a plentiful supply of oxygen, did not proceed

to completion ($CO_2 + H_2O$) but that a portion of the substrate was assimilated by the cells. He represented the oxidative assimilation of acetate by the organism *Prototheca zopfii* as follows:

$$\underset{\text{Acetic acid}}{CH_3 \cdot COOH} + O_2 \rightarrow (CH_2O) + CO_2 + H_2O$$

The acetic acid was oxidized to carbon dioxide, water, and a compound having the empirical formula of a carbohydrate. Barker concluded that the oxidation of organic compounds by *P. zopfii* was a process of oxidative assimilation leading to the synthesis of a carbohydrate that was stored in the cells.

Clifton (1937) experimented with the organism *Pseudomonas calcoacetica* and obtained results similar to those reported by Barker. The oxidative assimilation of acetate and butyrate by the organism was represented by the equations:

$$\underset{\text{Acetate}}{2CH_3 \cdot COOH} + 3O_2 \rightarrow (CH_2O) + 3CO_2 + 3H_2O$$

$$\underset{\text{Butyrate}}{2C_3H_7 \cdot COOH} + 7O_2 \rightarrow 3(CH_2O) + 5CO_2 + 5H_2O$$

On the addition of suitable concentrations of iodoacetate, sodium azide, 2,4-dinitrophenol, or methyl urethane, Clifton was able selectively to poison the cells in such a way that assimilation was prevented, but not respiration, thus effecting a complete oxidation of the substrate to $CO_2$ and $H_2O$. In the absence of the assimilatory poisons, the respiratory quotient of butyrate during the stage of rapid oxidation was found to be 0.68. In the presence of the poisons (complete oxidation), the respiratory quotient was 0.8.

Others who have reported similar results include Giesberger (1936) in his studies on three species of *Spirillum;* Clifton and Logan (1939) on *Escherichia coli;* Stier, Newton, and Sprince (1939), Stier and Newton (1939), Winzler and Baumberger (1938), Pickett and Clifton (1943), and van Niel and Anderson (1941) on the yeast *Saccharomyces cerevisiae;* and Burris and Wilson (1942) on species of rhizobia.

## CHROMOGENESIS

A large number of bacteria produce colored compounds known as pigments. This is especially true of the strictly aerobic bacteria. Many colors are produced ranging from red to violet.

Very little is known concerning the chemical composition of pigments. This is due largely to the fact that it is difficult to obtain sufficient pigment in a high state of purity. Some of the pigments remain confined within the bacterial cells; others are secreted into the surrounding media giving them a characteristic appearance. Only a few of them are water-soluble.

The majority of them are soluble in fat solvents such as alcohol, acetone, ether, and chloroform.

**Conditions Necessary for Pigment Production.**—It is probable that pigments are produced only in the presence of oxygen. When pigmented cultures are placed in an anaerobic environment, the color gradually fades until completely decolorized. On exposure of such cultures to oxygen, the color gradually returns. Certain special media are required for strong pigment production. Solid media are better than liquid preparations. In some cases, certain mineral salts are necessary. The hydrogen-ion concentration of the medium influences the color of some pigments. A temperature of 20°C. appears to be best for the production of most pigments. As the temperature increases, pigment production gradually decreases.

Chapman (1943) recommended the use of 10 per cent evaporated milk agar for the demonstration of pigment production by species of *Micrococcus*. Mayer (1944*a*, *b*) reported that a strain of *Mycobacterium tuberculosis* var. *hominis* formed a bright yellow pigment when grown in the presence of *p*-aminobenzoic acid (PABA). The pigment was formed from PABA by an enzyme that appeared to be a specific oxidase.

**Effect of Light.**—With few exceptions (notably the green and purple photosynthetic bacteria), pigment formation takes place best in the dark. Baker (1938) examined 185 strains of acid-fast organisms and found that 12 of them produced their characteristic pigment only in the presence of light. Pigment was entirely absent when the organisms were cultivated in total darkness.

**Types of Bacterial Pigments.**—Most bacterial pigments may be classified as (1) carotenoids, (2) anthocyanins, and (3) melanins.

*Carotenoids.*—The carotenoids are red, orange, or yellow pigments, which are soluble in fat solvents such as alcohol, ether, or chloroform. They derive their name from the unsaturated hydrocarbon carotene, $C_{40}H_{56}$, the same coloring matter present in butter, yolks of eggs, flour, and carrots. Oxidized carotene, $C_{40}H_{56}O_2$, is known as xanthophyll. The two pigments are usually found together in nature.

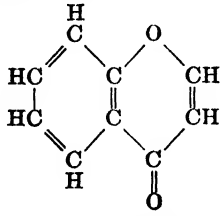
Some organisms that produce carotenoid pigments include the following:

1. *Sarcina lutea* produces bacterioxanthophyll.
2. *S. aurantiaca* shows the presence of $\beta$-carotene and zeaxanthin.
3. *Micrococcus pyogenes* var. *aureus* gives zeaxanthin.
4. *Mycobacterium phlei* produces lutein, kryptoxanthin, and $\alpha$-, $\beta$-, and $\gamma$-carotenes.
5. *Torula rubra*, a red yeast, produces $\beta$-carotene and torulene.

Wheeler and Foley (1942), by means of spectrophotometric analysis, reported the presence of carotenoid pigments in hemolytic streptococci. Sobin and Stahly (1942) extracted pigments from 12 strains of *M. pyogenes* var. *aureus* and found that $\gamma$-carotene and rubixanthin were present in all

of them. Haas and Bushnell (1944) isolated a strain of *Mycobacterium lacticola* from mud in contact with crude oil and found it to produce carotenoid pigments when cultivated in a mineral salts medium with paraffin oil.

*Anthocyanins.*—The anthocyanins include the red and blue pigments and intermediate shades found in the petals of many flowers and some bacteria. They are soluble in water and alcohol but not in ether. Most, if not all, of them are natural indicators, changing usually from red in acid solutions to green in alkaline solutions. They are glucosides and on hydrolysis yield a sugar and some derivative of benzopyrilium:



Benzopyrilium

The chief derivatives are said to be pelargonidin, cyanidin, delphinidin, and oenidin.

Several species of *Actinomyces* have been reported as being capable of producing anthocyanin pigments.

*Melanins.*—The melanins include certain brown, black, orange, and red pigments that are insoluble in almost all solvents. They are dissolved by concentrated sulfuric acid and reprecipitated by the addition of water. The pigments are produced from the decomposition of proteins by boiling concentrated mineral acids, or by the action of the enzyme tyrosinase on the amino acid tyrosine. In addition to tyrosine, the enzyme attacks several other compounds including tryptophane.

The following organisms produce melanin pigments:

1. *Azotobacter chroococcum* forms a black melanin pigment.
2. *Clostridium perfringens* is said to elaborate a black pigment.
3. *Aspergillus niger* produces a black melanin pigment known as aspergillin.
4. Many species of *Actinomyces* produce various colored melanin pigments.

**Function of Pigments.**—Little is known concerning the physiological functions of nonphotosynthetic pigments. Because pigment production generally takes place in the presence of oxygen, some believe that they act as respiratory carriers. Evidence available at the present time, however, is not sufficient to support this view.

For more information, see Bancroft (1943) and Conn (1943).

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## CHAPTER XIV

## DECOMPOSITION AND PUTREFACTION OF PROTEINS

Proteins are extremely complex compounds, being essential constituents of all living cells, both animal and vegetable. The name protein is derived from the Greek πρῶτος, which means preeminence, or of first importance. All proteins contain carbon, hydrogen, nitrogen, and oxygen. Sulfur is found in all except certain basic proteins known as the protamines. The proportions of these constituents are approximately as follows: C, 51 per cent; H, 7 per cent; N, 16 per cent; O, 25 per cent; and S, 0.4 per cent.

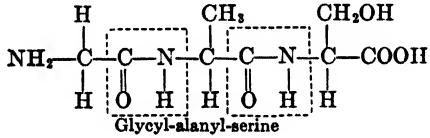
All proteins form colloidal solutions which means either that they are composed of very large molecules or that the molecules form aggregates of colloidal dimensions. One of the properties of colloids in solution is their inability to pass through certain membranes, such as parchment, collodion, and animal. Most proteins form opalescent solutions—another indication that their molecules are large. They are usually amorphous, but some have been obtained in crystalline form. Colloidal substances are classed either as suspensoids or emulsoids. Suspensoids (lyophobic colloids) do not show any affinity for the dispersion medium. On the other hand, emulsoids (lyophilic colloids) show a strong affinity for the dispersion medium. Proteins form solutions of the emulsoid type. Proteins are precipitated from solution by salts of heavy metals, such as mercuric chloride and silver nitrate, by tannic acid, phosphotungstic acid, etc. Proteins are amphoteric compounds, being capable of reacting with both acids and bases to form ionizable salts.

Another property of proteins is that, when they are treated with proteolytic enzymes, or heated for some time with mineral acids, the molecules are decomposed into a mixture of compounds known as amino acids, which are sometimes referred to as the building stones of the protein molecule. The hydrolysis of proteins to the stage of amino acids results in a complete loss of colloidal characteristics.

About 20 amino acids have been recognized as constituents of protein molecules. They are all soluble in water, with the exception of tyrosine and cystine. Tyrosine is sparingly soluble in cold water but more soluble in hot water, and cystine is sparingly soluble in both hot and cold water. They are all soluble in dilute acids and alkalies, with the exception of cystine, which dissolves with difficulty in dilute ammonia water. All the amino acids possess at least one free amino group ($NH_2$) and one free

carboxyl group (COOH), except proline and oxyproline, which contain an imino (NH) group instead of an amino group. This means that they are amphoteric compounds, being capable of reacting with both acids and bases (page 192). Most of the acids have one free amino group and one free carboxyl group. These are known as mono-amino-mono-carboxylic acids and are neutral in reaction. Some have two amino groups to one carboxyl group. These are di-amino-mono-carboxylic acids and are alkaline in reaction. Others have one amino to two carboxyl groups. These are mono-amino-di-carboxylic acids and are acid in reaction.

Many theories have been advanced to explain how the amino acids are linked together to produce a protein molecule. It is generally agreed that the amino acids in proteins are linked together principally through their $\alpha$-amino and carboxyl groups to produce the so-called peptid linkage (R·CONH·R′). Other types of linkages probably occur but, from the known facts, the peptid linkage is the principal one. For example, if a tripeptid is composed of glycine, alanine, and serine, the amino acids would be joined together in the following manner, according to the peptid linkage:



Glycyl-alanyl-serine

The hydrolysis of proteins by proteolytic enzymes results in the formation of the following fractions and in the order named:

Proteins → proteoses (albumoses) → peptones → peptids → amino acids

## PROTEOLYTIC ENZYMES

Enzymes that open up or hydrolyze peptid linkages are grouped under the proteases. The proteases are further subdivided into the proteinases and peptidases. The proteinases attack the true proteins. They do not hydrolyze proteins beyond the polypeptid or dipeptid stage. The peptidases hydrolyze polypeptids and dipeptids to the stage of amino acids. This may be schematically respresented as shown in Fig. 150.

Berger, Johnson, and Peterson (1938*a*, *b*) investigated the peptidases elaborated by a number of organisms. The specificity of the peptidase systems of *Bacillus megatherium* and *Escherichia coli* was studied in some detail. Dipeptids and tripeptids were readily hydrolyzed, but acylated or decarboxylated peptids were hydrolyzed very slowly or not at all. Substitution of a methyl group for a hydrogen atom of the free amino group on a peptid resulted in a very marked decrease in hydrolysis. The peptidase system from *Leuconostoc mesenteroides* was capable of hydrolyzing

both optical components of the racemic peptids leucylglycine, leucyldiglycine, alanylglycine, and alanyldiglycine. The peptidase complex appeared to contain at least two dipeptid-splitting enzymes, at least three polypeptidases, and an acylase but no carboxypeptidase. Appreciable amounts of peptidases were found in culture filtrates of *E. coli* and *B. megatherium*. With *E. coli* more enzymes could be extracted from the cells than from the medium on which they were grown. This was to be expected with a nonproteolytic organism. With the proteolytic organism, *B. megatherium*, more peptidases were consistently found in a given volume

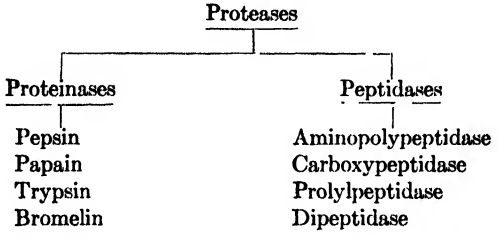



FIG. 150.—Classification of some of the proteases.

of medium than could be obtained from the cells grown on the same volume of medium.

**Protein Decomposition.**—Protein decomposition is defined in various ways, but the term is used here to indicate the action of proteolytic enzymes on a protein resulting in the disruption of the colloidal molecule into diffusible substances. The bacterial enzymes responsible for the action are probably all extracellular and hydrolytic in nature. As has already been explained (page 303), the function of the extracellular enzymes is to convert insoluble, indiffusible compounds into soluble, diffusible substances which can be absorbed by the bacterial cell. The compounds produced are probably without odor. The extracellular enzymes play no part in the respiratory activities of the cell, such function being performed by the intracellular enzymes.

**Putrefaction.**—The term "putrefaction" may be defined as the anaerobic decomposition of proteins, protein split products, and nitrogenous compounds of a similar nature, with the formation of foul-smelling compounds. Probably all the reactions take place inside the cell by means of the intracellular or respiratory enzymes. The putrefactive changes are the result of the action of organisms on the individual amino acids. The compounds produced include methyl mercaptan, hydrogen sulfide, amines, alcohols, organic acids, hydroxy acids, indole, phenol, cresol, ammonia, methane, carbon dioxide, and hydrogen.

Bacteria vary considerably in their ability to degrade proteins or pro-

tein split products. Organisms are usually designated as putrefactive or fermentative, depending upon whether they act more vigorously on proteins or on fermentable substances. Many of the putrefactive compounds are produced only from specific amino acids whereas others may be produced from more than one acid.

**Decay.**—Decay may be defined as the aerobic decomposition of proteins in which the products of putrefaction are completely oxidized to stable compounds having no foul odors. The process takes place in nature by aerobic organisms in the presence of a plentiful supply of air after the putrefactive changes have occurred. If the substrate is well aerated from the start, the reactions will be aerobic without the formation of foul-smelling compounds.

A practical application of this principle is employed in the disposal of sewage. In one process, the sewage is first digested by the anaerobic organisms resulting in the liberation of offensive odors. The anaerobic digestate is then well aerated, after which the aerobic organisms digest the foul-smelling compounds, resulting in the disappearance of the offensive odors. In another process, the sewage is kept well aerated from the start, thus preventing the growth of anaerobic organisms. The aerobic digestion occurs without the formation of ill-smelling compounds.

For more information on proteolytic enzymes, see Bergmann and Fruton (1941), Fruton (1938, 1941), Gale (1940, 1943), and Grob (1946*a*,*b*).

## ACTION OF BACTERIA ON PROTEINS

Bainbridge (1911) first reported that native proteins are resistant to bacterial attack, even by the most proteolytic species. He employed an inorganic medium to which was added egg albumin and other proteins as the only source of nitrogen and carbon. The test organisms included *Escherichia coli*, *Salmonella typhosa*, *Proteus vulgaris*, *Micrococcus pyogenes* var. *aureus*, *Pseudomonas aeruginosa*, *S. enteritidis*, and *Neisseria gonorrhoeae*. The organisms not only failed to degrade the pure proteins but showed no increase in numbers. If, however, a small amount of peptone was added to the medium, multiplication of the organisms and degradation of the proteins took place.

Later Sperry and Rettger (1915) confirmed and extended the observations of Bainbridge. They employed aerobic, anaerobic, and facultative species in both nonsynthetic and synthetic media. None of the species employed was capable of degrading the proteins. The addition of a trace of peptone resulted in the decomposition of the proteins.

The results appear to indicate that an extracellular enzyme is necessary to convert the indiffusible proteins into diffusible compounds. In the absence of an available nitrogen and carbon source, the organisms are unable to multiply and elaborate the necessary enzyme or enzymes. The addition of peptone furnishes a utilizable source of nitrogen and carbon,

thus permitting the organisms to multiply and elaborate the necessary extracellular proteolytic enzymes.

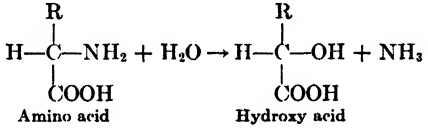
Berman and Rettger (1916, 1918) showed that proteoses are also immune to attack by bacteria. Proteoses or albumoses are intermediate products in the digestion of proteins by proteolytic enzymes. Here again the addition of a small amount of peptone to the medium stimulated the organisms to secrete the proteolytic enzyme or enzymes necessary to attack the proteoses.

The results indicate that proteins and higher split fractions such as the proteoses are not available as sources of nitrogen and carbon. Peptids and amino acids appear to be essential nitrogenous food constituents. This explains why commercial peptones are so extensively employed in bacteriological culture media.

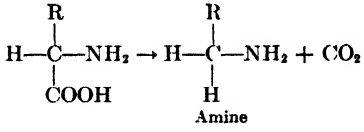
## ACTION OF BACTERIA ON AMINO ACIDS

Amino acids may be attacked by bacteria in a variety of ways. The types of chemical reactions involved include the following:

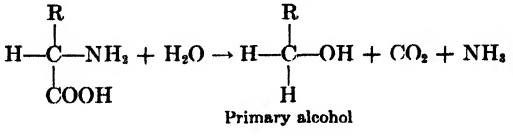
1. Hydrolytic deaminization resulting in the formation of a hydroxy acid:



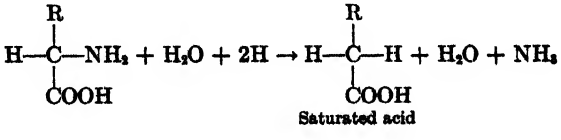

2. Decarboxylation or elimination of $CO_2$, resulting in the formation of an amine with one less carbon atom:



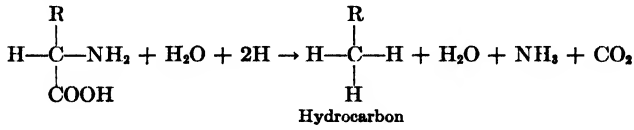

3. Deaminization and decarboxylation, resulting in the formation of a primary alcohol with one less carbon atom:



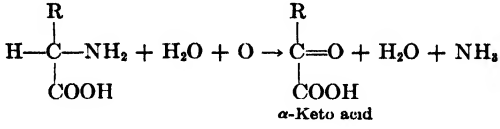

4. Deaminization and reduction, resulting in the formation of a saturated acid:



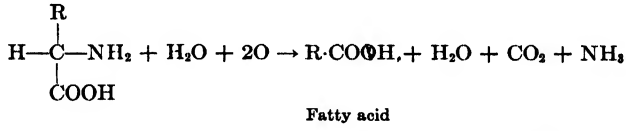
5. Deaminization, decarboxylation, and reduction, resulting in the formation of a hydrocarbon:



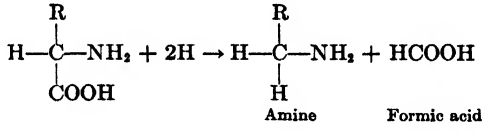

6. Deaminization and oxidation, resulting in the formation of an $\alpha$-keto acid:



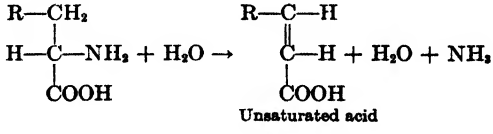

7. Deaminization, decarboxylation, and oxidation, resulting in the formation of a fatty acid with fewer C atoms:




8. Reduction and elimination of formic acid, resulting in the formation of an amine with one less C atom:




9. Deaminization and desaturation at the $\alpha$-$\beta$-linkage, resulting in the formation of an unsaturated acid:




10. Anaerobic decomposition resulting in the liberation of hydrogen.

The foregoing types of chemical reactions or their combinations are able to account for all or nearly all the products of decomposition and putrefaction produced by bacteria from amino acids.

In Table 37 are included the names and formulas of almost all the amino acids present in proteins, and the products formed by bacterial action. The formulas of the amino acids are given at the top of each division. The list does not include all possible compounds but most of those reported to be produced by bacterial action. The numbers refer to the types of chemical reactions already given.

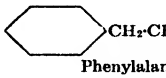
TABLE 37.—ACTION OF BACTERIA ON THE AMINO ACIDS

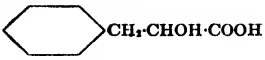
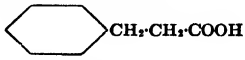
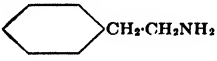
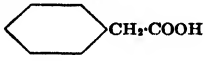
| | |
|---|---|
| $CH_2NH_2 \cdot COOH$<br>Glycocoll or glycine | |
| (2) Methylamine, $CH_3NH_2$<br>(4) Acetic acid, $CH_3 \cdot COOH$ | (5) Methane, $CH_4$ |
| $CH_3 \cdot CHNH_2 \cdot COOH$<br>Alanine | |
| (2) Ethylamine, $C_2H_5NH_2$<br>(3) Ethyl alcohol, $C_2H_5OH$ | (4) Propionic acid, $C_2H_5 \cdot COOH$<br>(7) Acetic acid, $CH_3 \cdot COOH$ |
| $(CH_3)_2CH \cdot CHNH_2 \cdot COOH$<br>Valine | |
| (2) Isobutylamine, $(CH_3)_2CH \cdot CH_2NH_2$<br>(4) Isovaleric acid, $(CH_3)_2CH \cdot CH_2 \cdot COOH$ | (7) Isobutyric acid, $(CH_3)_2CH \cdot COOH$ and<br>Acetic acid, $CH_3 \cdot COOH$<br>Formic acid, $HCOOH$ |
| $(CH_3)_2CH \cdot CH_2 \cdot CHNH_2 \cdot COOH$<br>Leucine | |
| (1) Leucic acid, $(CH_3)_2CH \cdot CH_2 \cdot CHOH \cdot COOH$ | (2) Isoamylamine, $(CH_3)_2CH \cdot CH_2 \cdot CH_2NH_2$ |
| $CH_3 \cdot C_2H_5CH \cdot CHNH_2 \cdot COOH$<br>Isoleucine | |
| (4) Methylethylpropionic acid (*d*-caproic acid), $CH_3 \cdot C_2H_5CH \cdot CH_2 \cdot COOH$ | (7) Methyl ethyl acetic acid, $CH_3 \cdot C_2H_5CH \cdot COOH$ |

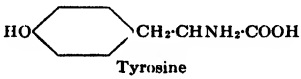
TABLE 37.—(*Continued*)

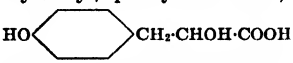
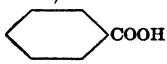
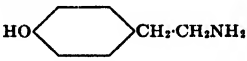
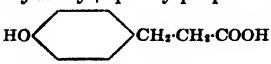
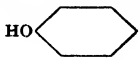
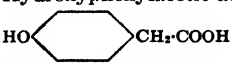
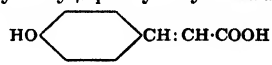
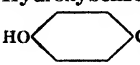
$CH_2OH \cdot CHNH_2 \cdot COOH$
Serine

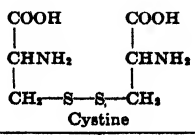
| | |
|---|---|
| (2) Aminoethyl alcohol, $CH_2OH \cdot CH_2NH_2$ | (4) Propionic acid, $C_2H_5 \cdot COOH$ |
| (3) Ethylene glycol, $CH_2OH \cdot CH_2OH$ | (7) Formic acid, HCOOH |


Phenylalanine

| | |
|---|---|
| (1) Phenyllactic acid,  | (4) Phenylpropionic acid,  |
| (2) Phenylethylamine,  | (7) Phenylacetic acid,  |


Tyrosine

| | |
|---|---|
| (1) *p*-Hydroxy-β-phenyllactic acid,  | (7) Benzoic acid,  |
| (2) *p*-Hydroxy-β-phenylethylamine (tyramine),  | (7) *p*-Cresol,  |
| (4) *p*-Hydroxy-β-phenylpropionic acid,  | (7) Phenol (carbolic acid),  |
| (7) *p*-Hydroxyphenylacetic acid,  | (9) *p*-Hydroxy-β-phenylacrylic acid,  |
| (7) *p*-Hydroxybenzoic acid,  | |


Cystine

| | |
|---|---|
| Cysteine, $COOH \cdot CHNH_2 \cdot CH_2SH$ | Acetic acid, $CH_3 \cdot COOH$ |
| Thioglycollic acid, $COOH \cdot CH_2SH$ | Formic acid, HCOOH |
| Methyl mercaptan, $CH_3SH$ | Hydrogen and carbon dioxide, $H_2$ and $CO_2$ |
| Hydrogen sulfide, $H_2S$ | |

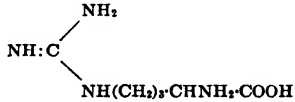
TABLE 37.—(*Continued*)

**$COOH{\cdot}CH_2{\cdot}CHNH_2{\cdot}COOH$**
**Aspartic acid**

| | |
|---|---|
| (1) Malic acid, $COOH{\cdot}CH_2{\cdot}CHOH{\cdot}COOH$ | (7) Acetic acid, $CH_3{\cdot}COOH$, and formic acid, $HCOOH$ |
| (2) β-Alanine, $COOH{\cdot}CH_2{\cdot}CH_2NH_2$ | (9) Fumaric acid, $COOH{\cdot}CH{:}CH{\cdot}COOH$ |
| (4) Succinic acid, $COOH{\cdot}CH_2{\cdot}CH_2{\cdot}COOH$ | (10) Hydrogen, $H_2$, and carbon dioxide, $CO_2$ |
| (5) Propionic acid, $CH_3{\cdot}CH_2{\cdot}COOH$ | |

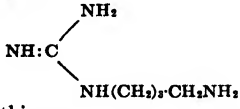
**$COOH{\cdot}CH_2{\cdot}CH_2{\cdot}CHNH_2{\cdot}COOH$**
**Glutamic acid**

| | |
|---|---|
| (2) γ-Aminobutyric acid, $COOH{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2NH_2$ | (7) Succinic acid, $COOH{\cdot}CH_2{\cdot}CH_2{\cdot}COOH$ |
| (4) Glutaric acid, $COOH{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}COOH$ | (7) Acetic acid, $CH_3{\cdot}COOH$, and formic acid, $HCOOH$ |
| (5) Butyric acid, $CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}COOH$, and formic acid, $HCOOH$ | (10) Succinic acid, $COOH{\cdot}CH_2{\cdot}CH_2{\cdot}COOH$, Acetic acid, $CH_3{\cdot}COOH$, Hydrogen, $H_2$, and carbon dioxide, $CO_2$ |

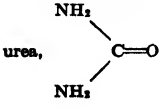


**Arginine**

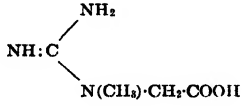
(2) Agmatine,



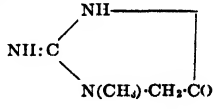

Ornithine, $NH_2CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CHNH_2{\cdot}COOH$, and




Creatine,




Creatinine,




Further decomposition of arginine is the result of the action of organisms on ornithine.

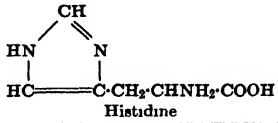
**$NH_2CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CHNH_2{\cdot}COOH$**
**Ornithine**

| | |
|---|---|
| (2) Tetramethylenediamine (putrescine), $NH_2CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2NH_2$ | (4) *d*-Aminovaleric acid (putridin), $NH_2CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}COOH$ |

**$NH_2CH_2{\cdot}(CH_2)_3{\cdot}CHNH_2{\cdot}COOH$**
**Lysine**

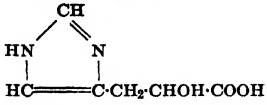
| | |
|---|---|
| (2) Pentamethylenediamine (cadaverine), $NH_2CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2NH_2$ | (4) ε-Aminocaproic acid, $NH_2CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}COOH$ |

TABLE 37.—(*Continued*)

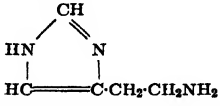



Histidine

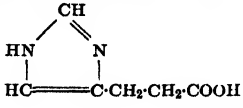
(1) β-Imidazole lactic acid,



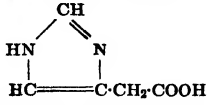

(2) β-Imidazole ethylamine (histamine),



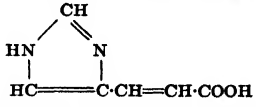

(4) β-Imidazole propionic acid,



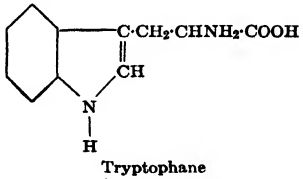

(7) Imidazole acetic acid,




(9) β-Imidazole acrylic acid (urocanic acid),

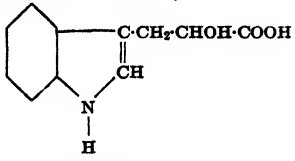



(10) Hydrogen and carbon dioxide

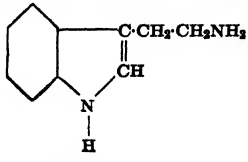



Tryptophane

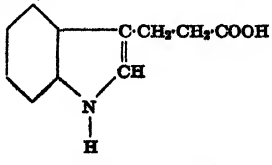
(1) β-Indole lactic acid,



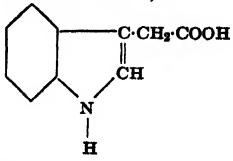

(2) β-Indole ethylamine (tryptamine),



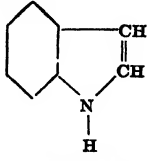

(4) β-Indolepropionic acid (skatoleacetic acid),




(7) Indoleacetic acid,




Indole,




Anthranilic acid,

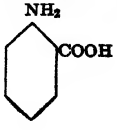
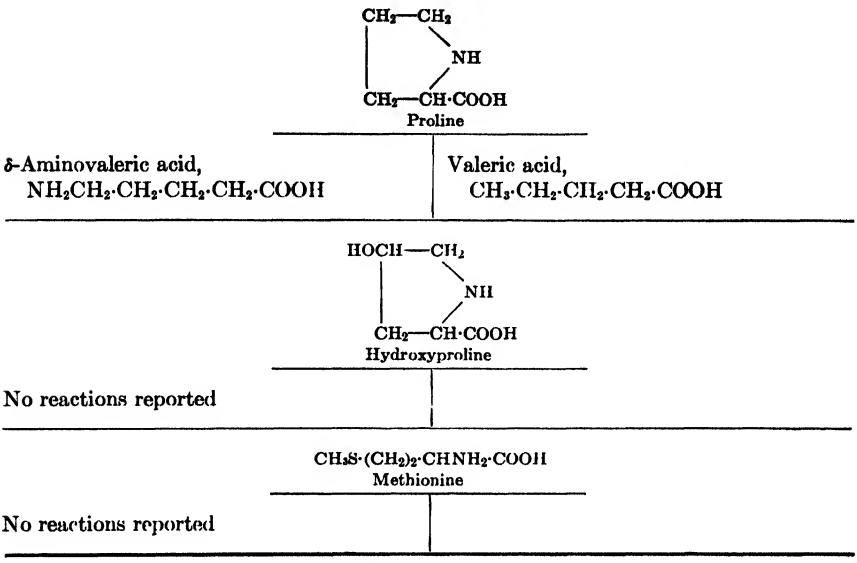


TABLE 37.—(*Continued*)




## PTOMAINES

Ptomaines may be defined as basic amines and diamines, that are formed by the action of putrefactive bacteria on proteins, amino acids, and organic bases. They are produced from amino acids by a process of decarboxylation.

TABLE 38.—SOME PTOMAINES FORMED BY BACTERIAL ACTION

| Amino Acid | Ptomaine |
|---|---|
| Glycocoll (glycine) | Methylamine |
| Alanine | Ethylamine |
| Valine | Isobutylamine |
| Leucine | Isoamylamine |
| Phenylalanine | Phenylethylamine |
| Tyrosine | *p*-Hydroxy-*β*-phenylethylamine (tyramine) |
| Serine | Hydroxyethylamine |
| Cystine (cysteine) | Thioethylamine |
| Arginine | Agmatine |
| Ornithine | Tetramethylenediamine (putrescine) |
| Lysine | Pentamethylenediamine (cadaverine) |
| Histidine | *β*-Imidazole ethylamine (histamine) |
| Tryptophane | *β*-Indole ethylamine (tryptamine) |
| Choline | Trimethylamine |

The ptomaines formed by the decarboxylation of some of the amino acids are given in Table 38. The organic base choline is included as an example of a compound that is not an amino acid.

## PROTEIN-SPARING ACTION

Kendall and Walker (1915) found that the presence of a deficient amount of glucose in a gelatin medium inhibited the secretion of a gelatinolytic enzyme until all of the carbohydrate was utilized. In the presence of an excess of glucose, however, the enzyme never appeared. Control experiments ruled out the possibility that glucose inhibited the action of the enzyme subsequent to its secretion. The question of whether the enzyme was inhibited in its action by the presence of the high acidity was also ruled out, since the enzyme appeared after all of the carbohydrate was fermented. They concluded that organisms preferred a fermentable carbohydrate to protein for energy purposes. The nitrogenous constituents were utilized for structure only. In the absence of a fermentable carbohydrate, the bacteria were forced to utilize the protein constituents for both structure and energy.

Berman and Rettger (1918) and others criticized the conclusions of Kendall and his school. They found that organisms like *Bacillus subtilis*, which ferment glucose very slowly, or those like *Aerobacter cloacae*, which do not produce strongly acid products, failed to show a protein-sparing action. Protein breakdown occurred as though there was no carbohydrate present. On the other hand, organisms such as *Escherichia coli* and *Proteus vulgaris*, which produced considerable amounts of acid in a short period of time, were inhibited in growth and chemical activity after a brief incubation period. Organisms in this group showed a definite protein-sparing action. In the presence of an excess of buffer, such as a mixture of primary and secondary phosphates, the limiting hydrogen-ion concentration was never reached and the course of protein breakdown occurred as rapidly in the presence of a fermentable carbohydrate as in its absence. The result was a negative, protein-sparing action.

Stephenson and Gale (1937) showed that glucose had no effect on the metabolism of *E. coli*, once the cell had formed its enzymes, but that the action was on the formation of the enzymes during growth. The inhibitory effect was not altered by bubbling oxygen through the cultures, indicating it was not due to anaerobic conditions produced by the fermentation gases. Likewise, the control of pH of the medium during growth, by the addition of calcium carbonate, was without effect on the inhibitory action.

It may be concluded that the presence of a fermentable carbohydrate exerts a protein-sparing action only when it is rapidly utilized with the accumulation of a relatively large amount of acid in a short period of time. The protein-sparing action appears to be due to the acid that is evolved during fermentation. If steps are taken to neutralize the acidity by strongly buffering the medium, the inhibitory effect of carbohydrate is prevented.

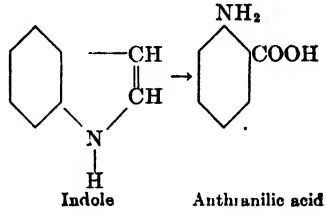
## SOME ROUTINE PHYSIOLOGICAL REACTIONS EMPLOYED FOR THE IDENTIFICATION OF BACTERIA

Many biochemical reactions are employed for the identification and classification of bacteria. Some of them are based on the breakdown of carbohydrates; others depend upon certain changes in the nitrogenous constituents of the medium.

**Production of Indole.**—Indole is a putrefactive compound produced by the action of some bacteria on the amino acid tryptophane (page 358). Since no other amino acid contains the indole ring, the test is specific for the presence of tryptophane.

Tryptophane is not present in all proteins. Manufacturers select only those proteins for peptone production which are certain to yield a relatively high content of tryptophane. Casein, the principal protein of milk, contains a large amount of this acid and is often used for the preparation of peptone where a strong indole test is required.

There is evidence to show that some organisms are capable of oxidizing the indole ring. Supniewski (1924) reported that *Pseudomonas aeruginosa* oxidized indole to anthranilic acid:




Indole Anthranilic acid

Sasaki (1923) showed that *B. subtilis* not only produced indole from tryptophane but also oxidized it to anthranilic acid. Gray (1928) isolated *P. indoloxidans* from soil which oxidized indole to indigotin (indigo blue). Crystals of the compound appeared around colonies of the organisms on agar plates. Surprisingly, this organism is unable to oxidize tryptophane to indole. Kotake (1933) showed that *B. subtilis* was capable of producing kynurenic acid and anthranilic acid from tryptophane in the presence of glycerol and aluminum phosphate.

In order to prevent the possible occurrence of a protein-sparing action, noncarbohydrate media should be employed for the detection of indole in bacterial cultures.

The test is of value in identifying and classifying bacteria.

**Production of Ammonia.**—The development of ammonia in bacterial cultures results largely from the deaminization of the amino acids present in culture media.

It is generally believed that amino acids must be first deaminized prior

to assimilation. In the absence of a fermentable carbohydrate, organisms utilize the ammonia for structure, and the deaminized carbon chains for energy. Since more carbon is required for energy than is nitrogen for structure, ammonia will accumulate in the medium. In the presence of a fermentable carbohydrate, a protein-sparing action might occur in which case the organisms utilize the amino acids for structure but not for energy. A carbohydrate medium inoculated with a vigorous fermenter usually shows less free ammonia than the same medium without carbohydrate. This may be attributed to two factors: (1) Organisms utilize carbohydrate in preference to the deaminized amino acids for energy. Since the carbon of the amino acids is not required for energy, less ammonia is produced. (2) The addition of a fermentable carbohydrate to a medium stimulates bacterial growth (increase in numbers), resulting in an increased utilization of ammonia. This causes a decrease in the free ammonia content of the medium. It is generally believed that both factors apply. This means that, in the case of the strongly fermentative organisms, the addition of a carbohydrate to a culture results in the accumulation of less free ammonia than in the same culture medium not containing carbohydrate.

To prevent the possible occurrence of a protein-sparing action, non-carbohydrate media should be employed for the detection of ammonia in bacterial cultures.

**Peptonization and Fermentation of Milk.**—According to Van Slyke and Bosworth (1915), the constituents of milk may be placed in three groups on the basis of their solubilities. Their classification, with additions by Hawk, Oser, and Summerson (1947), is as follows:

I. Milk constituents in true solution:
   1. Lactose.
   2. Citric acid.
   3. Potassium.
   4. Sodium.
   5. Chlorine.
   6. Vitamin $B_1$.
   7. Vitamin G (riboflavin).
   8. Vitamin C.

II. Milk constituents partly in solution and partly in suspension or colloidal solution:
   1. Albumin.
   2. Inorganic phosphate.
   3. Calcium.
   4. Magnesium.

III. Milk constituents entirely in suspension or colloidal solution:
   1. Fat.
   2. Casein.
   3. Vitamin A.
   4. Vitamin D.

The use of milk as a culture medium dates back to the beginning of bacteriology. It is used as a differential medium to demonstrate the ability of an organism to produce a fermentation or a peptonization, or a simultaneous fermentation and peptonization.

Casein is a protein capable of reacting both as a weak acid and as a weak base. It is present in milk entirely in colloidal suspension. Some bacteria secrete a rennin-like enzyme capable of hydrolyzing casein to soluble *p*-casein and a compound similar to peptone. The soluble *p*-casein

then reacts with the calcium salts in solution, resulting in the formation of a precipitate of *p*-casein or calcium *p*-caseinate. The clear liquid surrounding the curd of *p*-casein is known as whey. This may be schematically represented as follows:

Casein + rennin

- Peptone-like body
- Soluble *p*-casein + Calcium salts → *p*-casein (Calcium *p*-caseinate)

Bacteria capable of vigorously attacking the lactose produce a high degree of acidity, resulting in the precipitation or curdling of the casein. The clear supernatant fluid is also known as whey. The final pH is sufficient to prevent further bacterial action. Since carbohydrate spares protein, no putrefactive changes occur (Fig. 151).

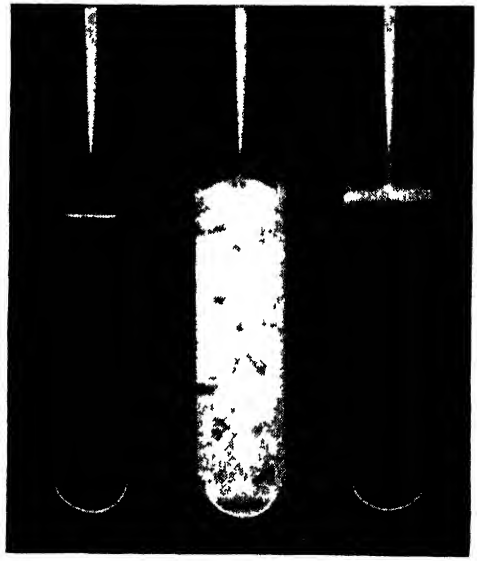


FIG. 151.—Fermentation and peptonization of milk. Left, litmus milk inoculated with *Bacillus subtilis*. The casein is completely peptonized to a clear purplish solution. Center, litmus milk inoculated with *Escherichia coli*. The lactose is fermented, followed by a curdling of the casein, and the indicator is completely reduced. Right, litmus milk uninoculated.

A rennin curd is usually followed by a peptonization of the casein. This means that the organisms either do not attack the lactose or ferment it very slowly. In the former instance, the casein is first hydrolyzed and then decomposed with the formation of various soluble products. In the latter case, a slow action on the milk sugar results in a simultaneous fermentation and protein decomposition, owing to the fact that the lactose exhibits no protein-sparing action.

The indicator litmus is frequently added to milk to detect acid production and also the ability of an organism to decolorize it to its colorless form. The newer, more brilliant indicators, such as bromocresol purple, are superior to litmus for detecting acid production, owing to their greater sensitivity, but they do not exhibit reduction phenomena that are often of diagnostic importance.

**Production of Hydrogen Sulfide.**—Cystine and methionine are the two sulfur-containing amino acids found in proteins. Cystine is probably of greater occurrence and importance from a bacteriological standpoint. Ransmeier and Stekol (1942*a*,*b*) found that *E. coli*, *Klebsiella pneumoniae*, *B. subtilis*, *P. aeruginosa*, and *Pasteurella tularensis* were capable of dissimilating cystine to hydrogen sulfide but failed to produce the compound from methionine. Cystine does not occur in all proteins. Only those proteins are selected for peptone production which are certain to contain relatively large amounts of cystine.

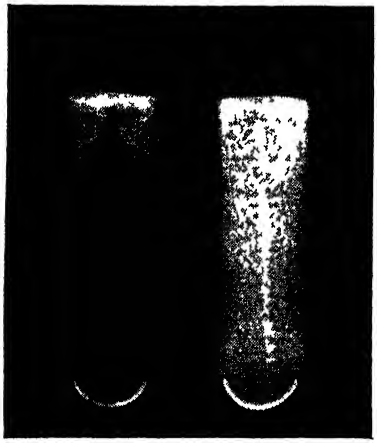


FIG. 152.—Production of hydrogen sulfide. Left, peptone iron agar medium inoculated with *Proteus vulgaris*. Hydrogen sulfide formed which reacted with the iron to give black iron sulfide; right, same inoculated with *Escherichia coli*. Hydrogen sulfide not produced.

Some organisms are capable of dissimilating cystine with the production of hydrogen sulfide as one of the putrefactive products. It is usually stated that the first change that occurs is a reduction of one molecule of cystine to two molecules of cysteine (page 356). Tarr (1933) believed that cystine undergoes an anaerobic reduction to give hydrogen sulfide and other products.

Hydrogen sulfide reacts with heavy metals to produce colored compounds. The metal salts are incorporated in solid media. The presence of hydrogen sulfide is detected by a darkening of the medium along the line of inoculation (Fig. 152). The metals commonly employed include lead, iron, bismuth, cobalt, and nickel. Tittsler and Sandholzer (1937) found iron to be more sensitive than lead. Hunter and Crecelius (1938) reported that bismuth was more sensitive than either lead or iron for detecting the presence of hydrogen sulfide. Organisms that produced small amounts of hydrogen sulfide gave negative results with iron and lead, and definite browning with bismuth sulfite. Utermohlen and Georgi (1940) employed a medium containing a mixture of cobalt and nickel salts and found it to be as good as one containing bismuth. Georgi and McMaster (1944) made a study of 20 different peptones and 3 metallic ions, namely, lead, iron, and bismuth, and concluded that bismuth was superior as a detector of hydrogen sulfide production by a number of bacteria. The sensitivity of the detector ions to hydrogen sulfide followed the order, Bi > Pb > Fe.

It may be concluded that it is not enough to state merely that an organism does or does not produce hydrogen sulfide, without giving the conditions under which it was investigated.

Organisms may be placed into two groups on the basis of hydrogen sulfide production. The test is of value in identifying and classifying bacteria.

**Liquefaction of Gelatin.**—The liquefaction of gelatin by bacteria is the result of the action of an enzyme known as gelatinase. It is an extracellular enzyme concerned with the hydrolysis of the indiffusible protein prior to intracellular utilization.

The presence of the enzyme may be demonstrated by inoculating a tube of gelatin with the organism in question and incubating the culture at the proper temperature. If the temperature of incubation is above 20°C., the gelatin will melt. Under these conditions, the presence of gelatinase may be determined by placing the gelatin culture in the refrigerator and noting whether or not hardening occurs. If the gelatin remains liquid, it shows that the organism under examination secreted a gelatinase into the culture medium.

The extracellular nature of the enzyme may be demonstrated by filtering a culture of an appropriate organism and adding some of the filtrate to a tube of gelatin medium. The presence of a gelatinase will result in a liquefaction of the gelatin.

Another method used for demonstrating the presence of the enzyme in a culture is to add just sufficient germicide (phenol) to kill the organisms but not enough to have any appreciable effect on the enzyme. The addition of some of this phenolated culture to a tube of gelatin medium will produce a liquefaction, if the organism under examination is capable of elaborating a gelatinase.

A protein-sparing action results in a negative test for gelatin liquefaction. Therefore, noncarbohydrate media should be employed for demonstrating the ability of an organism to secrete a gelatinase. The test is of value in identifying and classifying bacteria.

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## CHAPTER XV

## FERMENTATION OF CARBOHYDRATES AND RELATED COMPOUNDS

During the early part of the nineteenth century, the biological theory of fermentation made slow progress owing to the fact that the majority of scientists of that period were chemists and physicists. Berzelius and his pupils Liebig and Wöhler believed that all vital phenomena could be explained on purely chemical grounds. They believed that fermentation and putrefaction were the result of the action of compounds known as ferments (enzymes). The ferments were believed to arise from some constituent of the solution, when exposed to air, but, after fermentation had started, oxygen was no longer required.

Pasteur believed that living yeast cells were always present in fermentation. In their absence, fermentation did not occur.

Later, Buchner demonstrated that yeast juice, when added to sugar, was capable of inducing alcoholic fermentation. This observation proved that both views were correct, namely, that fermentation was produced by enzymes but that living cells were necessary for their elaboration.

The term "fermentation" has undergone many changes in meaning since the time of Pasteur, but it is generally defined as the incomplete oxidation of carbohydrates or carbohydrate-like compounds by microorganisms. The various types of fermentations result in the formation of many kinds of organic acids and other compounds.

Bacterial action on carbohydrates may be either aerobic or anaerobic. In the presence of sufficient oxygen, carbohydrate breakdown may proceed to the final end products, carbon dioxide and water. In the absence of free oxygen, incomplete combustion and anaerobic breakdown occur. In anaerobic breakdown, organisms are unable to obtain the maximum amount of energy available in fermentable compounds.

If glucose is taken as an example of a fermentable compound that is utilized for purposes of energy, the (1) complete oxidation, (2) partial oxidation, and (3) anaerobic decomposition yield the following number of calories:

1. Complete oxidation:
   $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + 674$ Cal.
2. Partial oxidation:
   $C_6H_{12}O_6 + 4\frac{1}{2}O_2 \rightarrow \underset{\text{Oxalic acid}}{3(COOH)_2} + 3H_2O + 493$ Cal.

3. Anaerobic decomposition:

(*a*) $C_6H_{12}O_6 \rightarrow \underset{\text{Lactic acid}}{2CH_3{\cdot}CHOH{\cdot}COOH} + 22\frac{1}{2}$ Cal.

(*b*) $C_6H_{12}O_6 \rightarrow \underset{\text{Ethyl alcohol}}{2CH_3{\cdot}CH_2OH} + 2CO_2 + 22$ Cal.

(*c*) $C_6H_{12}O_6 \rightarrow \underset{\text{Acetic acid}}{3CH_3{\cdot}COOH} + 15$ Cal.

The fermentation of glucose by *Escherichia coli* yields formic, acetic, lactic, and succinic acids, ethyl alcohol, hydrogen, and carbon dioxide. Grey (1919) showed that 100 gm. of the above products on complete oxidation yielded about 316 Cal. but only about 58 Cal. were liberated in the reaction. The incomplete oxidation of glucose by *E. coli* yielded only about 16 per cent of the potential energy.

**Fermentation of Proteins.**—It is generally stated that in the process of fermentation only carbohydrates or carbohydrate-like compounds are involved, but this statement is not strictly correct. It is now known that a few species of anaerobes, or facultative forms growing under anaerobic conditions, are able to satisfy all or part of their energy requirements by the fermentation of single amino acids or other nitrogenous compounds. The amino group of amino acids is probably first removed as ammonia, followed by a fermentation of the carbon residue.

Cardon (1942) reported that the anaerobes *Clostridium tetanomorphum* and *C. cochlearium* fermented glutamic acid, and *C. acidi-urici* and *C. cylindrosporum* fermented uric acid as sources of energy. An unidentified anaerobe species was found to ferment alanine according to the equation:

$$\underset{\text{Alanine}}{3CH_3{\cdot}CHNH_2{\cdot}COOH} + 2H_2O \rightarrow 3NH_3 + \underset{\text{Propionic acid}}{2C_2H_5{\cdot}COOH} + \underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + CO_2$$

Clifton (1942) found that aspartic acid, glutamic acid, and serine were fermented by *C. tetani* with the production of ammonia, acetic acid, lactic acid, butyric acid, alcohol, and carbon dioxide. Essentially the same products were produced during the dissimilation of pyruvic acid, an intermediary product in the fermentation of carbohydrates. Pickett (1943), working with the same organism, reported similar results.

Barker (1943) cultivated *Streptococcus allantoicus* in the presence of allantoin under anaerobic conditions and found that the organism fermented the compound with the production of ammonia, urea, formic acid, acetic acid, lactic acid, oxamic acid, and carbon dioxide.

For more information see Cardon and Barker (1946).

## RETTING OF FLAX AND HEMP

Celluloses, hemicelluloses, and pectins are polysaccharides, which together form the framework of plants. The hemicelluloses differ from the celluloses in being more easily hydrolyzed with dilute mineral acids and in giving different products when hydrolyzed. The hemicelluloses include:

The hexosans:
1. The galactans yield *d*-galactose on hydrolysis.
2. The mannans yield principally *d*-mannose and a small amount of *d*-fructose on hydrolysis.

The pentosans:
1. The xylans yield *l*-xylose on hydrolysis.
2. The arabans yield *l*-arabinose on hydrolysis.

Hemicelluloses are attacked by bacteria with difficulty but are easily hydrolyzed by molds.

Pectins accompany celluloses and hemicelluloses in making up the framework of plants. They are compounds of a gelatinous consistence which form the middle lamellar layer of plant cells. Pectins act as cement-like substances that bind the cells together. Pectins from different sources appear to have the same general chemical composition, being calcium and magnesium salts of a complex carbohydrate association. Hydrolysis of pectins yields chiefly galacturonic acid, *l*-arabinose, and *d*-galactose, accompanied by small amounts of methyl alcohol, acetic acid, calcium, magnesium, etc.

The bacterial hydrolysis of pectin is of great importance in the retting or rotting of flax and hemp. This may be accomplished by both anaerobic and aerobic methods.

**Anaerobic Retting.**—Retting is carried out by immersing the flax or hemp stalks in water and weighting them down. Water is absorbed by the tissues, causing swelling and the extraction of water-soluble substances. The substances that are extracted amount to about 12 per cent of the weight of the plants and consist of sugars, glucosides, tannins, soluble nitrogenous compounds, and coloring matter. The highly colored water now becomes a good culture medium for the growth of many kinds of organisms. The aerobic organisms reduce the concentration of dissolved oxygen and create an environment compatible to the growth of the anaerobes. The pectin is slowly fermented and dissolved by the anaerobes, leaving the fibers intact. During the fermentation, various organic acids and gases are produced. These include chiefly acetic and butyric acids, carbon dioxide, and hydrogen. About 10 days are required for the reaction to go to completion. The flax or hemp should be removed from the water when the reaction has gone to completion, otherwise overretting will result. The bundles are thoroughly washed, to remove the organic acids, odors, and other undesirable substances, and then spread out in the sun or air to dry. The dried material is then ready for scutching.

The organism responsible for the hydrolysis and fermentation of pectin is known as *Clostridium butyricum*. It is a motile, anaerobic, spore-bearing, Gram-positive rod. The organism ferments a number of carbohydrates and carbohydrate-like compounds with the production of acid and gas. The fermentable products include butyl, ethyl, and isopropyl alcohols, acetone,

organic acids, hydrogen, and carbon dioxide. The rods are capable of utilizing atmospheric nitrogen. The organism grows best at a temperature of 30 to 37°C.

Recent improvements on the process make use of the anaerobic organism *C. felsineum*. A culture of the organism is added to the water of the retting vat in the proportion of 1000 cc. to 10,000 gm. of dry tissue. The vat is kept at a temperature of 37 to 38°C. for a period of from 50 to 75 hr. The process requires closer supervision than the preceding method.

According to McClung (1942), *C. felsineum* is a long, slender bacillus producing oval spores and an orange pigment. It forms ethyl alcohol, butyl alcohol, and acetone by fermentation of carbohydrate-containing material.

**Aerobic Retting.**—An aerobic process known as dew retting is also employed. The stalks are spread on the ground in the fall and allowed to remain throughout the winter months. A disadvantage to the method is that it is too slow, months instead of days being required. The organism chiefly responsible for the reaction is the mold *Mucor stolonifer*. Other organisms, both molds and bacteria, are doubtless concerned in the reaction.

## ALCOHOLIC FERMENTATION

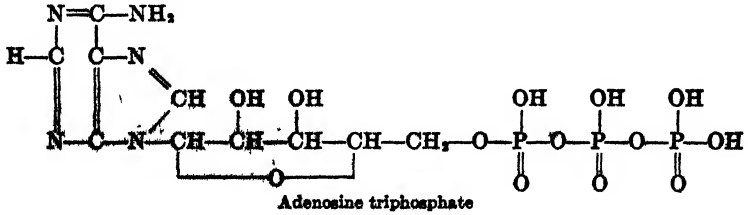
Alcohol is produced commercially by means of yeasts. The species of yeast that is generally used for this purpose is *Saccharomyces cerevisiae*, the ordinary baker's or brewer's yeast. The organism converts approximately 90 per cent of the sugar into equimolecular quantities of alcohol and carbon dioxide. The final equation may be written:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \rightarrow 2CO_2 + 2\underset{\text{Ethyl alcohol}}{C_2H_5OH}$$

Alcoholic fermentation proceeds in the presence of air as well as in its absence. However, the velocity of the reaction is decreased by aeration. In the presence of oxygen, respiration (oxygen uptake) is increased while fermentation is decreased. In the absence of oxygen, the reverse is true. This is sometimes referred to as the "Pasteur effect."

A number of theories have been proposed to explain the mechanism of alcoholic fermentation. It is generally agreed that it proceeds in the following manner:

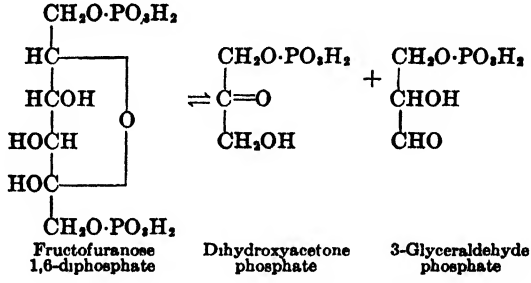
The first step in the reaction involves the phosphorylation of the hexose by the coenzyme adenosine triphosphate:



Adenosine triphosphate

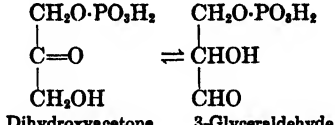
The coenzyme is present in yeast juice and is capable of transferring phosphate to the hexose, converting the sugar first to hexosemonophosphate and then to hexosediphosphate. The loss of one molecule of phosphate converts the coenzyme to adenosine diphosphate. The phosphate is not utilized in the reaction. When the fermentation reaches the pyruvic acid stage, the phosphate is liberated and becomes available again for phosphorylating additional sugar.

The phosphorylation of glucose, fructose, or mannose, in the presence of the coenzyme adenosine triphosphate, yields the same compound, namely, fructofuranose 1,6-diphosphate. This compound splits to form one molecule of dihydroxyacetone phosphate and one molecule of 3-glyceraldehyde phosphate which are in equilibrium with the hexose diphosphate:



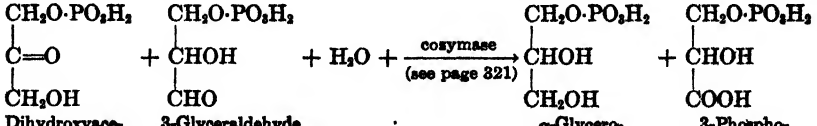

Fructofuranose 1,6-diphosphate — Dihydroxyacetone phosphate — 3-Glyceraldehyde phosphate

Dihydroxyacetone phosphate and 3-glyceraldehyde phosphate are also in equilibrium with each other, being converted largely into the former compound:



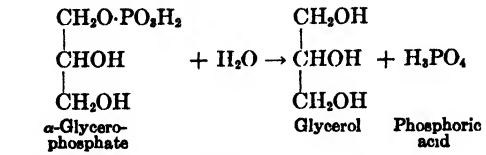

Dihydroxyacetone phosphate — 3-Glyceraldehyde phosphate

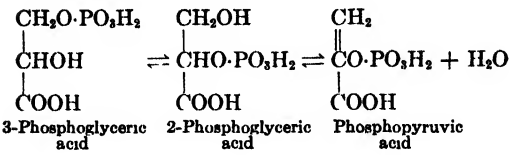
In the presence of cozymase or coenzyme I and water, one molecule of 3-glyceraldehyde phosphate is oxidized to 3-phosphoglyceric acid, and one molecule of dihydroxyacetone phosphate is reduced to $\alpha$-glycerophosphate:



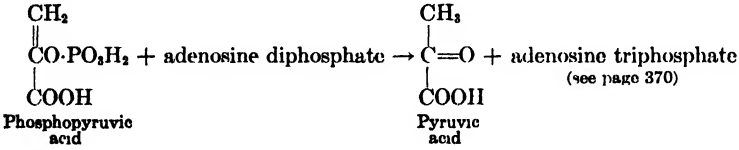

Dihydroxyacetone phosphate — 3-Glyceraldehyde phosphate — $\alpha$-Glycerophosphate — 3-Phosphoglyceric acid

In the next step the $\alpha$-glycerophosphate is hydrolyzed to glycerol and phosphoric acid, and the 3-phosphoglyceric acid is converted first to 2-phosphoglyceric acid and finally to phosphopyruvic acid. The reactions in the latter case are reversible:



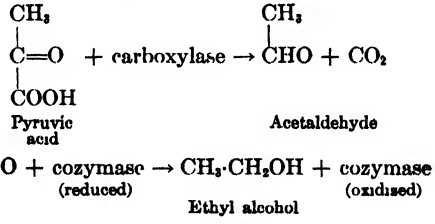




The phosphopyruvic acid is now dephosphorylated by adenosine diphosphate to give pyruvic acid and adenosine triphosphate:

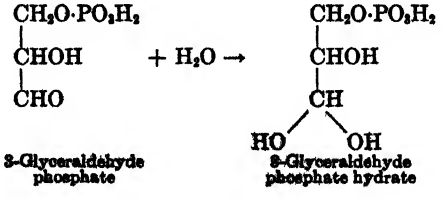


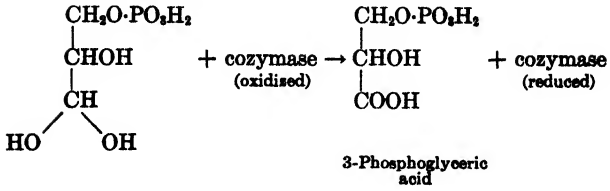

The adenosine triphosphate transfers phosphate to more molecules of hexose to give first hexosemonophosphate, then hexosediphosphate and the above series of reactions is repeated.

In the final steps, the pyruvic acid is decarboxylated to acetaldehyde and carbon dioxide by the enzyme carboxylase. Then the reduced form of cozymase or coenzyme I transfers hydrogen to the acetaldehyde to give ethyl alcohol, and the cozymase becomes oxidized:




The oxidized cozymase now acts on the 3-glyceraldehyde phosphate oxidizing it to 3-phosphoglyceric acid, and the cozymase becomes reduced again. The function of the coenzyme is that of a hydrogen acceptor and hydrogen donator to produce reactions of oxidation and reduction:





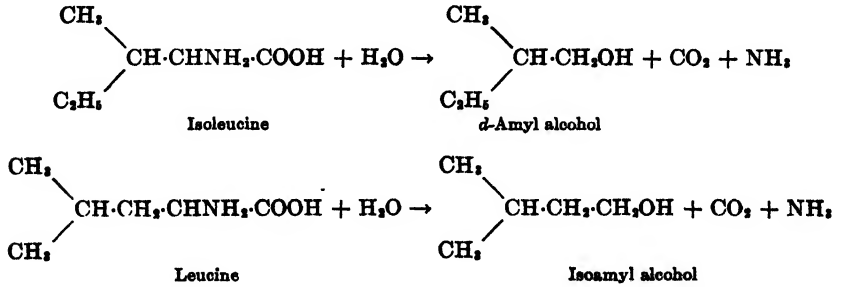

The reduced cozymase is now capable of acting on more acetaldehyde and reducing it to ethyl alcohol:

$$CH_3{\cdot}CHO + \underset{\text{(reduced)}}{\text{cozymase}} \rightarrow \underset{\text{Ethyl alcohol}}{CH_3{\cdot}CH_2OH} + \underset{\text{(oxidised)}}{\text{cozymase}}$$

The 3-phosphoglyceric acid is then carried through the same series of reactions already outlined to give more acetaldehyde, which reacts with more 3-glyceraldehyde phosphate to give 3-phosphoglyceric acid and ethyl alcohol. This cycle continues until fermentation is complete.

In addition to alcohol and carbon dioxide, small amounts of *d*-amyl alcohol, isoamyl alcohol, succinic acid, glycerol, and other compounds are also produced.

***d*-Amyl and Isoamyl Alcohols.**—A mixture of *d*-amyl and isoamyl alcohols together with traces of other higher alcohols and compound ethers obtained from fermented liquors is sometimes referred to as fusel oil. Ehrlich showed that *d*-amyl alcohol and isoamyl alcohol are derived from the amino acids isoleucine and leucine, respectively. These acids may originate from the constituents of the medium or from the protein of dead and autolyzed yeast cells. The alcohols are produced by a deaminization and a decarboxylation of the amino acids:




The amounts of the two alcohols produced depend upon the concentrations of the specific amino acids present in the medium, upon the species of yeast employed, and upon the nutritional requirements of the yeast cells. Since the organisms derive their nitrogen from the deaminization of amino acids, the presence of a more easily available source of nitrogen will prevent or delay the hydrolysis of amino acids for this purpose. The reaction occurs only in the presence of a fermentable carbohydrate. Small

amounts of alcohols are produced from other amino acids, such as tyrosol from tyrosine and tryptophol from tryptophane.

**Succinic Acid.**—Succinic acid is produced from glutamic acid during alcoholic fermentation. Ehrlich found that, of all the amino acids added to a fermentation medium, glutamic acid was the only one that gave an increase in the concentration of succinic acid. The most probable course of the decomposition of glutamic acid to succinic acid is the following:

Oxidative deaminization:

$$\underset{\text{Glutamic acid}}{COOH\cdot CH_2\cdot CH_2\cdot CHNH_2\cdot COOH} + \tfrac{1}{2}O_2 \rightarrow \underset{\alpha\text{-Ketoglutaric acid}}{COOH\cdot CH_2\cdot CH_2\cdot CO\cdot COOH} + NH_3$$

Decarboxylation:

$$\underset{\alpha\text{-Ketoglutaric acid}}{COOH\cdot CH_2\cdot CH_2\cdot CO\cdot COOH} \rightarrow \underset{\text{Succinic semialdehyde}}{COOH\cdot CH_2\cdot CH_2\cdot CHO} + CO_2$$

Oxidation:

$$\underset{\text{Succinic semialdehyde}}{COOH\cdot CH_2\cdot CH_2\cdot CHO} + \tfrac{1}{2}O_2 \rightarrow \underset{\text{Succinic acid}}{COOH\cdot CH_2\cdot CH_2\cdot COOH}$$

Succinic acid is produced either from the glutamic acid of the medium or from the proteins of dead and autolyzed yeast cells. As in the case of fusel oil, the production of succinic acid does not occur in the absence of a fermentable carbohydrate.

**Glycerol.**—Glycerol is prepared commercially chiefly by the saponification of fats and oils in the manufacture of soaps. It is produced in small amounts during the alcoholic fermentation of carbohydrates by yeasts. The yield rarely amounts to more than about 3.8 per cent of the sugar fermented. Neuberg and Reinfurth (1918) were probably the first to show that the yield of glycerol could be greatly increased by adding an appropriate alkali or sodium sulfite to the fermenting mixture.

As has already been shown, alcohol results from a reduction of the intermediary compound acetaldehyde by means of hydrogen from reduced cozymase. If the hydrogen is prevented from reducing the acetaldehyde, an increased yield of glycerol will result. This is accomplished by adding sodium sulfite to the fermenting mixture. The sulfite reacts with the acetaldehyde to produce an addition product, preventing it from accepting hydrogen from the reduced cozymase:

$$\underset{\text{Acetaldehyde}}{\begin{matrix}CH_3\\|\\CHO\end{matrix}} + Na_2SO_3 + H_2O \rightarrow \underset{\substack{\text{Aldehyde-bisulfite}\\\text{compound}}}{\begin{matrix}CH_3\\|\\C{-}H\\|\ \diagdown\\OH\\SO_3Na\end{matrix}} + NaOH$$

A second molecule in the fermenting medium, a triose, produced from the hexose sugar, acts as a hydrogen acceptor and becomes reduced to glycerol.

Sodium carbonate reacts with the acetaldehyde in a similar manner to prevent its reduction to alcohol. The yield of glycerol varies, depending upon the amount of sulfite or carbonate added. In the sulfite process, the yield of glycerol may be as high as 37 per cent of the sugar fermented.

For further reading on alcoholic fermentation and its chief by-products, consult Colowick and Kalckar (1943), Cori (1941, 1942), Gottschalk (1942), Gould, Tytell, and Jaffe (1942), Guillaudeu (1937), Kalckar (1943), Lipmann (1942), Meyerhof (1941, 1942*a,b*, 1943, 1945), Nord and Mull (1945), Ochoa (1942), Pickett and Clifton (1943), Prescott and Dunn (1940), Stephenson (1939), and Sumner and Somers (1947).

The fermentation of carbohydrates by yeasts with the production principally of alcohol and carbon dioxide has been fairly well worked out. However, the pathway of carbohydrate breakdown in some special forms of bacterial fermentations is still unsettled. The most important of these are now discussed.

## BUTYRIC ACID FERMENTATION

The organism involved in this type of fermentation is the anaerobic, spore-forming rod known as *Clostridium butyricum*. It is the same organism that is concerned in the retting of flax and hemp.

The reaction scheme, according to Kluyver and associates (1931), is as follows:

$$\underset{\text{Hexose}}{C_6H_{12}O_6} \rightarrow 2C_3H_6O_3$$

$$CH_3{\cdot}CO{\cdot}C(H)(OH){-}OH \rightarrow \underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + \underset{\text{Formic acid}}{HCOOH}$$

Methylglyoxal hydrate

$$\underset{\text{Formic acid}}{HCOOH} \rightarrow CO_2 + H_2$$

$$2\underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + H_2O \rightleftharpoons \underset{\text{Acetaldehyde hydrate}}{CH_3{\cdot}C(H)(OH){-}OH} + \underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + H_2$$

$$\underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + \underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} \rightarrow \underset{\text{Acetaldol}}{CH_3{\cdot}CHOH{\cdot}CH_2{\cdot}CHO}$$

$$CH_3{\cdot}CHOH{\cdot}CH_2{\cdot}CHO \rightleftharpoons CH_3{\cdot}CH{=}CH{\cdot}C(H)(OH){-}OH$$

Acetaldol

$$CH_3{\cdot}CH{=}CH{\cdot}C(H)(OH){-}OH \rightarrow \underset{\text{Butyric acid}}{CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}COOH}$$

Kluyver believed that the internal mechanism for the conversion of the sugar to methylglyoxal hydrate corresponded to that of alcoholic fermentation.

The important products of this type of fermentation are butyric acid, acetic acid, carbon dioxide, and hydrogen. The theoretical figures given by the above scheme agree remarkably well with the amounts of the final products found in actual tests.

## BUTYL ALCOHOL AND ACETONE FERMENTATION

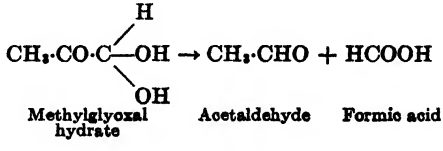
This type of carbohydrate breakdown is characterized by the production of more end products than was noted in the butyric acid fermentation. The two chief products, aside from carbon dioxide, are butyl alcohol and acetone. In addition, smaller amounts of hydrogen, formic acid, acetic acid, butyric acid, ethyl alcohol, and acetylmethylcarbinol have also been recovered.

The organism commonly employed for this purpose is the anaerobe *Clostridium acetobutylicum*. It is a large, motile, spore-forming, Gram-positive rod. The organism is capable of fermenting a large number of carbohydrates and carbohydrate-like compounds.

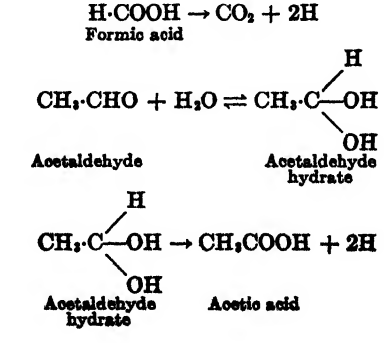
According to Kluyver (1931), the reaction scheme is as follows:

$$C_6H_{12}O_6 \rightarrow 2C_3H_6O_3$$

(The internal mechanism for this conversion corresponds to that of alcoholic fermentation.)




Dehydrogenation reactions:



Condensation reactions:

$$\underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + \underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} \rightarrow \underset{\text{Acetaldol}}{CH_3{\cdot}CHOH{\cdot}CH_2{\cdot}CHO}$$

$$CH_3{\cdot}CHOH{\cdot}CH_2{\cdot}CHO \rightleftharpoons CH_3{\cdot}CH{=}CH{\cdot}C(H)(OH){-}OH$$

Acetaldol

$$CH_3{\cdot}CH{=}CH{\cdot}C(H)(OH){-}OH \rightarrow \underset{\text{Butyric acid}}{CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}COOH}$$

$$\underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + \underset{\text{Acetic acid}}{CH_3{\cdot}COOH} \rightarrow CH_3{\cdot}C(OH)_2{\cdot}CH_2{\cdot}COOH$$

$$CH_3{\cdot}C(OH)_2{\cdot}CH_2{\cdot}COOH \rightleftharpoons \underset{\text{Acetoacetic acid}}{CH_3{\cdot}CO{\cdot}CH_2{\cdot}COOH} + H_2O$$

$$\underset{\text{Acetoacetic acid}}{CH_3{\cdot}CO{\cdot}CH_2{\cdot}COOH} \rightarrow \underset{\text{Acetone}}{CH_3{\cdot}CO{\cdot}CH_3} + CO_2$$

Hydrogenation reactions:

$$2H \rightarrow H_2$$

$$\underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + 2H \rightarrow \underset{\text{Ethyl alcohol}}{CH_3{\cdot}CH_2OH}$$

$$\underset{\text{Butyric acid}}{CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}COOH} + 4H \rightarrow \underset{\text{Butyl alcohol}}{CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2OH} + H_2O$$

$$\underset{\text{Acetone}}{CH_3{\cdot}CO{\cdot}CH_3} + 2H \rightarrow \underset{\text{Isopropyl alcohol}}{CH_3{\cdot}CHOH{\cdot}CH_3}$$

The amounts of the end products recovered from a fermenting mixture agree quite closely with theoretical calculations according to the above scheme.

Wood, Brown, Werkman, and Stuckwisch (1944) used heavy carbon ($C^{13}$) compounds as tracers in an attempt to determine the mechanism of the butyl alcohol fermentation. When acetic acid, $CH_3C^{13}OOH$ was added to a fermentation of corn mash by the butyl alcohol bacteria, butyl alcohol was formed which contained heavy carbon. Butyric acid, $CH_3{\cdot}C^{13}H_2{\cdot}CH_2{\cdot}C^{13}OOH$, was isolated which contained heavy carbon in the carboxyl and $\beta$ positions. On the basis of the distribution of the $C^{14}$ in the molecule, they concluded that butyl alcohol was formed by a condensation of acetic acid or its derivative.

## ACETONE AND ETHYL ALCOHOL FERMENTATION

In this type of fermentation the chief products are acetone, ethyl alcohol, and carbon dioxide. Smaller amounts of acetic acid, lactic acid, formic acid, and hydrogen are also produced.

The organism responsible for the acetone and ethyl alcohol fermentation is *Bacillus acetoethylicus*, a motile, aerobic, spore-bearing, Gram-

positive rod. Young cells are said to be Gram-negative. The spores are ovoid, terminal, and cause a bulging of the rod. The organism attacks a large number of carbohydrates and carbohydrate-like substances with the production of acid, gas, and other compounds. *B. acetoethylicus* produces about 2 parts of ethyl alcohol to 1 part of acetone. The optimum growth temperature of the organism is about 42 to 45°C. It is probably identical with *B. macerans.*

According to Speakman (1925), the scheme for the fermentation of carbohydrates to acetone and ethyl alcohol is as follows:

Scheme (Part 1):

$$\underset{\text{Hexose}}{C_6H_{12}O_6} \rightarrow 2\underset{\text{Pyruvic acid}}{CH_3\cdot CO\cdot COOH} + 2H_2$$

$$\underset{\text{Pyruvic acid}}{CH_3\cdot CO\cdot COOH} + H_2 \rightarrow \underset{\text{Lactic acid}}{CH_3\cdot CHOH\cdot COOH}$$

$$\underset{\text{Pyruvic acid}}{CH_3\cdot CO\cdot COOH} \rightarrow \underset{\text{Acetaldehyde}}{CH_3\cdot CHO} + CO_2$$

$$\underset{\text{Pyruvic acid}}{CH_3\cdot CO\cdot COOH} + H_2O \rightarrow \underset{\text{Acetic acid}}{CH_3\cdot COOH} + \underset{\text{Formic acid}}{H\cdot COOH}$$

$$\underset{\text{Acetaldehyde}}{CH_3\cdot CHO} + H_2 \rightarrow \underset{\text{Ethyl alcohol}}{CH_3\cdot CH_2OH}$$

Scheme (Part 2):

$$\underset{\text{Acetaldehyde}}{CH_3\cdot CHO} + \underset{\text{Acetaldehyde}}{CH_3\cdot CHO} \rightarrow \underset{\text{Acetaldol}}{CH_3\cdot CHOH\cdot CH_2\cdot CHO}$$

$$\left\{\begin{array}{l}\underset{\text{Acetaldol}}{CH_3\cdot CHOH\cdot CH_2\cdot CHO} \\ \\ \underset{\text{Acetaldehyde}}{CH_3\cdot CHO}\end{array}\right. + H_2O \xrightarrow[\text{reduction}]{\text{oxidation}} \begin{array}{l}\underset{\beta\text{-Hydroxybutyric acid}}{CH_3\cdot CHOH\cdot CH_2\cdot COOH} \\ \\ \underset{\text{Ethyl alcohol}}{CH_3\cdot CH_2OH}\end{array}$$

$$\left\{\begin{array}{l}\underset{\beta\text{-Hydroxybutyric acid}}{CH_3\cdot CHOH\cdot CH_2\cdot COOH} \\ \\ \underset{\text{Acetaldehyde}}{CH_3\cdot CHO}\end{array}\right. + H_2O \xrightarrow[\text{reduction}]{\text{oxidation}} \begin{array}{l}\underset{\text{Acetoacetic acid}}{CH_3\cdot CO\cdot CH_2\cdot COOH} + H_2O \\ \\ \underset{\text{Ethyl alcohol}}{CH_3\cdot CH_2OH}\end{array}$$

$$\underset{\text{Acetoacetic acid}}{CH_3\cdot CO\cdot CH_2\cdot COOH} \rightarrow \underset{\text{Acetone}}{CH_3\cdot CO\cdot CH_3} + CO_2$$

## PROPIONIC ACID FERMENTATION

The fermentation of certain carbon compounds to propionic acid is the result of the action of several species of organisms classified under the genus *Propionibacterium.*

**Propionibacterium.**—The members of this genus are characterized as nonmotile, nonspore-forming, Gram-positive organisms that grow under anaerobic conditions in neutral media as short, diphtheroid rods, sometimes resembling streptococci and under aerobic conditions as long, irregular, club-shaped, and branched rods. The organisms ferment a large number of carbohydrates and polyalcohols with the formation chiefly of propionic acid, acetic acid, and carbon dioxide. The organisms develop very slowly, visible colonies not being discernible until after 5 to 7 days. The cells

grow best in yeast extract media containing simple carbohydrates. The optimum temperature for growth is about 30°C.

All the species have been isolated from dairy products and are responsible for the characteristic appearance and flavor of Gruyère (Swiss) cheese.

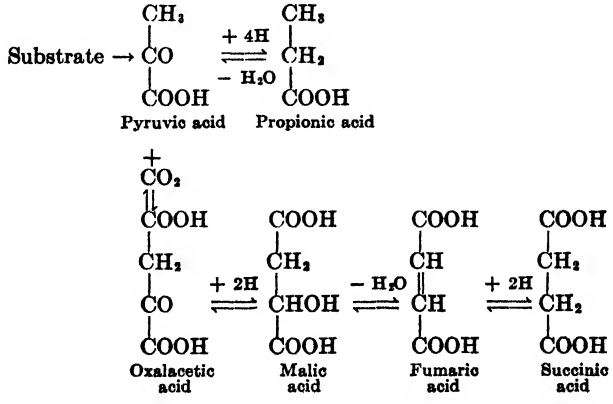
In a series of investigations, Wood and Werkman (1936, 1938, 1940*a*,*b*), Wood, Stone, and Werkman (1937), and Phelps, Johnson, and Peterson (1939) showed that propionic acid bacteria utilized carbon dioxide during the fermentation of glycerol. In the absence of carbon dioxide, the fermentation could be represented by the equation:

$$\underset{\text{Glycerol}}{CH_2OH\cdot CHOH\cdot CH_2OH} \rightarrow \underset{\text{Propionic acid}}{CH_3\cdot CH_2\cdot COOH} + H_2O$$

In the presence of carbon dioxide, the formation of propionic acid was accompanied by the appearance of succinic acid. If this were true, the carbon dioxide was converted into succinic acid by combination with a 3-carbon compound.

Ruben, Hassid, and Kamen (1939), Carson and Ruben (1940), Carson, Foster, Ruben, and Kamen (1940), Carson, Foster, Ruben, and Barker (1941), and Wood, Werkman, Hemingway, and Nier (1940) employed radioactive carbon dioxide as an indicator of its utilization by the propionic acid bacteria. On the basis of the information obtained from their tests, they concluded that propionic acid and succinic acid contained radioactive carbon only in the carboxyl groups.

Carson, Foster, Ruben, and Barker suggested the following scheme for the formation of propionic and succinic acids in the presence of $C^*O_2$:[1]




On the basis of their results they came to the following conclusions:

1. Both propionic and succinic acids formed in the presence of $C^*O_2$ are radioactive.
2. These acids contain the labeled carbon only in the carboxyl (COOH) groups.

[1] The symbol C* indicates radioactive carbon.

3. Pyruvic acid is an intermediate compound in the propionic acid fermentation.

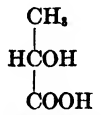
4. A radioactive $\alpha$-keto acid, besides pyruvic acid, is formed during the fermentation of pyruvic acid in the presence of $C^*O_2$. This acid contains most, if not all, of the $C^*$ in carboxyl groups.

5. The set of reversible reactions from oxalacetic acid to succinic acid have been found to occur in the propionic acid bacteria.

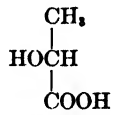
## LACTIC ACID FERMENTATION

Lactic acid was first discovered as one of the products resulting from the souring of milk. It is named after the milk constituent lactose, or milk sugar, which is the precursor of the compound. It is probably the oldest known acid, having been discovered by Scheele in 1780.

The acid produced by bacterial action on carbohydrates is known as fermentation acid. Since lactic acid has an asymmetric carbon atom, it exists in two modifications. These are known as dextrolactic acid and levolactic acid.



Dextrolactic acid



Levolactic acid

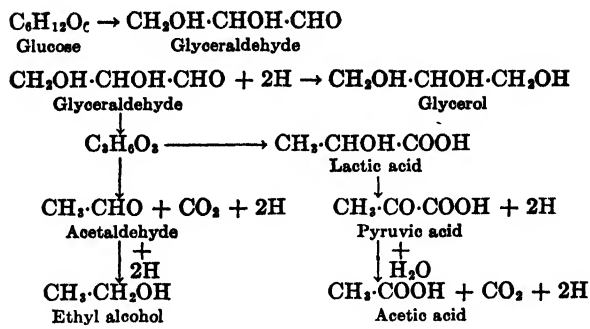
The organisms that are generally employed industrially for the production of lactic acid are members of the genus *Lactobacillus*, and *Streptococcus lactis*. These organisms differ in the kind of lactic acid produced: some produce the levo acid; others produce the dextro modification; still others produce inactive acid, *i.e.*, a mixture of the two forms in equal quantities.

**Lactobacillus.**—The various species consist of large rods, often long and slender and capable of producing lactic acid from carbohydrates and carbohydrate-like compounds. Some species produce gas; others do not. If gas is produced, it consists entirely of carbon dioxide. A number of species are somewhat thermophilic and some are microaerophilic.

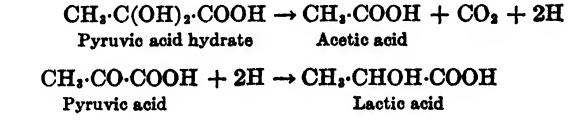
**Streptococcus lactis.**—The characteristics of this organism are given on page 497.

A large number of carbohydrates are employed for the production of lactic acid: generally, glucose, sucrose, and lactose. Starches of various kinds may be first hydrolyzed to sugars by means of acids or enzymes and then fermented to lactic acid. Molasses and whey are low-priced and excellent sources of carbohydrate for lactic acid production.

The scheme for the fermentation of glucose to lactic acid, according to Nelson and Werkman (1935), is as follows:




Acetaldehyde and pyruvic acid are important intermediary compounds. The addition of pyruvic acid to a fermentation medium results in the formation of equimolar quantities of acetic acid, lactic acid, and carbon dioxide:




For further reading on lactic acid fermentation by streptococci, see Smith and Sherman (1942).

## ACETIC ACID BACTERIA

Vinegar is a product of the oxidation of alcoholic liquids to acetic acid by certain microorganisms.

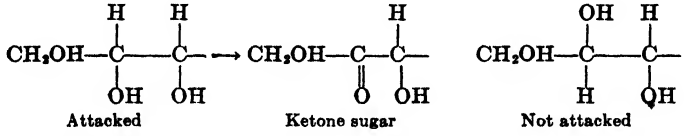
Any alcoholic liquid may be employed in the manufacture of vinegar. The fermentation of apple cider to hard cider (alcoholic) by means of yeasts and then to acetic acid by the specific bacteria yields a product known as cider vinegar. The oxidation of wines yields wine vinegar. The alcoholic fermentation of an infusion of barley malt followed by the acetic fermentation gives a product known as malt vinegar. Sugar vinegar is the result of the alcoholic fermentation of sugar followed by the oxidation of the alcohol to acetic acid. The final product is named after the raw materials used in its manufacture. All vinegars contain about 4 per cent of acetic acid.

When any alcoholic liquid is exposed to the air, a film appears on its surface. At the same time, the liquid becomes sour, owing to the oxidation of the alcohol to acetic acid. The film is composed of a viscous gelatinous substance, or zooglea, in which are embedded many bacteria. It is commonly known as "mother of vinegar" because a small portion of this material is capable of acting as a starter when added to more alcoholic liquid.

The bacteria present in a zoogleal mass are classed under the genus *Acetobacter*. They are the so-called acetic acid bacteria. These organisms are dependent upon a plentiful supply of oxygen for growth and multiplication. That is why their activities are confined to the surface of alcoholic liquids.

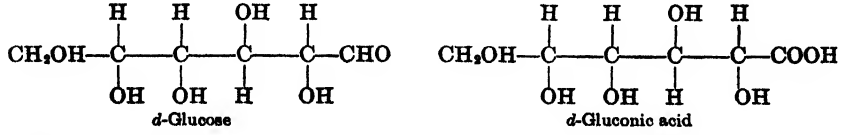
**Acetobacter.**—The cells are rod-shaped, frequently in chains, and motile by means of a single polar flagellum (Vaughn, 1943). Usually they grow on the surface of alcoholic liquids as obligate aerobes, securing growth energy by the oxidation of alcohol to acetic acid. They are also capable of utilizing many other carbonaceous compounds. Elongated, filamentous, club-shaped, swollen, and even branched cells may occur as involution forms.

The acetic acid bacteria are capable of oxidizing other primary alcohols and aldehydes to carboxylic acids and secondary alcohols to ketones. Bertrand (1904) found that *A. xylinum* (sorbose bacillus) was capable of oxidizing a secondary alcohol group of sorbitol to the corresponding ketone sugar sorbose. He noted that the organism oxidized some alcohols but failed to attack others. On careful examination of his results he concluded that (1) only secondary alcohol groups are attacked; (2) the group oxidized must be on the second carbon atom; and (3) the (OH) groups on the second and third carbon atoms must be adjacent to each other for oxidation to occur:



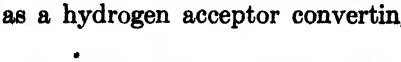

The effect of the configuration of a number of higher alcohols on fermentability by *A. xylinum* is given in the tabulation on page 383.

Hermann and Neuschul (1931) noted an exception to the above rule. They found that the aldehyde group of *d*-glucose was oxidized to a carboxyl group to give *d*-gluconic acid, even though the (OH) groups on the second and third carbon atoms are not adjacent to each other:

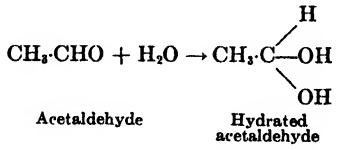



**Aerobic Oxidation of Alcohol.**—The aerobic oxidation of alcohol to acetic acid is generally believed to occur according to the following scheme:

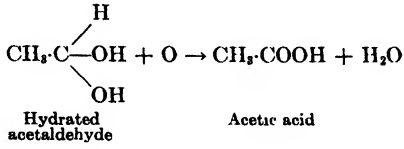
1. Oxygen acts as a hydrogen acceptor converting alcohol to acetaldehyde.



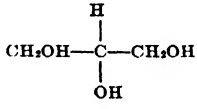
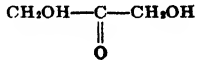
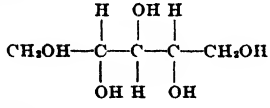
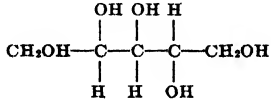
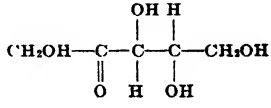
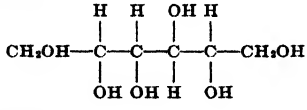
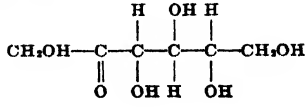
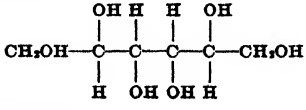
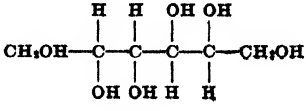
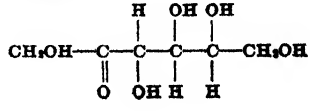
2. Acetaldehyde becomes hydrated.




3. Oxygen accepts two hydrogen atoms from the hydrated acetaldehyde to give acetic acid.

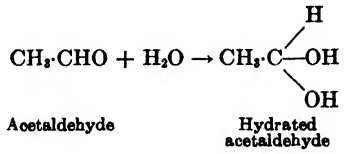



EFFECT OF CONFIGURATION ON OXIDATION BY *Acetobacter xylinum*

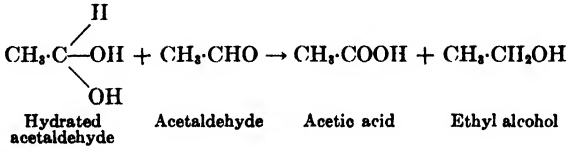
| Alcohol | Oxidation Product |
|---|---|
| Ethylene glycol, $CH_2OH \cdot CH_2OH$ | Not oxidized |
| Glycerol,  | Dihydroxyacetone,  |
| Xylitol,  | Not oxidized |
| Arabitol,  | Araboketose,  |
| Sorbitol,  | Sorbose,  |
| Dulcitol,  | Not oxidized |
| Mannitol,  | Fructose,  |

**Anaerobic Oxidation of Alcohol.**—In the absence of oxygen, 1 molecule of acetaldehyde may act as a hydrogen acceptor for a second hydrated molecule of acetaldehyde. This results in the formation of 1 molecule of acetic acid and 1 of ethyl alcohol from 2 molecules of acetaldehyde, according to the following scheme:

1. Hydration of acetaldehyde.



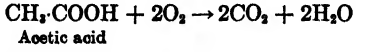

2. Cannizzaro reaction.




Neuberg and Windisch (1925) believed that ethyl alcohol was first oxidized to acetaldehyde, which in turn was dismutated to equimolar quantities of ethyl alcohol and acetic acid according to the foregoing reactions. Alternate oxidation and dismutation continued until all the ethyl alcohol was converted into acetic acid.

The acetic acid concentration in the final solution is diluted to 4 per cent to give commercial vinegar. In addition to acetic acid, traces of esters also produced in the fermentation are largely responsible for the pleasant odor and flavor of vinegar.

Vinegar may lose its strength on standing. This is due to the oxidation of the acetic acid to carbon dioxide and water by some species of *Acetobacter:*




This reaction takes place only in the presence of considerable oxygen. The oxidation may be prevented by storing vinegar in well-filled, tightly stoppered bottles or by the destruction of the organisms by pasteurization.

**Methods of Manufacture.**—Two general methods are employed in the manufacture of vinegar: (1) the Orleans method and (2) the quick method.

*Orleans Method.*—This is the oldest commercial method known for the preparation of vinegar. Barrels or vats are perforated near the top to permit free entrance of air and then filled about two-thirds full with a mixture composed of 2 parts of vinegar and 3 parts of wine. The wine may be raw or pasteurized, the latter being preferable since it greatly reduces the percentage of abnormal fermentations. The acetic acid bacteria grow

better in a strongly acid medium. For this reason, vinegar is added to the wine to speed up the reaction and at the same time to check the growth of undesirable organisms. At definite intervals some of the vinegar is drawn off and fresh wine added. In this manner, the process becomes continuous.

*Quick Method.*—The quick method depends upon the use of large wooden tanks or generators with perforated bottoms through which air

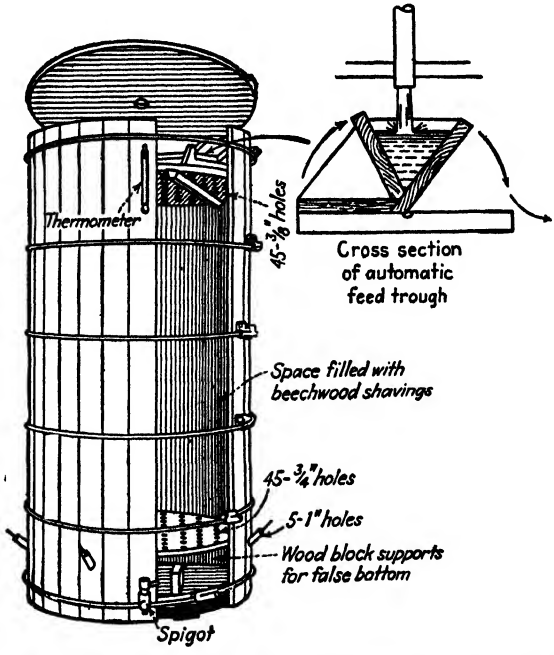



FIG. 153.—Generator used in the quick vinegar process. (*From Prescott and Dunn, Industrial Microbiology.*)

enters (Fig. 153). These tanks are filled with beechwood shavings, charcoal, etc., to give greater aeration of the alcoholic liquid by increasing the surface area. The shavings or charcoal is first soaked with a culture of acetic acid bacteria. Then the alcoholic solution is sprayed at the top of the tank and allowed to trickle over the surface of the shavings. By the time the liquid reaches the bottom of the tank, the alcohol becomes oxidized to acetic acid by the organisms present on the shavings. The temperature is kept at about 35 to 38°C. After the completion of the process, the vinegar is drawn off at the bottom of the tank. A generator 10 ft. in diameter and 20 ft. high is capable of producing from 80 to 100 gal. of vinegar per day.

Many modifications of the above generator are employed for the manufacture of vinegar. One of these, known as the Frings generator, possesses

many advantages (Fig. 154). It is cheap and simple to operate. It produces vinegars having higher concentrations of acetic acid than those produced by other methods. Since the tank is smaller, it utilizes less space. The generator is airtight, thus avoiding loss of vinegar or alcohol by evaporation.

For additional information, see Fulmer, Bantz, and Underkofler (1944).

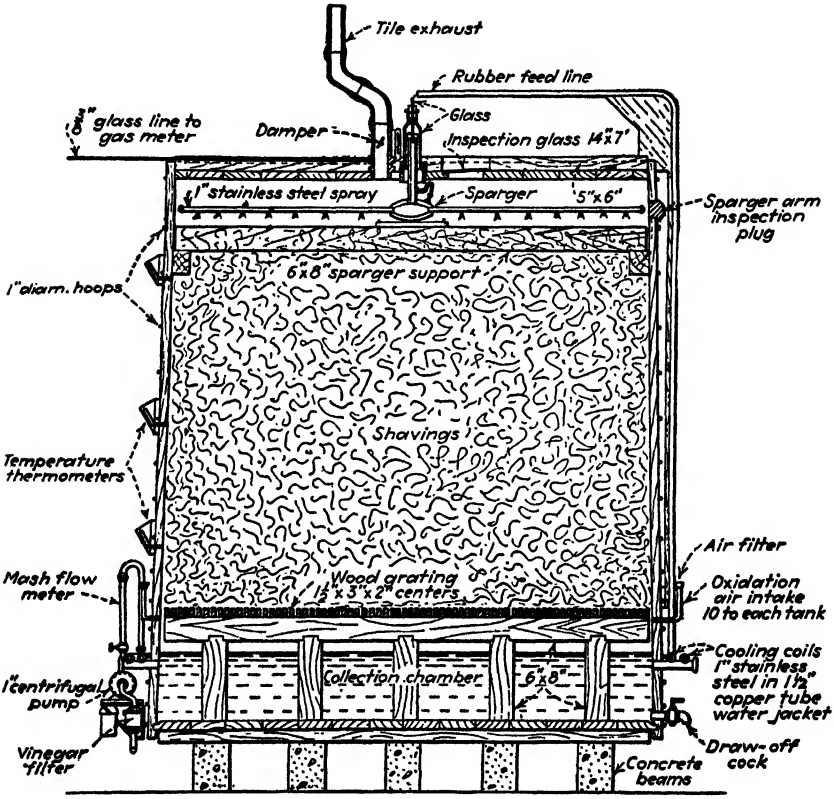



FIG. 154.—Cross section of the Frings generator. (*From Prescott and Dunn, Industrial Microbiology.*)

## GENERAL CARBOHYDRATE FERMENTATIONS

Carbohydrates and compounds of a similar nature are generally added to culture media for two important purposes: (1) to serve as readily available sources of energy, and (2) to aid in the identification and classification of bacteria.

Carbohydrates are more readily available as sources of energy than are proteins. This means that the rate of multiplication of an organism is generally increased in the presence of a fermentable carbohydrate.

Organisms vary considerably in their ability to ferment various carbohydrates. Some bacteria are able to attack one or more carbohydrates and produce acid and gas; others are able to produce acid but not gas; still others fail to ferment any carbohydrate (Fig. 155). Such information is of considerable value in the identification of organisms.

It is not clearly understood why an organism ferments one aldose sugar and not another having the same empirical formula. The sugars differ only in the arrangement of H atoms and OH groups around carbon atoms. There is no method for determining beforehand whether or not a particular organism is capable of fermenting a given carbohydrate. This can be

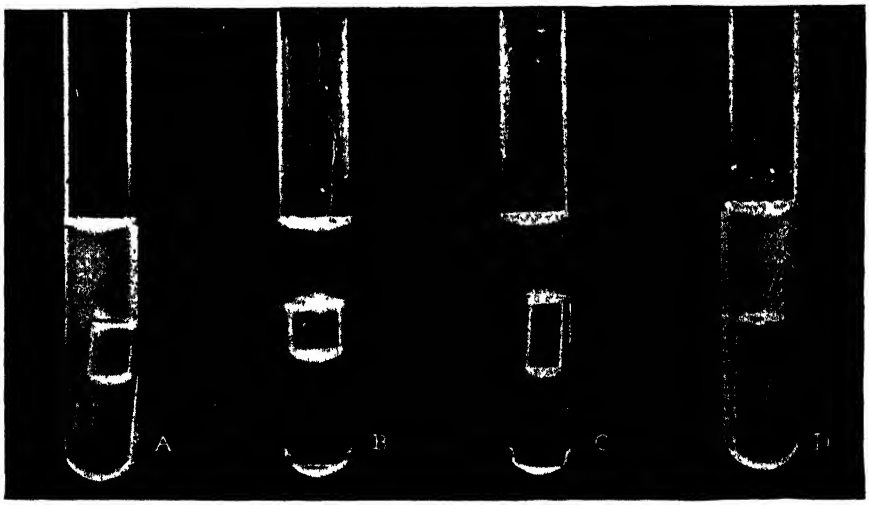



FIG. 155.—Fermentation of carbohydrates. *A*, glucose; *B*, lactose; *C*, sucrose, all inoculated with *Escherichia communior*. Acid and gas produced. *D*, sucrose inoculated with *E. coli*. No acid or gas.

determined only by making the test. As Kendall, Bly, and Haner (1923) stated, ". . . the carbohydrate, to be utilizable, must possess a stereoconfiguration which is compatible with a corresponding asymmetry of the protoplasm of the microbe." Emil Fischer in his studies on the chemistry of the carbohydrates expressed the above relationship as that of a key fitting its particular lock.

Sternfeld and Saunders (1937) concluded from their studies that any change from the structure of the aldose sugars resulted in a decreased frequency of fermentation of the derivatives. Robbins and Lewis (1940) in their studies on the fermentation reactions of a large number of organisms came to the same general conclusions. They found that *d*-gluconic, *d*-mannonic, and *d*-galactonic acids as well as *d*-sorbitol, *d*-mannitol, and dulcitol were fermented by fewer organisms than the corresponding aldoses.

Similarly, the dicarboxylic mucic and *d*-saccharic acids were attacked less frequently than the corresponding monocarboxylic *d*-galactonic and *d*-gluconic acids. Georgi and Ettinger (1941) obtained similar results in their studies on several species of *Rhizobium*. In general, mono- and disaccharides were more frequently attacked than tri- and polysaccharides and the sugar acids.

**Fermentations by Coliform Organisms.**—Members of the colon group and related species ferment glucose with the production of acetic, formic, succinic, and lactic acids, ethyl alcohol, acetylmethylcarbinol, 2,3-butanediol (2,3-butylene glycol), carbon dioxide, and hydrogen. These organisms produce probably the most common types of bacterial fermentations.

The products produced from the fermentation of glucose by *Escherichia coli* and *Aerobacter aerogenes* are given in Table 39.

TABLE 39.—FERMENTATION OF GLUCOSE BY *Escherichia coli* AND *Aerobacter aerogenes*.

| Compounds Produced | |
|---|---|
| *E. coli* | *A. aerogenes* |
| Formic acid | Formic acid |
| Acetic acid | Acetic acid |
| Lactic acid | Lactic acid |
| Succinic acid | Succinic acid |
| Ethyl alcohol | Ethyl alcohol |
| Carbon dioxide | Acetylmethylcarbinol |
| Hydrogen | 2,3-Butanediol |
| | Carbon dioxide |
| | Hydrogen |

In the case of *E. coli*, the compounds account for about 99 per cent of the carbon of the fermented glucose. A similar analysis on the same medium fermented by *A. aerogenes* does not account for such a high percentage of the carbon of the fermented glucose. The discrepancy is due to the production by *A. aerogenes*, but not by *E. coli*, of the two compounds acetylmethylcarbinol and 2,3-butanediol.

The production of acetylmethylcarbinol and 2,3-butanediol is not characteristic of *A. aerogenes* alone. A number of other bacteria and yeasts are also capable of producing the two compounds (see page 516). Since 2,3-butanediol contains two asymmetric carbon atoms, it exists in three isomeric forms: *d*-2,3-, *l*-2,3-, and meso-2,3-butanediols.

The mechanism for the formation of acetylmethylcarbinol and 2,3-butanediol is not clearly understood. The two compounds are generally found to be present in fermentation cultures. Some believe that acetylmethylcarbinol is formed first, then a part of it is reduced to 2,3-butanediol. Others maintain that 2,3-butanediol is first produced, after which some of it

is oxidized to acetylmethylcarbinol. Stahly and Werkman (1942) in their studies on the fermentation of glucose by *Aerobacillus polymyxa* suggested that acetylmethylcarbinol and 2,3-butanediol comprise a reversible oxidation-reduction (redox) system. A low redox potential favored the accumulation of 2,3-butanediol; a high potential favored the formation of acetylmethylcarbinol.

For additional information, see Adams and Stanier (1945), Fulmer, Underkofler, and Bantz (1943), Kalnitsky and Werkman (1943), Stanier and Fratkin (1944), and Underkofler, Fulmer, Bantz, and Kooi (1944).

## SOME ROUTINE FERMENTATION REACTIONS EMPLOYED FOR THE IDENTIFICATION OF BACTERIA

The ability of an organism to ferment a particular carbohydrate is determined by incorporating an appropriate indicator in a liquid or a solid medium. Gas production is detected by placing an inverted vial in a carbohydrate broth medium to trap the gas as it is evolved.

**Litmus Carbohydrate Media.**—Litmus is a weakly staining dye and is employed only as an indicator. Since it is not a delicate detector of changes in acidity or alkalinity, it has been largely replaced by the more sensitive and brilliant sulfonephthalein indicators.

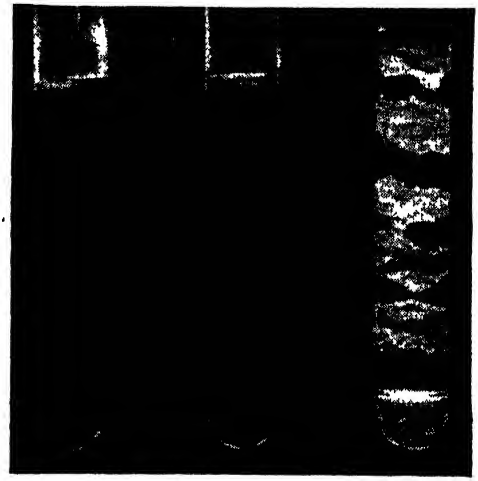


FIG. 156.—Action of organisms on lactose litmus agar. Left, *Bacillus subtilis;* center, *Micrococcus pyogenes* var. *aureus;* right, *Escherichia coli. B. subtilis* and *M. pyogenes* var. *aureus* produce neither acid nor gas; *E. coli* produces acid and gas with the result that the agar is separated into rings and the litmus is decolorized.

Litmus possesses an important advantage over the newer indicators in that it is sensitive to decolorization by organisms. It functions as an oxidation-reduction system. The dye accepts hydrogen and becomes reduced to the colorless compound. The decolorization of the indicator is first noted in the deeper portions of culture media where the dissolved oxygen is soon exhausted. In the surface layer of media exposed to air, the indicator seldom becomes completely decolorized, owing to the fact that the hydrogen becomes transferred to oxygen.

The fermentation of a tube of deep carbohydrate agar results in a splitting of the agar by the escaping gas. In some cases, the agar splits in the form of disks with a layer of gas separating each disk of agar (Fig. 156).

Under some conditions, the gas pressure may be sufficient to force some of the agar disks, together with the cotton stopper, out of the tube.

**Bromocresol Purple Carbohydrate Media.**—The fermentative ability of an organism can be determined very easily by streaking a loopful of the culture over the surface of nutrient agar containing carbohydrate and indicator. Fermentation of the carbohydrate results in an increase in the

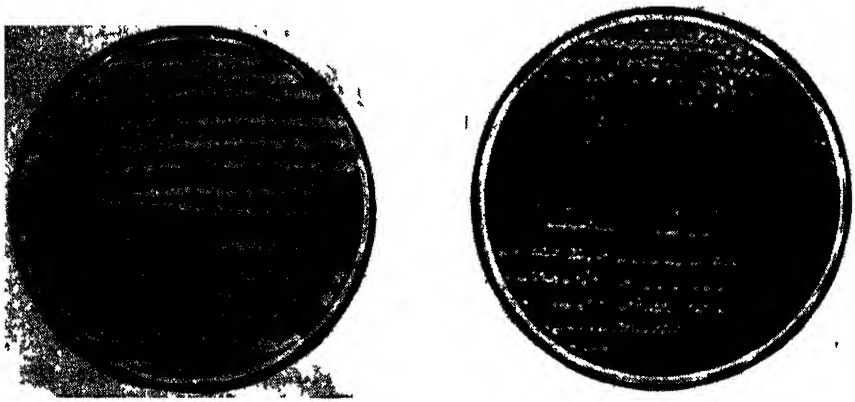


FIG. 157.—Bromocresol purple lactose agar. Left, plate streaked with a culture of *Bacillus subtilis*. The organism produced no visible change in the color of the agar in 24 hr.; right, plate streaked with a culture of *Escherichia coli* The organism produced considerable acid in 24 hr. changing the color of the agar from purple to yellow

hydrogen-ion concentration of the agar. The visible result is a change in the color of the indicator (Fig. 157).

If only a few well-isolated colonies appear on the plate, acid production remains confined to areas immediately surrounding the colonies. This results in a color change in the vicinity of each colony without affecting the agar free from colonies. The contrast in the colors in the acid and alkaline or neutral regions of the agar becomes very striking.

In the presence of a mixed culture composed of two organisms only one of which is capable of fermenting the carbohydrate, isolations of two different colonial forms may be successfully realized. This is possible only if the culture is diluted highly before being streaked and, also, if the two organisms are present in approximately equal numbers.

Bromocresol purple is frequently used as the indicator in carbohydrate media. This indicator is especially valuable for bacteriological work because of its brilliant colors at different hydrogen-ion concentrations and its resistance to decolorization by bacterial action. The sensitive range of the indicator is from pH5.2 to 6.8. It is yellow at pH5.2 and purple at pH6.8. The pK value, *i.e.*, the point at which the dye is 50 per cent dissociated, is pH6.3. Its pH range makes it suitable for the detection of bacterial fermentations in solid carbohydrate media.

**Reversal of Reaction.**—It sometimes happens that the acid reaction in carbohydrate media turns alkaline, with respect to the indicator employed, if the plates or tubes are stored for several days. This may be explained on the basis of (1) a lack of sufficient carbohydrate and (2) the oxidation of the organic acids to carbonates.

If a medium contains an insufficient amount of carbohydrate, an organism will not be able to produce its limiting hydrogen-ion concentration. Under these circumstances, the organism will continue to multiply by utilizing the nitrogenous constituents for both structure and energy. This results in the production of alkaline products with the consequent reversal in the reaction of the medium.

Reversal in the reaction of a medium sometimes occurs even in the presence of an amount of carbohydrate sufficient for an organism to produce its limiting hydrogen-ion concentration. It was shown by Ayers and Rupp (1918) that under these conditions simultaneous acid and alkaline reactions occur. The carbohydrates are first fermented to organic acids, which are in turn oxidized to carbonates. The alkaline carbonates are responsible for the reversal in the reaction of the medium. The alkaline changes do not occur after all of the carbohydrate has been converted to acid, but run simultaneously with acid production.

## ENSILAGE FERMENTATION

The preparation of ensilage affords a rather simple method for the preservation of foods for stock animals. Any farm crop is satisfactory for ensilage production, provided it contains sufficient sugar to furnish the required amount of acid for its preservation.

The plants are cut into small pieces and packed into silos. Sufficient moisture must be present in the cut pieces, otherwise water is added. Since the surface of the plants contains the required kinds of organisms, it is not necessary to inoculate the material. Counts have shown that there are normally present from 10,000 to 400,000,000 bacteria per gram of plant tissue.

Because of the presence of carbohydrates in the plant sap, the changes that occur are fermentative rather than putrefactive. Within a few hours, the aerobic organisms utilize the free oxygen present in the silo. The changes that follow are anaerobic rather than aerobic in character. After a period of about 48 to 72 hr., from 70 to 80 per cent of the gas present in the silo is carbon dioxide. Considerable heat is generated during the fermentation process. When fermentation is active, each gram of the silage may contain as many as 2,000,000,000 organisms. Each cubic centimeter of the silage juice may show a count as high as 4,000,000,000 bacteria. Fermentation is complete after a period of about 1 month. The organisms responsible for the fermentation are certain streptococci and several species

of the genus *Lactobacillus*. The organisms produce chiefly lactic acid, acetic acid, butyric acid, propionic acid, and small amounts of alcohol. The quantities and proportions of each vary, depending upon the kinds of plants used.

### TOBACCO FERMENTATION

Tobacco is cured in order to improve its aroma and texture. During this treatment, about 28 per cent of the nicotine is lost, accompanied by an increase in citric acid.

The leaves are stacked in piles and allowed to ferment. During this stage, heat is generated, the temperature going as high as 60°C. Oxygen is consumed and carbon dioxide, ammonia, and other volatile substances are released. The flavors of tobaccos are improved by moistening the leaves with sugars, sirups, malt extract, honey, etc., which are responsible for the development of aromatic esters and other compounds. After the completion of the fermentation, the leaves are dried and then used in the manufacture of various kinds of tobaccos. The course of the fermentation appears to be greatly influenced by variations in the composition of the tobacco leaf. Failure of certain crops to undergo a satisfactory fermentation has been responsible for great losses in the industry.

The nature of the chemical changes that occur during fermentation are not clearly understood. As stated by Reid, McKinstry, and Haley (1938*a*),

> Three theories have been advanced to account for the chemical changes occurring during the fermentation process by workers who have studied the problem. The first theory advanced maintained that the reactions were purely of an oxidative character and were not catalyzed by enzymes. That the fermentation of cigar leaf may be explained in this manner is no longer the belief of those familiar with the process, but the literature of recent years on the subject of the fermentation of other types of tobacco implies acceptance of this theory. The second theory advanced ascribed the chemical reactions as due to the activities of microorganisms. Although 50 years have passed since the inception of this theory, the support accorded it in recent years has been vague and somewhat contradictory. The third theory attempts to account for the chemical reactions during fermentation on the basis of catalysis by leaf enzymes, and this appears to be accepted by the majority of those engaged in the processing of cigar-leaf tobacco at the present time.

Recently, considerable evidence has been brought forward in support of the bacterial theory. Reid, McKinstry, and Haley (1938*a*,*b*) showed that the major changes were brought about by the activity of certain bacteria that usually developed in large numbers during the fermentation process, and that leaf enzymes played only a subordinate role. The predominant forms upon cured leaf were bacteria of the *Bacillus megatherium* group and molds of the genera *Penicillium* and *Aspergillus*. A satisfactory fermenta-

tion was found to be associated with a rapid increase in numbers of organisms of the *Micrococcus candicans* type and of the *B. subtilis-mesentericus-vulgatus* group. The predominant types upon cured leaf played little if any part in a satisfactory fermentation. Viable fungi disappeared during the early stages of the fermentation, and bacteria of the *B. megatherium* group failed to show any significant increase in number during the process. Some cellulose-decomposing species of the genus *Clostridium* were occasionally encountered on cured tobacco. They were usually responsible for the rotting that sometimes occurred during the fermentation process.

In a later report Gribbins, Haley, and Reid (1944) found that the addition of yeast to cigar-leaf tobacco produced a relatively high initial increase in temperature during the preliminary stages of fermentation. A number of true yeast cells were present 24 hr. after treatment but later examinations failed to show their presence. However, the presence of certain yeast-like organisms were always observed whether or not the samples were treated with yeast. Their numbers were definitely increased as a result of the yeast treatment, which appeared to be beneficial to the fermentation process. The addition of yeast stimulated the development of organisms considered necessary to the fermentation process. With few exceptions, a high bacterial count was paralleled by a low mold content, which is considered highly desirable. There appeared to be little, if any, increase in mold development as a result of yeast treatment.

For further reading, consult Bernhauer (1938, 1939), Burton (1937), Butlin (1936), Hann, Tilden, and Hudson (1938), Hansen (1935), Pan, Peterson, and Johnson (1940), Prescott and Dunn (1940), Prescott and Proctor (1937), and Werkman and Wood (1942).

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## CHAPTER XVI

## DIFFERENTIATION AND CLASSIFICATION OF BACTERIA

In order that reference may be made to an organism it must be given a name. Two kinds of names are employed. Each organism possesses a scientific name, which is more or less international in meaning. In addition, an organism may possess one or more common names, which are of local interest. The common names are responsible for considerable confusion in bacteriology. For this reason, it is better to refer to organisms by their scientific names which are supposed to be the same in all countries.

**General Principles of Nomenclature.**—The method followed in naming organisms was first introduced by the Swedish botanist Karl von Linné and is known as the binomial system of nomenclature.

Each kind of organism or species has two names. The first word is the name of the genus and the second that of the species. The name of the genus is usually taken from the Latin or, rarely, the Greek language. It is a noun and is always capitalized. It may be masculine, or feminine, or neuter. Examples of the three genders are *Bacillus* (masculine); *Sarcina* (feminine); and *Bacterium* (neuter).

The second or species name is generally an adjective and is not capitalized. It may be capitalized if it is derived from a proper noun but that is not necessary. For the sake of consistence, it is better to capitalize only the name of the genus.

Sometimes a species is subdivided into varieties. These are also given Latin designations. For example, *Mycobacterium tuberculosis* has been subdivided into the two varieties, *M. tuberculosis* var. *hominis* (human tuberculosis) and *M. tuberculosis* var. *bovis* (tuberculosis of cattle).

**General Principles of Taxonomy.**—The term "taxonomy" may be defined as the classification of plants and animals according to their natural relationships. It is compounded from the two Greek words τάξις, an arrangement, order, and νομος, a law.

A satisfactory development of taxonomy is dependent upon a sound nomenclature. Regardless of whether bacteriologists will ever be able to agree on the exact classification to be employed, they should agree on some of the fundamental characteristics necessary for the development of a satisfactory bacteriological classification.

Each kind of plant or animal is referred to as a species (plural, species). The term is defined in various ways, but in bacteriology it is usually stated

to be the lowest member of a classificatory system. A bacterial species is a plant that occupies a place in a classification between the genus and the variety. Since the differences between varieties are often very difficult to recognize, it is the species that to the untrained observer usually seem to represent the simplest distinct assemblages or kinds in the plant or animal kingdoms.

The first described specimen of a species is spoken of as the type of the species. It is used as the type species for all other species regarded as sufficiently like the type to be placed together in the same group or genus.

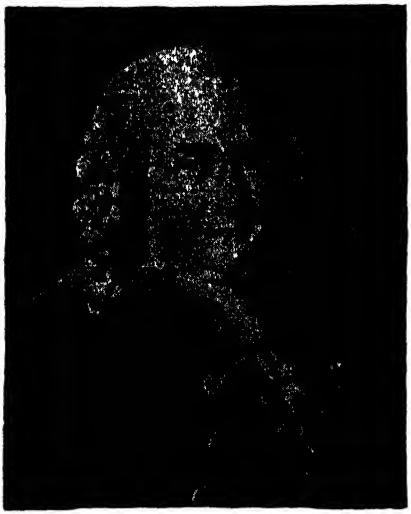

FIG. 158.—Karl von Linné.

A group of closely related species is spoken of as a genus (plural, genera). It ranks between the family and the species. Closely related genera, then, are grouped into families. Finally, closely related families are grouped into orders.

**Differentiating Bacteria.**—Hundreds of species of bacteria have been recognized and studied. They represent probably only a small percentage of the total number of species in existence. Since bacteria are so small and undifferentiated, it is an exceedingly difficult matter to identify and classify them.

During the early years of bacteriology, organisms were classified entirely on the basis of morphology. Only a few species were recognized, and their classification was comparatively a simple matter. The morphological characters employed include size and shape of an organism; arrangement of the cells; presence or absence of well-defined capsules; presence or absence of spores; size and shape of the spore and position in the cell; presence, number, and arrangement of flagella; irregular forms; presence or absence of characteristic metachromatic granules; acid fastness; Gram reaction and other differential staining procedures; cultural and colonial characteristics; etc. As more and more species were recognized, the problems of classification became increasingly more difficult. At the present time, it would be a hopeless task to attempt to identify and classify bacteria entirely on morphology because there are so few characteristics on which to base a classification.

Higher plants are differentiated almost entirely on the basis of morphology. One tree may be easily distinguished from another by differences in such characteristics as the size, shape, and color of the tree; size, shape,

and color of the leaves and seeds; etc. In the case of bacteria, it is quite evident that the problems of classification are more difficult, owing to the fact that such minute organisms are comparatively simple spheres, rods, and spirals.

It soon became apparent that a classification based entirely on morphology was inadequate and that more characteristics were necessary. Physiological reactions were, therefore, introduced into the newer classifications. They include such reactions as temperature relations; chromogenesis or pigment production; effect of a change in the reaction of the environment on growth; production of indole; production of hydrogen sulfide; relation to oxygen; reduction of nitrate to nitrite and even to ammonia, and finally to free nitrogen; fermentation of carbohydrates; etc. At the present time, physiological reactions are probably more important than morphological differences in the classification of bacteria.

Sometimes it is necessary to resort to animal inoculation and serological reactions to separate similarly appearing and reacting organisms. Serological methods are discussed in Chap. XXIV, Infection and Immunity.

Bacteria are placed in the class *Schizomycetes* (fission fungi). This class is composed of five orders. The true bacteria are placed in the order *Eubacteriales*. Organisms placed in the other four orders show characteristics intermediate between the true bacteria and higher plants or animals.

The abridged classification that follows was prepared by a committee appointed by the Society of American Bacteriologists. This committee, now known as the Board of Editor-Trustees, has had the cooperation of a group of approximately 100 bacteriologists, interested in developing the systematic relationships of the various groups of bacteria. The work of the committee is published under the title of "Bergey's Manual of Determinative Bacteriology" in honor of Dr. D. H. Bergey who was responsible for developing the first edition of the manual.

The Board of Editor-Trustees of the sixth edition of the manual is composed of E. G. D. Murray, A. P. Hitchens, and R. S. Breed.[1]

Class: *Schizomycetes*. Typically unicellular plants. Cells usually small, sometimes ultramicroscopic. Frequently motile. Cells lack the definitely organized nucleus found in the cells of higher plants and animals. However, bodies containing chromatin which may represent simple nucleuses are demonstrable in some cases. Individual cells may be spherical; or straight, curved, or spiral rods. Cells may occur in regular or irregular masses or even in cysts. Where they remain attached to each other after cell division, they may form chains or even definite filaments. The

[1] The author is greatly indebted to Dr. R. S. Breed for his kindness in making available this outline classification of the manual in advance of its date of publication. The names of the organisms used throughout the text are based on this latest classification insofar as it was possible to do so.

latter may show some differentiation into holdfast cells, and into motile or nonmotile reproductive cells (conidia). Some grow as branching mycelial threads whose diameter is not greater than that of ordinary bacterial cells, i.e., about one micron. Some species produce pigments. The sulfur purple and green bacteria possess pigments much like or related to the true chlorophylls of higher plants. These pigments have photosynthetic properties. The phycocyanin found in the blue-green algae does not occur in the *Schizomycetes*. Multiplication is typically by cell division. Endospores are formed by some species included under the *Eubacteriales*. Sporocysts are found in the *Myxobacteriales*. Ultramicroscopic reproductive bodies are found in the *Borrelomycetaceae*. The bacteria are free-living, saprophytic, parasitic, or even pathogenic. The latter types cause diseases of either plants or animals. Five orders are recognized.

**Key to the Orders and Suborders of the Class Schizomycetes**

A. Cells rigid, not flexuous. Motility by means of flagella or by a gliding movement.
  1. Cells single, in chains or masses. Not branching and mycelial in character. Not arranged in filaments. Not acid-fast. Motility when present by means of flagella.
     Order I. *Eubacteriales*, page 401.
     *a*. Do not possess photosynthetic pigments. Cells do not contain free sulfur.
        *b*. Not attached by a stalk. Do not deposit iron oxide.
           Suborder I. *Eubacterineae*, page 401.
        *bb*. Attached to substrate, usually by a stalk. Some deposit iron oxide.
           Suborder II. *Caulobacteriineae*, page 412.
     *aa*. Possess photosynthetic purple or green pigments. Some cells contain free sulfur.
        Suborder III. *Rhodobacteriineae*, page 413.
  2. Organisms forming elongated usually branching and mycelial cells. Multiply by special oidiospores and conidia. Sometimes acid-fast. Nonmotile.
     Order II. *Actinomycetales*, page 415.
  3. Cells in filaments frequently enclosed in tubular sheath with or without a deposit of iron oxide. Sometimes attached. Motile flagellate and nonmotile conidia. Filaments sometimes motile with a gliding movement. Cells sometimes contain free sulfur.
     Order III. *Chlamydobacteriales*, page 416.

B. Cells flexuous, not rigid.
  1. Cells elongate. Motility, by creeping on substrate.
     Order IV. *Myxobacteriales*, page 418.
  2. Cells spiral. Motility, free swimming by flexion of cells.
     Order V. *Spirochaetales*, page 419.

C. Supplement: Groups whose relationships are uncertain.
  1. Obligate intracellular parasites or dependent directly on living cells.
     *a*. Not ultramicroscopic. More than 0.1$\mu$ in diameter. Adapted to intracellular life in arthropod tissues.
        Group I. Family *Rickettsiaceae*, page 421.
     *aa*. Ultramicroscopic. Less than 0.1$\mu$ in diameter.
        Group II. Order *Virales*, page 421.
  2. Grow in cell-free culture media with the development of polymorphic structures including rings, globules, filaments, and minute reproductive bodies (less than 0.3 $\mu$ in diameter).
     Group III. Family *Borrelomycetaceae*, page 421.

**An Abridged Classification of the Organisms into Orders, Suborders, Families, Tribes and Genera**

Order I. *Eubacteriales.* Simple and undifferentiated rigid cells which are either spherical or rod-shaped. The rods may be short or long, straight, curved, or spiral. Some are nonmotile, others show locomotion by means of flagella. Elongated cells divide by transverse fission and may remain attached to each other in chains. Spherical organisms divide either by parallel fission producing chains, or by fission alternating in two or three planes producing either tetrads or cubes of 8 and multiples of 8 cells. Many spherical cells form irregular masses in which the plane of division cannot be ascertained. Endospores occur in some species. Some species are chromogenic, but only in a few is the pigment photosynthetic (bacteriopurpurin and chlorophyll).

Suborder I. *Eubacteriineae.* These are the true bacteria in the narrower sense of the word. The cells are rigid and free. Branching occurs only under abnormal conditions of life. They are not attached by holdfasts nor stalks. They form no sheaths. One-third of the species form pigments of the nonphotosynthetic type. Endospores occur in the family *Bacillaceae*, rarely in others.

Family I. *Nitrobacteriaceae.* Cells without endospores. Rod-shaped or ellipsoidal except for one spherical species (*Nitrosococcus nitrosus*). Spiral rods in *Nitrosospira* and in one species of *Thiobacillus*. Flagella either polar or absent. Gram stain uncertain, but presumably Gram-negative for all of the polar flagellate, rod-shaped species except for *Nitrosomonas monocella* which is reported to be Gram-positive. Capable of growing without organic compounds, using $CO_2$ as a source of carbon, and obtaining their energy by oxidation of ammonia, nitrite, hydrogen, sulfur, or thiosulfate. Some species can also utilize organic compounds. Nonparasitic, usually soil or water forms.

Tribe I. *Nitrobactereae.* Organisms deriving energy from the oxidation of ammonia to nitrite or from nitrite to nitrate and depend on this oxidation for growth. Do not grow on media containing organic matter in the absence of the specific inorganic materials used as sources of energy. Many organic compounds commonly used in standard culture media are toxic to this group.

Genus I. *Nitrosomonas.* Cells ellipsoidal, nonmotile or with a single polar flagellum, occurring singly, in pairs, short chains, or irregular masses, which are not enclosed in a common membrane. Oxidize ammonia to nitrite more rapidly than the other genera of this tribe.

Type species: *Nitrosomonas europaea.*

Genus II. *Nitrosococcus.* Cells large spheres, nonmotile, do not produce zoogloea. Oxidize ammonia to nitrite.

Type species: *Nitrosococcus nitrosus.*

Genus III. *Nitrosospira.* Cells spiral-shaped. Oxidize ammonia to nitrite very slowly.

Type species: *Nitrosospira briensis.*

Genus IV. *Nitrosocystis.* Cells ellipsoidal or elongated, uniting in compact, rounded aggregates surrounded by a common membrane to form cysts. The cysts disintegrate to free the cells, particularly when transferred to fresh media. Within the cyst, the cells are embedded in slime. Oxidize ammonia to nitrite at a rate intermediate between *Nitrosomonas* and *Nitrosospira.*

Type species: *Nitrosocystis javanensis.*

Genus V. *Nitrosogloea.* Cells ellipsoidal or rod-shaped. Embedded in slime to form zoogloea. No common membrane surrounds the cell aggregates. Oxidize ammonia to nitrite.

Type species: *Nitrosogloea merismoides.*

Genus VI. *Nitrobacter.* Cells rod-shaped. Oxidize nitrite to nitrate.

Type species: *Nitrobacter winogradskyi.*

Genus VII. *Nitrocystis.* Cells ellipsoidal or rod-shaped. Embedded in slime and united into compact zoogloeal aggregates. Oxidize nitrite to nitrate.

Type species: *Nitrocystis sarcinoides.*

Tribe II. *Hydrogenobactereae.* Short rods, nonmotile, or with a single polar flagellum. Organisms capable of deriving energy from oxidation of hydrogen. They probably grow well on organic media without hydrogen, although this has not been shown to be true for all species.

Genus I. *Hydrogenomonas.* Description same as for the tribe.

Type species: *Hydrogenomonas pantotropha.*

Tribe III. *Thiobacilleae.* Organisms capable of deriving their energy from oxidation of sulfur or sulfur compounds. Most species do not grow on organic media.

Genus I. *Thiobacillus.* Small, Gram-negative, rod-shaped cells. Nonmotile or motile by means of a single polar flagellum. Derive their energy from the oxidation of incompletely oxidized sulfur compounds, principally from elemental sulfur and thiosulfate but in some cases also from sulfide, sulfite, and polythionates. The principal product of oxidation is sulfate, but sulfur is sometimes formed. They grow under acid or alkaline conditions and derive their carbon from $CO_2$ or from bicarbonates in solution. Some are obligate and some facultative autotrophic. One species is facultative anaerobic.

Type species: *Thiobacillus thioparus.*

Family II. *Pseudomonadaceae.* Cells without endospores, elongate rods, straight or more or less spirally curved. The genus *Mycoplana* has branched cells. Usually motile by polar flagella which are either single or in small or large tufts. A few species are nonmotile. Gram-negative (a few doubtful Gram-positive tests are recorded in *Pseudomonas*). Grow well on the surface of ordinary culture media excepting *Methanomonas* and some vibrios which attack cellulose. They are preferably aerobic, only certain vibrios including *Desulfovibrio* being anaerobic. Either water or soil forms, or plant or animal pathogens.

Tribe I. *Pseudomonadeae.* This tribe includes all the straight and branching rods of the family.

Genus I. *Pseudomonas.* Cells monotrichous, lophotrichous, or nonmotile. If pigments produced they are of greenish hue, fluorescent, and water-soluble. Gram-negative, with few exceptions. Frequently ferment glucose. Nitrates frequently reduced either to nitrites or ammonia, or to free nitrogen. Some species hydrolyze fat and attack hydrocarbons. Soil, water, and plant pathogens; very few animal pathogens.

Type species: *Pseudomonas aeruginosa.*

Genus II. *Xanthomonas.* Cells usually monotrichous, with yellow, water-insoluble pigment. Proteins usually readily digested. Milk usually becomes alkaline. Hydrogen sulfide is produced. Asparagin is not sufficient as an only source of carbon and nitrogen. Acid produced from mono- and disaccharides. Mostly plant pathogens causing necrosis.

Type species: *Xanthomonas hyacinthi.*

Genus III. *Methanomonas.* Cells monotrichous, capable of obtaining energy from oxidation of methane to carbon dioxide and water.
Type species: *Methanomonas methanica.*

Genus IV. *Acetobacter.* Cells ellipsoidal to long and rod-shaped, occurring singly, in pairs, or in short or long chains. Motile with polar flagella, or nonmotile. Involution forms may be spherical, elongated, filamentous, club-shaped, swollen, curved or even branched. Young cells Gram-negative; old cells often Gram-variable. Obligate aerobes. As a rule, strongly catalase positive. Oxidize various organic compounds to organic acids and other oxidation products that may undergo further oxidation. Common oxidation products include acetic acid from ethyl alcohol, gluconic and sometimes ketogluconic acid from glucose, dihydroxyacetone from glycerol, sorbose from sorbitol, etc. Nutritional requirements vary from simple to complex. Development generally best in yeast infusion or yeast autolysate media with added ethyl alcohol or other oxidizable substrate. Optimum temperature variable with the species. Widely distributed in nature where they are particularly abundant in plant materials undergoing alcoholic fermentation. Of importance for the production of vinegar.
Type species: *Acetobacter aceti.*

Genus V. *Protaminobacter.* Cells motile or nonmotile. Capable of dissimilating alkylamines. Frequently produce pigment. Soil or water forms.
Type species: *Protaminobacter alboflavum.*

Genus VI. *Mycoplana.* Cells branching, especially in young cultures. Frequently banded when stained. Capable of oxidizing phenol and similar aromatic compounds as a sole source of energy. Grow well on standard culture media.
Type species: *Mycoplana dimorpha.*

Tribe II. *Spirilleae.* More or less spirally curved cells.

Genus I. *Vibrio.* Cells short, curved, single or united into spirals. Motile by means of a single polar flagellum which is usually relatively short; rarely 2 or 3 flagella in one tuft. Grow well and rapidly on the surface of standard culture media. Aerobic to anaerobic species. Mostly water forms, a few parasites.
Type species: *Vibrio comma.*

Genus II. *Desulfovibrio.* Slightly curved rods of variable length, usually occurring singly, sometimes in short chains which have the appearance of spirilla. Swollen pleomorphic forms are common. Actively motile by means of a single polar flagellum. Strict anaerobes which reduce sulfates to hydrogen sulfide. Found in sea water, marine mud, fresh water, and soil.
Type species: *Desulfovibrio desulfuricans.*

Genus III. *Cellvibrio.* Long slender rods, slightly curved, with rounded ends, show deeply staining granules which appear to be concerned in reproduction. Monotrichous. Most species produce a yellow or brown pigment with cellulose. Oxidize cellulose, forming oxycellulose. Growth on ordinary culture media is feeble. Found in soil.
Type species: *Cellvibrio ochraceus.*

Genus IV. *Cellfalcicula.* Short rods or spindles, not exceeding 2$\mu$ in length, with pointed ends, containing metachromatic granules. Old cultures show coccoid forms. Monotrichous. Oxidize cellulose to oxycellulose. Growth on ordinary culture media is feeble. Soil bacteria.
Type species: *Cellfalcicula viridis.*

Genus V. *Thiospira.* Colorless, motile, slightly bent, somewhat pointed at the ends with granules of sulfur within the cells and a small number of flagella at the ends.

Type species: *Thiospira winogradsky.*

Genus VI. *Spirillum.* Cells form either long screws or portions of a turn. Volutin granules usually present. Usually motile by means of a tuft of polar flagella which may occur at one or both ends of the cells. Aerobic, growing well on ordinary culture media, except for one saprophyte and the pathogenic species. These have not yet been cultivated. Usually found in fresh and salt water containing organic matter.

Type species: *Spirillum undula.*

Family III. *Azotobacteriaceae.* Cells without endospores. Relatively large rods or even cocci, sometimes almost yeast-like in appearance. Motile by means of peritrichous flagella. Gram-negative. Obligate aerobes, usually growing in a film on the surface of the culture medium. Capable of fixing atmospheric nitrogen when provided with carbohydrate or other source of energy. Grow best on media deficient in nitrogen. Soil and water bacteria.

Genus I. *Azotobacter.* Characterization of genus identical with that of the family.

Type species: *Azotobacter chroococcum.*

Family IV. *Rhizobiaceae.* Cells without endospores, rod-shaped, one polar or lateral flagellum, or 2 to 4 peritrichous flagella. Some species nonmotile. Usually Gram-negative. The genus *Chromobacterium* produces a violet pigment. Grow aerobically on ordinary culture media containing glucose. Carbohydrates are utilized without appreciable acid formation. Saprophytes, symbionts, and pathogens. The latter are usually plant pathogens forming abnormal growths on roots and stems.

Genus I. *Rhizobium.* Cells rod-shaped. Motile when young, commonly changing to bacteroidal forms (*a*) upon artificial culture media containing alkaloids or glucosides, or in which acidity is increased; or (*b*) during symbiosis within the nodule. Gram-negative. Aerobic, heterotrophic, growing best with extracts of yeast, malt, or other plant materials. Nitrates may be reduced to nitrites. Nitrites are not utilized. Gelatin is not liquefied, or is very slightly liquefied, after long incubation. Optimum temperature 25°C. Group capable of producing nodules on the roots of Leguminosae, and of fixing free nitrogen during this symbiosis.

Type species: *Rhizobium leguminosarum.*

Genus II. *Agrobacterium.* Small, short rods, typically motile with 1 to 4 peritrichous flagella. If only 1 flagellum, lateral attachment is as common as polar. Ordinarily Gram-negative. On ordinary media, they do not produce visible gas nor sufficient acid to be detectable by litmus. In synthetic media, enough $CO_2$ may be produced to show acid with bromothymol blue, or sometimes with bromocresol purple. Gelatin is either very slowly liquefied or not at all. Free nitrogen cannot be fixed. Optimum temperature 25 to 30°C. Found in the soil, on plant roots in the soil, or on the stems of plants where they produce hypertrophies.

Type species: *Agrobacterium tumefaciens.*

Genus III. *Chromobacterium.* Cells rod-shaped. Motile with 1 to 4 or more flagella. Gram-negative. A violet pigment is formed which is soluble in alcohol, but not in water or chloroform. Grow on ordinary culture media, usually forming acid from glucose, sometimes from maltose, but not from lactose. Gelatin is liquefied. Indole is not produced. Nitrate

usually reduced to nitrite. Optimum temperature 20 to 25°C. but some grow well at 37°C. Usually saprophytic soil and water bacteria.
Type species: *Chromobacterium violaceum.*

Family V. *Micrococcaceae.* Cells without endospores except in *Sporosarcina.* Cells in their free state spherical; during division somewhat elliptical. Division in 2 or 3 planes. If the cells remain in contact after division, they are frequently flattened in the plane of last division. They occur singly, in pairs, tetrads, packets, or irregular masses. Motility rare. Generally Gram-positive. Many species form a yellow, orange, pink, or red pigment. Most species are preferably aerobic producing abundant growth on ordinary culture media, but capable of slight anaerobic growth. A few species are strictly anaerobic. Metabolism heterotrophic. Carbohydrates are frequently fermented to acid. Gelatin is often liquefied. Facultative parasites and saprophytes. Frequently live on the skin.

Genus I. *Micrococcus.* Cells in plates or irregular masses. Never in long chains or packets. Gram-positive to Gram-negative. Growth on agar usually abundant, some species nonpigmented, others form yellow or less commonly orange, or red pigment. Glucose broth slightly acid, lactose broth generally neutral. Gelatin frequently liquefied, but not rapidly. Facultative parasites and saprophytes.
Type species: *Micrococcus luteus.*

Genus II. *Gaffkya.* Occur in animal body and on special media as tetrads. On ordinary culture media, they occur in pairs and irregular masses. Aerobic to anaerobic. Gram-positive. Parasites.
Type species: *Gaffkya tetragena.*

Genus III. *Sarcina.* Division occurs, under favorable conditions, in three planes, producing regular packets. Usually Gram-positive. Growth on agar abundant, usually with formation of yellow or orange pigment. Glucose broth slightly acid, lactose broth generally neutral. Gelatin frequently liquefied. Nitrate may or may not be reduced to nitrite. Saprophytes and facultative parasites.
Type species: *Sarcina ventriculi.*

Family VI. *Neisseriaceae.* Cells spherical, in pairs or in masses. Nonmotile. Gram-negative. Pigment formation rare. The family contains aerobic and anaerobic species. Some grow poorly or not at all in the absence of mammalian body fluids. Optimum temperature 37°C. All known species are parasitic.

Genus I. *Neisseria.* Paired, Gram-negative cocci, with adjacent sides flattened. Four of the eleven species produce yellow pigment. Genus contains aerobic and anaerobic species. Growth on ordinary media may be poor. Biochemical activities are limited. Few carbohydrates are utilized. Indole is not produced. Nitrate is not reduced. Catalase is produced in abundance. Parasites of mammals so far as known.
Type species: *Neisseria gonorrhoeae.*

Genus II. *Veillonella.* Small, Gram-negative cocci, occurring in masses, rarely in pairs or short chains. Cells undifferentiated. United by an interstitial substance of ectoplasmic nature. The known species are anaerobic. Good growth on ordinary culture media. Biochemical activity pronounced. Harmless parasites in mouth and intestine of man and animals.
Type species: *Veillonella parvula.*

Family VII. *Lactobacteriaceae.* Long or short rods, or cocci that divide like rods in one plane only, producing chains, but never tetrads or packets. Nonmotile, except for certain cultures of streptococci. Gram-positive. Pigment production is rare; a few species form yellow, orange, red, or rusty brown pigment. Surface growth on all media is poor or absent. Some species are strictly anaerobic. Carbohydrates are essential for good development; being fermented to lactic acid, sometimes with volatile acids, alcohol, and $CO_2$ as by-products, except for nonfermenting *Diplococcus magnus.* Gelatin is very rarely liquefied. Nitrate not reduced to nitrite. Found regularly in the mouth and intestinal tract of man and other animals, dairy products fermenting vegetable juices. A few species are highly pathogenic.

Tribe I. *Streptococceae.* Cells spherical or elongated, dividing in one plane only, usually occurring in pairs or chains. A few species are strict anaerobes; none grow abundantly on solid media. Carbohydrates and polyalcohols are changed either by homofermentation to lactic acid or by heterofermentation to lactic and acetic acids, alcohol, and carbon dioxide. Some pathogenic species grow poorly without blood serum or other enrichment fluids. Catalase negative.

Genus I. *Diplococcus.* Cells usually in pairs, sometimes in chains or more rarely in tetrads or small clumps. Young cells Gram-positive. Parasites sometimes growing poorly or not at all on artificial media. Fermentative powers usually high, most strains forming acid from glucose, lactose, sucrose, and inulin. The aerobic species are bile-soluble; the anaerobic species are bile-insoluble.

Type species: *Diplococcus pneumoniae.*

Genus II. *Streptococcus.* Cells spherical or ovoid, rarely elongated into rods, occurring in pairs, or short or long chains, never in packets or zoogloeal masses. Capsules are not regularly formed but become conspicuous with some species under certain conditions. Gram-positive, some species decolorizing readily. A few cultures produce a rusty red growth in deep agar stab, or a yellow or orange pigment in starch broth. Growth on artificial media is slight. Agar colonies are small. Surface colonies are small. Surface colonies are translucent. Colonies may be effuse, convex, or mucoid. Mostly facultative anaerobes, with little surface growth in stab cultures. A few are strict anaerobes. Some of the latter attack proteins with production of gas and foul odors. Carbohydrate fermentation by all others is homofermentative, with dextrolactic acid as the dominant product, while volatile acids, other volatile products and $CO_2$ are either absent or produced in very small amounts. Inulin is rarely attacked. Nitrate is not reduced to nitrite. Not soluble in bile. Found regularly in the mouth and intestine of man and animals, dairy products, fermenting plant juices. Some species are highly pathogenic.

Type species: *Streptococcus pyogenes.*

Genus III. *Leuconostoc.* Cells normally spherical. Under certain conditions, as in acid fruits and vegetables, the cells may lengthen and become pointed or even elongated into a rod. Certain types grow with a characteristic slime formation in sucrose media. Growth on ordinary culture media is enhanced by the addition of yeast, tomato, or other vegetable extracts. Generally a limited amount of acid is produced, consisting of lactic and acetic acids. Alcohol is also formed and about one-fourth of the fermented glucose is changed to $CO_2$. Levolactic acid is always produced, and sometimes dextrolactic acid also. Milk is rarely curdled.

Fructose is reduced to mannitol. Found in milk and plant juices.
Type species: *Leuconostoc mesenteroides.*

Tribe II. *Lactobacilleae.* Rods, often long and slender. Nonmotile. Gram-positive. Pigment formation rare; when present, yellow or orange, to rust or brick-red in color. Surface growth poor, except in genus *Microbacterium*, because these organisms are generally microaerophilic or anaerobic. Carbohydrates and polyalcohols are changed either by homofermentation to lactic acid or by heterofermentation to lactic, acetic, propionic, or butyric acids, alcohol, and carbon dioxide. Growth on potato is poor or absent. Gelatin is not liquefied. Nitrate is not reduced to nitrite, except in genus *Microbacterium.* Several species grow at relatively high temperatures. May or may not produce catalase.

Genus I. *Lactobacillus.* Rods, usually long and slender. Microaerophilic. Carbohydrates and polyalcohols are changed by homofermentation to lactic acid, or by heterofermentation to lactic and acetic acids, alcohols, and $CO_2$. Catalase negative. Found in fermenting dairy and plant products.
Type species: *Lactobacillus caucasicus.*

Genus II. *Microbacterium.* Small rods, nonmotile, Gram-positive. Produce lactic acid but no gas from carbohydrates. Produce catalase. Usually heat-resistant. Found in dairy products and utensils, fecal matter, and soil.
Type species: *Microbacterium lacticum.*

Genus III. *Propionibacterium.* Nonmotile, nonspore-forming, Gram-positive bacteria growing under anaerobic conditions in neutral media as short diphtheroid rods, sometimes resembling streptococci; under aerobic conditions with heavy inoculum growing as long, irregular, club-shaped and branched cells. Metachromatic granules demonstrable with Albert's stain. Ferment lactic acid, carbohydrates, and polyalcohols with the formation of propionic and acetic acids, and $CO_2$. As a rule, strongly catalase positive. Strong tendency toward anaerobiosis. Development very slow, macroscopically visible colonies generally not discernible in less than 5 to 7 days. Nutritional requirements complex. Development best in yeast extract media with addition of lactates or simple carbohydrates. Optimum temperature 30°C. Found in dairy products, especially hard cheeses.
Type species: *Propionibacterium freudenreichii.*

Genus IV. *Butyribacterium.* Nonmotile, anaerobic to microaerophilic, straight or slightly bent rods. Gram-positive. Ferment carbohydrates and lactic acid forming acetic and butyric acids, and $CO_2$. Generally catalase negative but sometimes weakly positive. Intestinal parasites.
Type species: *Butyribacterium rettgeri.*

Family VIII. *Corynebacteriaceae.* Nonmotile rods, except in *Listeria*, frequently banded or beaded with metachromatic granules. May show marked diversity of form. Branching cells have been observed in a few species but these are uncommon. Generally Gram-positive but this reaction may vary depending on the nature of the cells. Where pigment is formed, it is grayish yellow to orange or pink in color. Aerobic to microaerophilic. Anaerobic species have been reported. Gelatin may be liquefied and nitrites may be produced from nitrates. Animal and plant parasites and pathogens. Also found in dairy products, soil, and water.

Genus I. *Corynebacterium.* Slender, straight to slightly curved rods, with

irregularly stained segments or granules. Frequently show pointed or club-shaped swellings at the ends. Snapping division produces angular and palisade arrangements of cells. Nonmotile with few exceptions. Gram-positive to variable, sometimes young cells and sometimes old cells being Gram-negative. Granules invariably Gram-positive. Generally quite aerobic, but microaerophilic or even anaerobic species occur. Catalase positive. They may or may not ferment sugars, but they seldom produce a high acidity. Many species oxidize glucose completely to $CO_2$ and $H_2O$ without producing visible gas. Some pathogenic species produce a powerful exotoxin. This group is widely distributed in nature. The best known species are parasites and pathogens on man and domestic animals. Other species have been found in birds and insects and the group is probably more widely distributed in the animal kingdom than this. Several species are well-known plant pathogens, others are found in dairy products, water, and soil.

Type species: *Corynebacterium diphtheriae.*

Genus II. *Listeria.* Small rods, Gram-positive. Flagellation peritrichous. Aerobic. Catalase positive. Grow freely on ordinary media. Acid but no gas from glucose and a few additional carbohydrates. Pathogenic parasites. Infection characterized by a monocytosis. Parasitic on warm-blooded animals.

Type species: *Listeria monocytogenes.*

Genus III. *Erysipelothrix.* Rod-shaped organisms with a tendency to the formation of long filaments. The filaments may also thicken and show characteristic granules. Nonmotile. Gram-positive. Microaerophilic. Catalase negative. Grow freely on ordinary media. Acid but no gas from glucose and a few additional carbohydrates. Parasitic on mammals.

Type species: *Erysipelothrix rhusiopathiae.*

Family IX. *Achromobacteriaceae.* Rods, small to medium in size, usually uniform in shape. No branching on ordinary media, if at all. Gram-negative, rarely Gram-variable. Peritrichous or nonmotile. Growth on agar slants non-chromogenic to grayish yellow, brownish yellow, or yellow to orange. The pigment does not diffuse through the agar. Attack carbohydrates feebly or not at all. May form acid but no gas. May or may not reduce nitrate. May or may not liquefy gelatin. Do not liquefy agar or attack cellulose, and are not phosphorescent. Litmus milk may become faintly acid but not sufficiently acid to curdle. Usually the reaction remains unchanged or becomes alkaline. Generally salt water, fresh water, and soil forms, less commonly parasites. Some plant pathogens may belong here.

Genus I. *Alcaligenes.* Peritrichous to monotrichous, or nonmotile rods. Gram-negative to Gram-variable. Do not produce acid or gas from carbohydrates. May or may not liquefy gelatin and solidified blood serum. Milk is alkaline in reaction, with or without peptonization. Do not produce acetylmethylcarbinol. May produce grayish-yellow, brownish-yellow, or yellow pigment. Generally occur in the intestinal tract of vertebrates or in dairy products.

Type species: *Alcaligenes faecalis.*

Genus II. *Achromobacter.* Nonpigment-forming rods. Motile with peritrichous flagella or nonmotile. Gram-negative to Gram-variable. Litmus milk faintly acid to unchanged or alkaline. Occur in salt to fresh water and in soil.

Type species: *Achromobacter liquefaciens.*

Genus III. *Flavobacterium.* Rods of medium size forming a yellow to orange pigment on culture media. Motile with peritrichous flagella or nonmotile. Generally Gram-negative. Feeble action on carbohydrates occasionally forming acid from hexoses but no gas. Occur in water and soil.
Type species: *Flavobacterium aquatile.*

Family X. *Enterobacteriaceae.* Straight rods. Gram-negative. Motile with peritrichous flagella, or nonmotile. Grow well on artificial media. Glucose attacked with formation of acid, or acid and visible gas. With the exception of *Erwinia* nitrate reduced to nitrite. Antigenic composition best described as mosaic which results in serological interrelationships among the several genera, even extending to other families. Many animal parasites, and some plant parasites causing blights and soft rots. Frequently occur as saprophytes causing decomposition of plant materials containing carbohydrates.

Tribe I. *Escherichieae.* Ferment glucose and lactose with the formation of acid and visible gas within 24 hr. at 37°C., or within 48 hr. at 25 to 30°C. Some forms produce acid and gas from lactose slowly. They do not liquefy gelatin, except slowly in *Aerobacter cloacae.*

Genus I. *Escherichia.* Short rods fermenting glucose and lactose with acid and gas production. Acetylmethylcarbinol is not produced. Methyl red test is positive. Carbon dioxide and hydrogen produced in approximately equal volumes from glucose. Generally not able to utilize uric acid as the only source of nitrogen. Found in feces, and is occasionally pathogenic in man (colitis, cystitis). Widely distributed in nature.
Type species: *Escherichia coli.*

Genus II. *Aerobacter.* Short rods, fermenting glucose and lactose with acid and gas production. Methyl red test is negative. Voges-Proskauer test is positive. Form two or more times as much carbon dioxide as hydrogen from glucose. Trimethylene glycol is not produced from glycerol by anaerobic fermentation. Citrates utilized as sole source of carbon. Grow readily on ordinary media. Facultative anaerobes. Widely distributed in nature.
Type species: *Aerobacter aerogenes.*

Genus III. *Klebsiella.* Short rods, somewhat plump with rounded ends, mostly occurring singly. Encapsulated in the mucoid phase. Nonmotile. Gram-negative. Aerobic, growing well on ordinary culture media. Encountered frequently in the respiratory, intestinal, and genitourinary tracts of man, but may be isolated from a variety of animals and materials.
Type species: *Klebsiella pneumoniae.*

Tribe II. *Erwineae.* Motile rods which normally require organic nitrogen compounds for growth. Produce acid with or without visible gas from a variety of sugars. In some species, the number of carbon compounds attacked is limited and lactose may not be fermented. May or may not liquefy gelatin. May or may not reduce nitrate. Invade the tissues of living plants and produce dry necrosis, galls, wilts, and soft rots. In the latter case, a protopectinase destroys the middle lamellar substance.

Genus I. *Erwinia.* Description same as for the tribe.
Type species: *Erwinia amylovora.*

Tribe III. *Serrateae.* Small, aerobic rods, usually producing a bright red or pink pigment on agar and gelatin.

Genus I. *Serratia.* Small, aerobic, rapidly liquefying, nitrate-reducing, Gram-negative, peritrichous rods which produce characteristic red pigments. White to rose-red strains that lack brilliant colors are common.

Coagulate and peptonize milk. Liquefy blood serum. Typical species produce $CO_2$ and frequently $H_2$ from glucose and other sugars, also acetic, formic, succinic, and lactic acids, acetylmethylcarbinol, and 2:3-butylene glycol. Saprophytic on decaying plant or even animal materials.

Type species: *Serratia marcescens.*

Tribe IV. *Proteae.* Ferment glucose but not lactose with the formation of acid and usually visible gas.

Genus I. *Proteus.* Straight rods, Gram-negative. Generally actively motile at 25°C. Motility may be weak or absent at 37°C., peritrichous, occasionally very numerous flagella. Generally produce amoeboid colonies, swarming phenomenon on moist medium. Marked pleomorphism characteristic only of very young, actively swarming culture. Ferment glucose and usually sucrose but not lactose. Urea decomposed and trimethylamine oxide reduced by all species.

Type species: *Proteus vulgaris.*

Tribe V. *Salmonelleae.* Rods either motile with peritrichous flagella or nonmotile. Attack numerous carbohydrates with the formation of acid, or acid and gas. Lactose, sucrose, and salicin are not ordinarily attacked. Acetylmethylcarbinol not produced. Gelatin not liquefied. Urea not hydrolyzed. Milk not peptonized. Spreading growth absent on ordinary 2 to 3 per cent agar. Live in the bodies of warm-blooded animals, occasionally in reptiles, and frequently in foods eaten by these animals.

Genus I. *Salmonella.* Usually motile, but nonmotile forms occur. Produce acid and gas from glucose, maltose, mannitol, and sorbitol, except that in *S. typhosa* and *S. gallinarum* no gas is produced. Lactose, sucrose, and salicin not attacked. Do not coagulate milk, form indole, or liquefy gelatin. Reduce trimethylamine oxide to trimethylamine. All species are pathogenic for warm-blooded animals, causing infections and enteric fevers. A few are found in reptiles. Some or all may also live in decomposing foods.

Genus II. *Shigella.* Nonmotile rods, although cultures of some of the less well-known species have been reported as motile. Produce acid but no gas from carbohydrates except with some type of *S. paradysenteriae.* Do not liquefy gelatin. Some species produce acid from lactose and indole. Some species reduce trimethylamine, others do not. Some species grow at 45.5°C. (Eijkman test.)

Type species: *Shigella dysenteriae.*

Family XI. *Parvobacteriaceae.* Small, motile or nonmotile rods. Some grow on ordinary media, but the majority either require or grow better on media containing body fluids or growth-promoting substances. Some invade living tissues. Gram-negative. Usually do not liquefy gelatin. No gas formed in fermenting carbohydrates. Infection in some cases may take place by penetration of organisms through mucous membranes or skin. Parasitic to pathogenic on warm-blooded animals, including man.

Tribe I. *Pasteurelleae.* Small, motile or nonmotile, ellipsoidal to elongated rods showing bipolar staining.

Genus I. *Pasteurella.* Small, Gram-negative, ellipsoidal to elongated rods showing bipolar staining by special methods. Facultative aerobic, requiring low oxidation-reduction potential on primary isolation. Majority ferment carbohydrates. Lactose not fermented. Gas not produced.

Gelatin not liquefied. Milk not coagulated. Parasitic on man, other mammals and birds.

Type species: *Pasteurella multocida.*

Genus II. *Malleomyces.* Short rods with rounded ends, sometimes forming threads and showing a tendency toward branching. Motile or nonmotile. Gram-negative. Tendency to bipolar staining. Milk slowly coagulated. Gelatin may be liquefied. Specialized for parasitic life. Grow well on blood serum and other body-fluid media.

Type species: *Malleomyces mallei.*

Genus III. *Actinobacillus.* Medium-sized, aerobic, Gram-negative rods which frequently show much pleomorphism. Coccus-like forms frequent. Tendency to bipolar staining. Acid but no gas produced from carbohydrates. Grow best, especially when freshly isolated, under increased $CO_2$ tension. Pathogenic for animals; some species attack man. The outstanding characteristic of the group is the tendency to form aggregates in tissues or culture which resemble the so-called sulfur granules of actinomycosis.

Type species: *Actinobacillus lignieresi.*

Tribe II. *Brucelleae.* Small, nonmotile rods or coccoids which grow on special media.

Genus I. *Brucella.* Short rods with many coccoid cells. Nonmotile, capsulated, Gram-negative. Gelatin not liquefied. No acid or gas from carbohydrates. Urea utilized. Parasitic, invading all animal tissues producing infection of the genital organs, the mammary gland, the respiratory and intestinal tracts. Pathogenic for various species of domestic animals and man.

Type species: *Brucella melitensis.*

Tribe III. *Bacteroideae.* Motile or nonmotile rods without endospores. May or may not require enriched culture media. Obligate anaerobes. Gram-negative.

Genus I. *Bacteroides.* Description same as for the tribe.

Type species: *Bacteroides fragilis.*

Genus II. *Fusobacterium.* Gram-negative, anaerobic rods, usually with tapering ends. Usually nonmotile. Stain with more or less distinct granules.

Type species: *Fusobacterium plauti-vincenti.*

Tribe IV. *Hemophileae.* Minute parasitic forms growing on first isolation only in the presence of hemoglobin, ascitic fluid, or other body fluids, or in the presence of certain growth accessory substances found in sterile, unheated plant tissue (potato). Motile or nonmotile. Commonly found in the mucosa of respiratory tract or conjunctiva.

Genus I. *Hemophilus.* Minute rod-shaped cells, sometimes thread-forming and pleomorphic. Nonmotile. Gram-negative. Strict parasites growing best, or only, in the presence of hemoglobin and in general requiring blood serum, ascitic fluid, or certain growth accessory substances.

Type species: *Hemophilus influenzae.*

Genus II. *Moraxella.* Small, short, rod-shaped cells, usually occurring singly or in pairs. Nonmotile. Parasitic. Aerobic. Gram-negative.

Type species: *Moraxella lacunata.*

Genus III. *Noguchia.* Small, slender, Gram-negative rods present in the conjunctiva of man and animals affected by a follicular type of disease. Growth of mucoid type which on first isolation takes place with some

difficulty in ordinary media. Motile, encapsulated, aerobic, and facultative anaerobic. Optimum temperature 28 to 30°C.

Type species: *Noguchia granulosis.*

Genus IV. *Dialister.* Minute rod-shaped cells, occurring singly, in pairs, and short chains. Nonmotile. Strict parasites. Growth occurs only under anaerobic conditions in media containing fresh, sterile tissue or ascitic fluid.

Type species: *Dialister pneumosintes.*

Family XII. *Bacteriaceae.* Cells rod-shaped and do not produce endospores. Motile or nonmotile. Their metabolism is complex, amino acids are utilized, and generally carbohydrates.

Genus I. *Bacterium.* Cells rod-shaped and nonspore-forming. Gram-negative or Gram-positive.

Family XIII. *Bacillaceae.* Rod-shaped cells, capable of producing spores, either with peritrichous flagella or nonmotile. Endospores are cylindrical, ellipsoidal, or spherical, and are located centrally, subterminally, or terminally. Sporangia do not differ from the vegetative cells except when bulged by spores larger than the cell diameter. Such sporangia are spindle-shaped when spores are central, or wedge-, or drumstick-shaped when spores are terminal. Usually Gram-positive. Pigment formation is rare. Aerobic, microaerophilic, or anaerobic. Gelatin is frequently liquefied. Sugars are generally fermented, sometimes with the formation of visible gas. Some species are thermophilic, *i.e.*, will grow readily at 55°C. Mostly saprophytes, commonly found in soil. A few are animal, especially insect, parasites, or pathogens

Genus I. *Bacillus.* Rod-shaped bacteria, sometimes in chains. Sporangia usually not different from the vegetative cells. Catalase present. Aerobic, sometimes showing rough colonies and forming a pellicle on broth. Usually oxidize carbohydrates or proteins more or less completely, often producing slight acidity, without pronounced accumulation of characteristic products. Soil is the most common habitat.

Type species: *Bacillus subtilis.*

Genus II. *Clostridium.* Rods, frequently enlarged at sporulation, producing clostridial or plectridial forms. Catalase not present. Anaerobic or microaerophilic. Many species ferment carbohydrates producing various acids (frequently including butyric) and gas ($CO_2$, $H_2$, and sometimes $CH_4$). Others cause rapid putrefaction of proteins producing offensive odors. Commonly found in soil and in human or animal feces. Some species, when growing saprophytically on decomposing vegetable matter or on dead tissue within an animal host, form various toxic and lytic substances and are thereby pathogenic.

Type species: *Clostridium butyricum.*

Suborder II. *Caulobacteriineae.* The bacteria are nonfilamentous growing characteristically upon stalks.

Family I. *Gallionellaceae.* The bacteria are stalked, the long axis of the rod-shaped cells being set at right angles to the axis of the stalks. Stalks are slender, twisted bands, dichotomously branched, composed of ferric hydroxide, which completely dissolves in dilute hydrochloric acid. Multiplication takes place by transverse binary fission. They grow in iron-bearing waters.

Genus I. *Gallionella.* Description same as for the family.

Family II. *Nevskiaceae.* The bacteria are stalked, the long axis of the rod-shaped

cells being set at right angles to the axis of the stalk. Stalks are lobose, dichotomously branched, and composed of gum. Multiplication of cells takes place by transverse binary fission. They grow in zooglea-like masses in water or in sugar vats.

Genus I. *Nevskia.* Description same as for the family.

Family III. *Siderocapsaceae.*

Genus I. *Siderocapsa.*

Genus II. *Sideromonas.*

Family IV. *Caulobacteriaceae.* The bacteria are stalked, the long axis of the elongated cells coinciding with the long axis of the stalks. Stalks are slender, flagellum-like, often attached to the substrate by a button-like holdfast, and unbranched. Multiplication is by transverse fission. The outermost cell of a pair may form a stalk before cell division is complete. They are periphytic, growing upon submerged surfaces.

Genus I. *Caulobacter.* Description same as for the family.

Family V. *Pasteuriaceae.* The bacteria are stalked with spherical or pear-shaped cells. If the cells are elongated, the long axis of the cell coincides with the axis of the stalk. Stalks may be very short or absent but, when present, are usually very fine and at times arranged in whorls attached to a common holdfast. Cells multiply by longitudinal fission or by budding or by both. They are mostly periphytic, one species is parasitic.

Genus I. *Pasteuria.* The cells are pear-shaped and attached to each other or to a firm substrate by holdfasts secreted at the narrow end. They multiply by longitudinal fission and by budding of spherical or ovoid cells at the free end.

Genus II. *Blastocaulis.* Cells pear-shaped or globular, attached to a firm substrate by long slender stalks with a holdfast at the base. Stalks may occur singly or may arise in clusters from a common holdfast. They have not been cultivated in artificial media.

Suborder III. *Rhodobacteriineae.* The cells are of various types, not filamentous, and contain bacteriopurpurin, with or without sulfur granules.

Family I. *Chromaticidaceae.* Cells not filamentous and contain both sulfur granules and bacteriopurpurin. Cells divide in three directions of space. They are sometimes called the *Thiorhodaceae.*

Genus I. *Thiosarcina.* Cells are nonswarming and arranged in packet-shaped families corresponding to the genus *Sarcina.* Cells are red with sulfu granules.

Type species: *Thiosarcina rosea.*

Genus II. *Thiopedia.* Families are arranged in the form of plates. They are capable of swarming. Cells contain bacteriopurpurin and bacteriochlorin.

Type species: *Thiopedia rosea.*

Genus III. *Thiocapsa.* The cells are spherical, with thick confluent membranes which unite to form a structureless gelatinous layer. The cells are of a bright rose-red color. The cells do not swarm.

Type species: *Thiocapsa roseopersicina.*

Genus IV. *Thiodictyon.* Cells are rod- or spindle-shaped with sharply pointed ends, and united into a net. The compact mass of rods finally assumes an appearance like that of *Hydrodictyon.* The color is slightly violet.

Type species: *Thiodictyon elegans.*

Genus V. *Thiothece.* Cells are spherical, in families, and enclosed in a thick, gelatinous cyst. Cells are capable of swarming and are very loosely em-

bedded in a common gelatin. When the swarm stage supervenes, the cells lie more loosely, the gelatin is swollen, and the cells swarm out singly and rather irregularly.

Type species: *Thiothece gelatinosa.*

Genus VI. *Thiocystis.* Usually 4 to 30 cells are massed into small, compact families, enveloped singly or several together in a gelatinous cyst, and capable of swarming. When the families have reached a definite size, they escape from the gelatinous cyst, the latter swelling and softening uniformly or at some particular spot. The escaped cells either pass into the swarm stage or unite into a large fused complex of families from which they separate later. Cells are light colored; single cells are almost colorless. In masses the cells show a beautiful violet or red color.

Type species: *Thiocystis violacea.*

Genus VII. *Lamprocystis.* Cells are ellipsoidal, dividing at first in three planes to form spherical cell masses, later in two planes to form hollow sacks in which the cells lie embedded in a layer in the walls. Finally, the membrane ruptures and the whole mass becomes net-like, much as in the algal genus *Clathrocystis.* They are usually colored intensely violet. The cells are capable of swarming.

Type species: *Lamprocystis roseopersicina.*

Genus VIII. *Amoebobacter.* The cells are connected by plasma threads. Families are amoeboid and motile. The cell families slowly change form, the cells drawing together into a heap or spreading out widely, thus bringing about a change in the shape of the whole family. In a resting condition, a common gelatin is extruded; the surface becomes a firm membrane.

Type species: *Amoebobacter roseum.*

Genus IX. *Thiopolycoccus.* Families are solid, nonmotile, and consist of small cells closely appressed. Multiplication occurs by breaking up of the surface into numerous, short threads and lobes, which continue to split up into smaller heaps.

Type species: *Thiopolycoccus ruber.*

Genus X. *Thiospirillum.* The bacteria are spiral and motile.

Type species: *Thiospirillum sanguineum.*

Genus XI. *Rhabdomonas.* The cells are differentiated from *Chromatium* by the elongated, rod-, or spindle-shaped cells. The cells are motile and red in color.

Type species: *Rhabdomonas rosea.*

Genus XII. *Rhodothece.* Cells are usually spherical and in pairs, and each is surrounded by a spherical or elliptical capsule. They are nonmotile. The cells are not united into families.

Type species: *Rhodothece pendens.*

Genus XIII. *Chromatium.* Cells are cylindrical-elliptical or relatively thick cylindrical. They are red in color and motile.

Type species: *Chromatium okenii.*

Family II. *Chlorobacteriaceae.*

Genus I. *Chlorobium.*

Genus II. *Pelodictyon.*

Genus III. *Clathrochloris.*

Genus IV. *Chlorobacterium.*

Genus V. *Chlorochromatium.*

Genus VI. *Cylindrogloea.*

Family III. *Athiorhodaceae.* Cells not filamentous, contain bacteriopurpurin but no sulfur granules.

Genus I. *Rhodopseudomonas.* Cells rod-shaped or spherical, not spiral-shaped, motile by means of polar flagella.

Type species: *Rhodopseudomonas palustris.*

Genus II. *Rhodospirillum.* Cells spiral-shaped and motile by means of polar flagella. Nonspore-forming and Gram-negative. Contain bacteriochlorophyll and are potentially photosynthetic in the presence of extraneous oxidizable substances.

Type species: *Rhodospirillum rubrum.*

Order II. *Actinomycetales.* Organisms forming elongated cells which show a definite tendency to branch. Hyphae from 1.0 to 1.5$\mu$ in diameter. They usually produce a characteristic branching mycelium. Multiplication by means of special spores, as well as by oidiospores and conidia. Special spores are formed by fragmentation of the plasma within the spore-bearing hyphae, the latter being straight or spiral-shaped; the oidiospores are formed by segmentation, or by simple division of hyphae by means of transverse walls, similar to the formation of oidia among the true fungi; the conidia are produced singly, at the end of special, simple, or branching conidiophores. They grow readily on artificial media and form well-developed colonies. The surface of the colony may become covered with an aerial mycelium. Some of the organisms are colorless or white; others form a variety of pigments. Most species are mesophilic, some are thermophilic. Certain forms are capable of growing at low oxygen tension. They are either saprophytes or parasites.

Family I. *Mycobacteriaceae.* Slender filaments, straight or slightly curved rods, frequently irregular in form, with only slight and occasional branching. Often stain unevenly, *i.e.*, show variations in staining reaction within the cell. Conidia not formed. Growth on media slow for most species. Nonmotile, aerobic, Gram-positive, and acid-fast. Human and animal pathogens, and soil forms.

Genus I. *Mycobacterium.* Description same as for the family.

Type species: *Mycobacterium tuberculosis.*

Family II. *Actinomycetaceae.* Mycelium is nonseptate during the early stages of growth but later may become septate and break up into short segments, rod-shaped or spherical in shape, or the mycelium may remain nonseptate and produce spores on aerial hyphae. The organisms in culture media are either colorless or produce various pigments. Some species are partly acid-fast. This family is distinguished from the preceding by the formation of a true mycelium.

Genus I. *Nocardia.* Slender filaments or rods, frequently swollen and occasionally branched, forming a mycelium which, after reaching a certain size, assumes the appearance of bacterium-like growths. Shorter rods and coccoid forms are found in older cultures. Conidia not formed. Stain readily, occasionally showing a slight degree of acid fastness. Aerobic, nonspore-forming, Gram-positive. The colonies are similar in gross appearance to those of the genus *Mycobacterium.* Paraffin, phenol and *m*-cresol are frequently utilized as sources of energy.

In their early stages of growth on culture media, the structure of *Nocardia* is similar to that of *Actinomyces* in that a typical mycelium is formed. Hyphae branch abundantly the branching being true. At first, the mycelium is nonseptate. Later, the filaments form transverse walls and the whole mycelium breaks up into regularly cylindrical short cells, then into coccoid cells. On fresh culture media, the coccoid cells germinate into mycelia. The whole cycle in the development of *Nocardia* continues for 2 to 7 days.

Numerous chlamydospores may be found in older cultures. They are formed

in the same way as the chlamydospores in true fungi. In older cultures many coccoid cells are changed into resistant cells. On fresh media, they germinate like spores.

Multiplication proceeds by fission, budding, and occasionally by the formation of special spores. Buds are formed on the lateral surface of the cells. When they have reached a certain size, they fall off and develop into rod-shaped cells or filaments. Spores are formed by the breaking up of the cell contents into separate portions usually forming 3 to 5 spores. The membrane of the mother cell dissolves and disappears. The spores germinate in the same way as those of *Actinomyces*. They form germ tubes which develop into a mycelium. Many species form pigments, their colonies being violet, blue, red, yellow, and green in color.

Type species: *Nocardia farcinica.*

Genus II. *Actinomyces.* True mycelium produced. The vegetative mycelium breaks up into elements of irregular size and may exhibit angular branching. Conidia not produced. Nonacid-fast. Anaerobic to microaerophilic. Pathogenic to man and animals.

Type species: *Actinomyces bovis.*

Family III. *Streptomycetaceae.* Vegetative mycelium not fragmenting into bacillary or coccoid forms. Conidia borne on sporophores. Primarily soil forms, sometimes thermophilic in rotting manure. A few species are parasitic.

Genus I. *Streptomyces.* Organisms growing in the form of a much-branched mycelium with a typical aerial mycelium. Conidiospores formed in chains. Aerobic. Saprophytic soil forms, less commonly parasitic on plants or animals.

Type species: *Streptomyces albus.*

Genus II. *Micromonospora.* Well-developed, fine, nonseptate mycelium. Grow well into the substrate. Do not form a true aerial mycelium. Multiply by means of conidia, produced singly at ends of special conidiophores, on surface of substrate mycelium. Conidiophores short and either simple, branched, or produced in clusters. Strongly proteolytic and diastatic. Many are thermophilic, growing at 65°C. Usually saprophytes. These organisms occur mostly in hot composted manure, aerial dust, soil, and in lake bottoms.

Type species: *Micromonospora chalcea.*

Order III. *Chlamydobacteriales.* Organisms are filamentous, alga-like, typically water forms, and ensheathed. They may be unbranched, or show true or false branching. False branching arises from lateral displacement of the cells of the filament within the sheath, giving rise to a new filament. The sheath may be composed entirely of iron hydroxide, or of an organic matrix impregnated with iron, or it may be entirely organic. Conidia and motile swarmers may be developed, but never endospores. Sulfur granules are absent. Mature cells or filaments are not protozoa-like.

Family I. *Chlamydobacteriaceae.* Description same as for the order.

Genus I. *Sphaerotilus.* Organisms are attached, colorless threads, showing false branching, though this may be rare in some species. Filaments consist of rod-shaped or oval cells surrounded by a firm sheath. Multiplication occurs by nonmotile conidia and by motile swarmers, the latter with lophotrichous flagella.

Type species: *Sphaerotilus natans.*

Genus II. *Clonothrix.* Filaments are attached and show false branching. Sheaths are organic, encrusted with iron or manganese, broader at the base, and tapering toward the tip. The cells are colorless and cylindrical. Reproduction occurs by spherical conidia formed in chains by transverse fission of cells.

Conidia formation is acropetal and limited to short branches of the younger portion of the filaments.

Type species: *Clonothrix fusca.*

Genus III. *Leptothrix.* The filaments are cylindrical, colorless cells, with a sheath at first thin and colorless, later becoming thicker, yellow or brown, and encrusted with iron oxide. The iron may be dissolved by dilute mineral acid, whereupon the inner cells show up well. Multiplication takes place by division and abstraction of cells and by motile cylindrical swarmers. True branching may occur.

Type species: *Leptothrix ochracea.*

Family II. *Crenothricaceae.* Filaments are unbranched and attached to a firm substrate, showing differentiation of base and tip. Sheaths are plainly visible, thin and colorless at the tips, thick and encrusted with iron oxide at the base. Cells are cylindrical to spherical, dividing in three planes, to produce spherical, nonmotile conidia.

Genus I. *Crenothrix.* Description same as for the family.

Type species: *Crenothrix polyspora.*

Family III. *Beggiatoaceae.* Bacteria are filamentous and usually show an oscillating motion similar to *Oscillatoria.* Cells contain sulfur granules. Spore formation and conidia are not known.

Genus I. *Thiothrix.* Filaments are nonmotile, segmented, with a definite differentiation into base and tip, attached, and usually filled with sulfur granules. The threads produce rod-shaped conidia at the ends. Conidia are motile, exhibit a slow creeping movement, attach themselves and develop into threads. Occur in hot sulfur springs.

Genus II. *Beggiatoa.* The threads are sheathless, formed of flat, discoidal cells, and not attached. Multiplication occurs by transverse splitting of the threads. The threads show an undulatory creeping motion. Cells contain sulfur granules.

Type species: *Beggiatoa alba.*

Genus III. *Thioploca.* Filaments are *Beggiatoa*-like, with numerous sulfur granules. They are motile. Filaments lie parallel in considerable numbers or are united into bundles enclosed in a colorless layer of gelatin.

Type species: *Thioploca schmidlei.*

Family IV. *Achromatiaceae.* Organisms are unicellular, large, and motile. Cells contain sulfur granules.

Genus I. *Achromatium.* Cells are large and nearly spherical. Cells are closely packed with large granules, at first interpreted as sulfur but later as calcium oxalate. When granules are dissolved, the cells show a coarse structure. Cells are motile. Cell division resembles the constriction of flagellates rather than the fission characteristics of bacteria.

Type species: *Achromatium oxaliferum.*

Genus II. *Thiophysa.* Cells are spherical, and the cell membrane is loaded with sulfur granules. The protoplasmic layer surrounds a large central vacuole. The oxalate is contained in the vacuole. A cell nucleus is not recognized. Flagella are absent. Cells elongate before division and divide into biscuit-shaped cells. In the presence of an excess of oxygen, the sulfur granules disappear and only the oxalate remains. In the absence of oxygen and in the presence of hydrogen sulfide, the oxalate disappears and sulfur granules fill the cell.

Type species: *Thiophysa volutans.*

Genus III. *Hillhousia.* Cells are very large and motile by means of peritrichous flagella. Cells are packed with large globules of oily, amorphous sulfur.
Type species: *Hillhousia mirabilis.*

Order IV. *Myxobacteriales.* The relatively long, slender, flexible, nonflagellate, vegetative cells produce a thin, spreading colony (pseudoplasmodium, swarm). The cells are often arranged in groups of 2 or 3 to a dozen or more, their long axes parallel. The group moves as a unit, by means of a crawling or creeping motion, away from the center of the colony. The moving cells pave the substrate with a thin layer of slime on which they rest.

During sporulation (which occurs in all forms except in *Cytophaga*) the cells are much shortened, in some cases becoming spherical or coccoid, thick-walled, and highly refractile. Fruiting bodies are formed by all species except members of the family *Cytophagaceae* and the genus *Sporocytophaga* of the family *Myxococcaceae.* The fruiting bodies may consist of aggregations of cysts in which the spores (resting cells) are enclosed, or of masses of mucilaginous slime surrounding large numbers of shortened, rod-shaped, or coccoid spores. Fruiting bodies may be sessile or stalked. They are usually pigmented a bright shade of orange, yellow, red, or brown, though colorless fruiting bodies, as well as black, have been described. Members of the genus *Sporocytophaga* are not known to produce fruiting bodies as such, but often dense agglomerations of shortened rods or cocci have been noted. These may be interpreted as primitive forms of fruiting bodies.

Physiologically most species show great similarity, preferring substrates rich in cellulosic or other complex carbohydrate materials.

Most of the known species are saprophytic or coprophilic and may be found on dung, in soil, on rotten wood, straw, leaves, etc. They frequently appear to live in close association with various true bacteria and are probably parasitic on them. Many have been cultivated on dung.

Family I. *Cytophagaceae.* Flexible, sometimes pointed rods, showing creeping motility. Fruiting bodies or spores (microcysts) not formed.

Genus I. *Cytophaga.* Description same as for the family.
Type species: *Cytophaga hutchinsonii.*

Family II. *Archangiaceae.* The swarm (pseudoplasmodium) produces irregular, swollen, or twisted fruiting bodies, or develops columnar or finger-like growths, usually without a definitely differentiated membrane.

Genus I. *Archangium.* The mass of shortened rods embedded in slime forms a pad-shaped or more rounded, superficially swollen or tuberous fruiting body, even with horny divisions. The fruiting body has no membrane. In the interior can be seen a mass resembling coiled intestines. The windings of this coil may be uniform, or irregularly jointed, free or stuck together; the ends may be extended and horny. Instead of a membrane there may be loosely enveloping slime.
Type species: *Archangium gephyra.*

Genus II. *Stelangium.* Fruiting bodies are columnar or finger-like, sometimes forked, without definite stalk, standing upright on the substrate.
Type species: *Stelangium muscorum.*

Family III. *Sorangiaceae.* The shortened rods of the fruiting body lie in angular, usually relatively small cysts of definite polygonal shape. Often many of these cysts are surrounded by a common membrane. The primary cyst may be differentiated from the angular or secondary cysts. Stalked forms are not known.

Genus I. *Sorangium.* The cysts are united into rounded fruiting bodies.
Type species: *Sorangium schroeteri.*

Family IV. *Polyangiaceae.* In the fruiting bodies the more or less shortened rods lie in rounded cysts of definite form. The well-defined wall is composed of hardened slime, and is yellow, red, or brownish. The cysts may be united by a definitely visible slime membrane, the remnant of the vegetative slime, or they may be tightly appressed and cemented by the scarcely visible remnants of the slime, or they may develop singly or in numbers on a stalk. In the more highly developed forms, the stalk branches and carries the cysts at the tips of the branches.

Genus I. *Polyangium.* Cysts rounded or coiled, surrounded by a well-developed membrane, either free or embedded in a second slimy layer.
Type species: *Polyangium vitellinum.*

Genus II. *Synangium.* Cysts provided with an apical point, united more or less completely to rosette-shaped, hemispherical, or spherical fruiting bodies.
Type species: *Synangium sessile.*

Genus III. *Melittangium.* Cysts brownish orange-red, on short white stalk, like a mushroom. Has appearance of a white-stalked *Boletus.* The rods inside stand at right angles to the membrane. Upon germination, the covering membrane is colorless and with an appearance of honeycomb.
Type species: *Melittangium boletus.*

Genus IV. *Podangium.* Cysts chestnut-brown or red-brown, single on a more or less definite white stalk.
Type species: *Podangium erectum.*

Genus V. *Chondromyces.* Cysts compactly grouped at the end of a colored stalk (cystophore). Cystophore simple or branched.
Type species: *Chondromyces crocatus.*

Family V. *Myxococcaceae.* The rods become shortened when fruiting occurs (resting cells are formed), and develop into spherical or ellipsoidal spores or microcysts. Upon germination, the vegetative cell develops from the spore by a process analogous to budding, pinching off at the point of emergence, leaving the spore wall entirely empty. In three of the genera, definite fruiting bodies are produced. In *Sporocytophaga,* the spores (microcysts) are produced from the vegetative cells without development of fruiting bodies.

Genus I. *Myxococcus.* Spherical spores in conical, or spherical, or occasionally ovoid upright fruiting bodies, united by a loose more or less mobile slime.
Type species: *Myxococcus fulvus.*

Genus II. *Chondrococcus.* Spores embedded in a viscous slime which hardens. Fruiting bodies divided by joints or constrictions, often branched, usually relatively small.
Type species: *Chondrococcus coralloides.*

Genus III. *Angiococcus.* Fruiting body consisting of numerous round (disk-shaped) cysts, cyst wall thin, spores within.
Type species: *Angiococcus disciformis.*

Genus IV. *Sporocytophaga.* Spherical or ellipsoidal microcysts formed loosely in masses of slime among the vegetative cells. Fruiting bodies absent.
Type species: *Sporocytophaga myxococcoides.*

Order V. *Spirochaetales.* Slender, flexuous cell body in the form of a spiral with at least one complete turn, 6 to 500$\mu$ in length. Some forms may show an axial filament, a lateral crista or ridge, or transverse striations; otherwise no significant protoplasmic pattern. Smaller forms may have a lower refractive index than bacteria, and so living organisms can be seen only with dark field illumination. Some forms take aniline dyes with difficulty. Giemsa's stain is uniformly successful.

Multiplication by transverse fission. Sexual cycle not known. Granules formed by some species in insect hosts. All forms are motile. Motility serpentine or by spinning on the long axis without polarity. Free-living, saprophytic, and parasitic.

Family I. *Spirochaetaceae.* Coarse spiral organisms, having definite protoplasmic structures. Found in stagnant, fresh, or salt water, and in the intestinal tract of bivalve molluscs.

Genus I. *Spirochaeta.* Nonparasitic, with flexible, undulating body and with or without flagelliform tapering ends. Protoplast wound spirally around a well-defined axial filament. No obvious periplast membrane and no cross striations. Motility by a creeping motion. Primary spiral permanent. Free living in fresh- or sea-water slime, especially in the presence of $H_2S$. Common in sewage and foul waters.

Type species: *Spirochaeta plicatilis.*

Genus II. *Saprospira.* Spiral protoplasm without evident axial filament. Spirals rather shallow. Transverse markings or septa (?) seen in unstained and stained specimens. Periplast membrane distinct. Motility active and rotating. Free-living in marine ooze.

Type species: *Saprospira grandis.*

Genus III. *Cristispira.* Flexuous cell bodies in coarse spirals, 28 to 120$\mu$ in length. Characterized by a crista or thin membrane of varying prominence on one side of the body extending the entire length of the organism. Cross striations. Actively motile. Found only in the intestinal tract of molluscs.

Type species: *Cristispira balbianii.*

Family II. *Treponemataceae.* Coarse or slender spirals, 4 to 16$\mu$ in length; longer forms due to incomplete or delayed division. Protoplasm with no obvious structural features. Some may show terminal filaments. Spirals regular or irregular, flexible or comparatively rigid. Some visible only with dark-field illumination. Parasitic on vertebrates with few exceptions.

Genus I. *Borrelia.* Coarse, shallow, irregular, with a few obtuse angled spirals. Length 8 to 16$\mu$. Generally taper terminally into fine filaments. Stain easily with ordinary aniline dyes. Refractive index approximately the same as that of true bacteria. Parasitic upon many forms of animal life. Some are pathogenic for man, other mammals, and birds. Generally hematophytes or found on mucous membranes. Some are transmitted by the bites of arthropods.

Type species: *Borrelia anserina.*

Genus II. *Treponema.* Length 3 to 18$\mu$. Longer forms due to incomplete division. Protoplasm in acute, regular, or irregular spirals. Terminal filament may be present. Some species stain only with Giemsa's stain. Weakly refractive by dark-field illumination in living preparations. Cultivated under strictly anaerobic conditions. Pathogenic and parasitic for man and animals. Generally produce local lesions in tissues.

Type species: *Treponema pallidum.*

Genus III. *Leptospira.* Finely coiled organisms 6 to 20$\mu$ in length. Spirals 0.3$\mu$ in depth and 0.4 to 0.5$\mu$ in amplitude. In liquid medium, one or both ends are bent into a semicircular hook each involving $\frac{1}{10}$ to $\frac{1}{8}$ of the organism. Spinning movements in liquid and vermiform in semisolid agar, forward or backward. Seen in living preparations only with dark field. Stain with difficulty except with Giemsa's stain and silver impregnation. Require oxygen for growth.

Type species: *Leptospira icterohaemorrhagiae.*

Supplement I.

Family: *Rickettsiaceae.* The rickettsias are a group of very small, Gram-negative bacillus-like rods measuring about 0.3$\mu$ in diameter and 2$\mu$ or less in length. The cells are stained readily by Giemsa's stain and possess a faintly stained bar through the middle, giving each organism the appearance of a diplobacillus. They are nonmotile. With one exception, they do not multiply in the absence of living tissue. The rickettsias cause a number of diseases including typhus fever, Rocky Mountain spotted fever, and tsutsugamushi disease.

Supplement II.

Order: *Virales.* Viruses. Etiological agents of disease, typically of small size and capable of passing filters that retain bacteria, increasing only in the presence of living cells, giving rise to new strains by mutation, not arising *de novo.* A considerable number of viruses have not been proved filterable; it is, nevertheless, customary to include these viruses with those known to be filterable because of similarities in other attributes and in the diseases induced. Some not known to be filterable are inoculable only by special techniques, as by grafting or by use of insect vectors, and suitable methods for testing their filterability have not been developed; moreover, it is not certain that so simple a criterion as size measured in terms of filterability will prove to be an adequate indicator of the limits of the natural group.

Supplement III.

Family: *Borrelomycetaceae.* Pleuropneumonia and pleuropneumonia-like organisms.

I. The Pleuropneumonia Group.

The organisms are soft and fragile. Without special precautions they are often distorted or entirely destroyed in microscopical preparations. The cultures contain pleomorphic elements: small granules, bacilli, bacillary filaments and round forms varying in size from a few tenths of a micron to 10$\mu$ or more. Autolyzed round forms may coalesce into large empty blebs. The round forms are part of a reproductive cycle. They are produced by the swelling of the bacillary forms and filaments and reproduce granules or filaments by inside segmentation or multiple germination. In freshly isolated bovine strains, the filaments show apparent or true branching and reproduce the small forms by segmentation. The smallest growing units may not be larger than 0.15 to 0.28$\mu$ and pass through filters that retain bacteria. On agar, tiny colonies (0.1 to 0.6 mm.) develop in great numbers. The colonies invade the agar, and after 2 to 5 days' growth have an opaque center embedded in the agar and a thin peripheral zone. The surface has a rugged or granular appearance due to the development and autolysis of the large forms. After a few days' growth, the cultures usually show pronounced autolysis. The parasitic strains require fresh animal serum for growth.

It may be seen that the classification of bacteria is a very difficult task. The work becomes more difficult as additional species are discovered and studied. Some organisms at one time placed in certain genera are now transferred to other genera or placed in new ones. This is to be expected when the difficulties encountered in studying such minute organisms are considered. It is highly probable that no single classification will ever be completely acceptable to all bacteriologists, but the one outlined here is undoubtedly the best of those that have been proposed and is in general use in this country.

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## CHAPTER XVII

## DISSOCIATION OF BACTERIA

During the early years of bacteriology, most bacteriologists were pleomorphists, believing that a bacterial species could exist in more than one cell form. Some years later, this concept was altered in favor of the monomorphic hypothesis, or fixity of bacterial cell form. Forms that departed more or less widely from the normal types were usually dismissed as being either involution forms, degenerate cells, or different species present as contaminants. At the present time, sufficient evidence has accumulated to support the original pleomorphic hypothesis, or variability in the morphological characteristics of an organism.

A considerable literature has accumulated concerning these aberrant forms, and the results have proved, without a doubt, that the concept of monomorphism can no longer be held. The question of the instability of bacterial species is of tremendous importance to all branches of bacteriology. The work of the systematic bacteriologist (classification of bacteria) becomes more difficult than was at first supposed because an organism may show variations in physiological reactions as well as in morphological characteristics. The phenomenon concerns also the questions of infection, immunity, virulence, and many other phases of bacteriology.

Several terms, in connection with the phenomenon of bacterial variation, have been used more or less loosely and, for this reason, are the cause of considerable confusion. It is, perhaps, appropriate at this time to define them according to their true meaning.

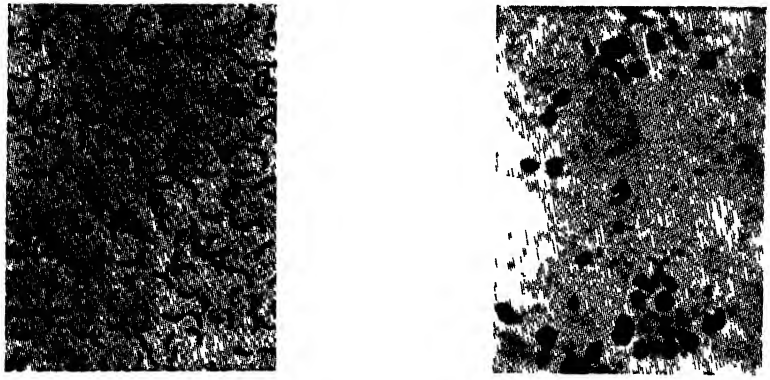
**Mutation.**—This term was first defined by De Vries as a sudden variation, the daughter cells differing from the mother cells in some well-marked character or characters, as distinguished from a gradual variation in which the new characters become developed only in the course of many generations. On the other hand, Dobell believed that mutation includes any permanent change transferred to the daughter cells regardless of whether it is a sudden or a gradual change. In bacteriology, mutation is generally defined as a gradual variation in which the new characters become developed in the course of several generations.

Roepke, Libby, and Small (1944) obtained eight different mutant strains by single-cell isolation from cultures of *Escherichia coli* which had been transferred serially in a complete medium with and without X-ray treatment. The isolated strains appeared to have lost the ability to

synthesize nicotinamide, thiamine, methionine, lysine, cystine, arginine, threonine, or tryptophane. A ninth strain grew well in a basal medium only after the addition of glycine or serine. The mutant strains tended to revert to the parent strain but the rate of reversion was very slow.

For more information, see Delbrück (1945), Luria (1945*a*,*b*), and Severens and Tanner (1945).

**Involution.**—This term is defined as a retrograde development or the appearance of degenerate cells. Culture media containing certain harmful substances will, when inoculated with some species of bacteria, cause



*A* *B*

FIG. 159.—*Vibrio comma*. *A*, Normal forms grown on nutrient agar; *B*, involution forms grown on nutrient agar containing 1 per cent glycine. (*Part B after Gordon and Gordon.*)

the appearance of forms which depart widely from the normal and which are sufficiently characteristic to be of diagnostic importance.

Several bacterial species have been shown to exhibit a peculiar pleomorphism, consisting of the gradual swelling of the organisms into large, round, or fusiform bodies. Sublethal doses of certain chemicals, such as lithium, calcium, penicillin, chromium, and glycine, often produced similar forms. The large bodies, if appropriately transplanted, germinated in certain cultures. Usually, they did not germinate into bacteria of normal shape, but into a peculiar granular growth which was first observed by Klieneberger (1935) in cultures of *Streptobacillus moniliformis* and designated as $L_m$. A similar type of germination was noted in a number of other organisms (Dienes, 1939, 1940, 1941, 1942, 1943, 1944; Dienes and Smith, 1942, 1943, 1944). The large bodies germinated only in spontaneously pleomorphic strains, not in cultures made pleomorphic by toxic influences. Dienes and Smith (1944) believed that the development of the large bodies in a culture was not a degenerative process but part of a reproductive process.

Gordon and Gordon (1943) found that the addition of glycine to culture

media in concentrations of from 0.5 to 1.5 per cent produced changes in the morphological appearances of the organisms of cholera and other vibrios and, also, in the consistency of their colonies. The chief morphological change was swelling of the organisms with the production of large, spherical, or oval bodies (Fig. 159). Alanine produced a similar effect.

**Variation.**—Variation may be defined as a divergence in the morphological or physiological characters (usually both) of a species from those observed in the original cells of the culture. The term is used synonymously with dissociation but is often regarded as opposed to heredity.

**Life Cycles in Bacteria.**—Bacterial dissociation is usually interpreted as being due either to changes in the environment or to a normal, orderly cyclogenic development, better known as a life cycle.

Lewis (1932, 1933, 1937, 1938), in a series of studies on *Bacillus mycoides*, *Azotobacter chroococcum*, *A. beijerinckii*, *Rhizobium meliloti*, and *R. trifolii*, concluded that there was no evidence to support the concept that variant forms in a culture represented phases of a complex pleomorphic life history through which an organism must pass in a cyclogenic method of development.

Burke, Swartz, and Klise (1943) brought forth evidence in support of the presence of life cycles in bacteria. They described hourly changes exhibited by a pleomorphic strain of a *Micrococcus pyogenes* var. *aureus*-like organism grown in flowing broth to eliminate the effect of an accumulation of metabolites. They stated that the organism regularly passed through a coccus motile-rod coccus cycle in 12 hr. Filaments occasionally appeared but represented a minor factor in the cycle. Filtration experiments failed to demonstrate a filter-passing stage.

Humphries (1944) in his work on the dissociation problem of *Klebsiella pneumoniae* concluded,

> The character changes that occur in the culture mass during bacterial dissociation have been perhaps most often explained as being due to changes in the hereditary mechanisms of some of the cells, either as the result of spontaneous mutations or because of impressed changes in these mechanisms by the direct action of certain environmental stimuli, followed by a selective action of the environment favorable to the mutant forms. However, in more recent years the dissociation process has been visualized by a number of workers as a normal cyclogenic development of the "species-microphyte"; the "ontogenetic" theory of Hadley.
>
> At the present time neither of these theories is adequately, or even satisfyingly, supported by experiment. Convincing evidence for the mutation theory is very difficult to obtain in the absence of a sexual cycle. Adequate support for the cyclogenic theory must necessarily continue to be lacking until knowledge concerning the conditions under which these changes may progress is obtained and a complete cycle is shown consistently to occur.
>
> However, if the assumption is made that one of these two theories is the correct explanation of culture-phase variation, then at least one fundamental difference

that is subject to experimental approach exists in these two concepts. If culture-phase transition is the result of mutations in the several characters involved, then, in the absence of character associations and in the presence of different selective

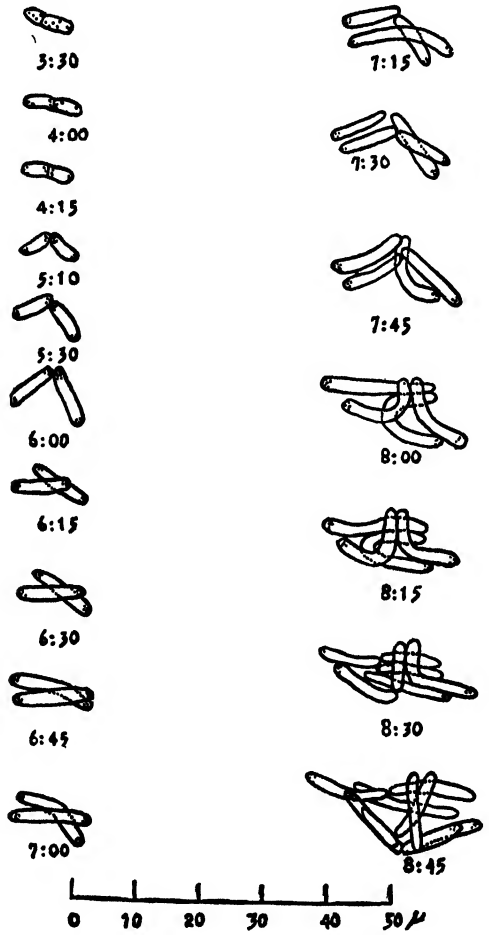



FIG. 160.—Morphologic variation in a growing microcolony of *Bacillus megatherium*. (*From Henrici, Morphologic Variation and the Rate of Growth of Bacteria, Charles C. Thomas, Springfield, Ill.*)

or impressing environments, the sequence of character changes might be expected to occur in a haphazard manner during the interphase period. On the contrary, if the process is a cyclogenic one, then the phase transition pattern should progress in an orderly fashion in those environments which allow a continuation of the phasic variation.

Humphries found that the character changes indicative of the various culture-phase transformations appeared in almost every conceivable order.

These experimental findings failed to support the "ontogenetic" theory of Hadley. The haphazard sequence would seem most readily explainable by the concept of cellular mutation aided by selective environmental conditions.

**Bacterial Instability.**—The instability of bacteria manifests itself in various ways. Coccus forms may, under some conditions, change to rods, and rod forms may change to cocci; motility may disappear only to return again; cells that take an even and uniform stain when young may appear granular when old; physiological reactions, such as the fermentation of carbohydrates, may become modified; spore-forming organisms may lose their ability to produce spores (asporogenous); capsulated organisms may lose their power to secrete a capsule, and species that normally do not produce capsules may be made to do so by modifying the composition of the culture medium or the temperature of incubation; the antigenic response may show marked variation; some species in culture agglutinate spontaneously, whereas others lose their ability to agglutinate; virulent cultures may lose their power to produce disease, and harmful avirulent strains may become virulent. These are some of the types of variations that have been reported. Thompson (1935) grouped bacterial variations as shown in Table 40.

**Variations in Cell Size with Age.**—It is well known that young cells are, in general, larger than old organisms. As a freshly inoculated culture ages, the cells become progressively larger and larger until a maximum is reached, after which the reverse effect takes place. Henrici (1928) measured the lengths of cells of *Bacillus megatherium* growing in a microcolony after gradually increasing periods of incubation. The results are shown in Fig. 160. The corresponding measurements of cell size, together with the number of cells produced in each time period, are recorded in Table 41 (see also page 256).

**Interrelationships of Variations.**—It is well known that many variations are interrelated. The loss of flagella not only results in the appearance of a nonmotile variant but affects also the antigenic response and colonial morphology. Weil and Felix (1917) applied the names "Hauch" (*H*) and "ohne Hauch" (*O*) to two types of *Proteus* $X_{19}$ colonies which appeared on agar plates. The *H* colonies were composed of motile organisms, whereas the *O* colonies showed only nonflagellated organisms. The flagella are capable of eliciting an antigenic response different from that of the bacterial bodies. The motile cells (*H*) produce a flocculent agglutination whereas the nonmotile organisms (*O*) give a granular agglutination. The nonmotile forms produce colonies that are discrete and domed as opposed to the flat, confluent growth displayed by the motile cells. The presence or absence of flagella affects the results in groups 5, 7, and 17 of Table 40.

TABLE 40.—TYPES OF BACTERIAL VARIATIONS

| Group | Character affected | Variations observed |
|---|---|---|
| 1 | Size of cells | Minute forms (filterable?)<br>Giant cells |
| 2 | Cell morphology | Coccoid forms in bacillary cultures<br>Bacillary forms in cultures of cocci<br>Filamentous forms<br>Club forms<br>Branched forms<br>Bizarre cells of various shapes<br>Ameboid forms—"symplasms" |
| 3 | Staining properties | Gram-negative forms in Gram-positive species<br>Nonacid-fast forms in acid-fast species<br>Irregular staining |
| 4 | Spore formation | Nonsporing variants in spore-forming species<br>Variations in method of germination of spores |
| 5 | Motility | Loss of flagella in motile species |
| 6 | Capsule | Increase or decrease in size of capsule<br>Complete loss of capsule (?) |
| 7 | Shape and structure of colonies on solid media | Smooth (*S*), rough (*R*), mucoid (*M*), dwarf (*D*) or gonidial (*G*)<br>Irregular, spreading (*H*) or raised, discrete (*O*)<br>Pigmented or nonpigmented colonies<br>Secondary papillae on colony surface<br>Moth-eaten colonies<br>Opaque or translucent colonies<br>Creamy or sticky colonies |
| 8 | Type of growth in broth | Diffusely turbid or granular sedimenting<br>Pellicle or no pellicle<br>Slimy sediment or diffuse clouding |
| 9 | Nutritional requirements | "Growth-promoting" substances necessary or not<br>Serum or other complex body substances necessary or not<br>Changes in $O_2$ requirements |
| 10 | Fermentation of carbohydrates | Loss of fermenting power typical for species.<br>Acquisition of a fermenting power not typical for species |
| 11 | Proteolysis | Loss of proteolytic power |
| 12 | Hemolysin production | Loss or gain of power to produce hemolysin |
| 13 | Toxin production | Loss or gain of power to produce toxin |
| 14 | Pigment production | Increase or decrease of pigment formation |
| 15 | Virulence | Decrease or increase of virulence in general<br>Decrease or increase of virulence for a particular animal species<br>Complete loss of virulence |
| 16 | Resistance to harmful influences. | Variations in resistance to heat, chemicals, autolysins, antibodies, bacteriophage |
| 17 | Antigenic components | Presence or absence of flagellar antigen<br>Flagellar antigen; group- or species-specific<br>Type-specific carbohydrate (*S* antigen) present or absent<br>Changes in *S* antigen |

## COLONY FORMS

Several types of colony forms have been recognized in bacterial cultures. These have been designated as mucoid (*M*), smooth (*S*), intermediate (*SR*), rough (*R*), dwarf (*D*), and gonidial (*G*) colony forms or phases.

**S and R Forms.**—Griffith (1923) noted two types of colonies when a pure culture of the pneumococcus was streaked over the surface of a solid

medium: one was dull and granular; the other was shiny and smooth. He designated the former type as the rough or *R* variant and the latter as the smooth or *S* variant. The *S* forms possessed distinct capsules, whereas the *R* variants were noncapsulated. Since then, *R* and *S* variants have been shown to be of general occurrence.

TABLE 41.—RATE OF GROWTH AND CELL SIZE OF *Bacillus magetherium*

| Minutes of growth | Number of cells in colony | Average length of cells, $\mu$ | Sum of lengths of cells, $\mu$ |
|---|---|---|---|
| 0 | 2 | 3.7 | 7.5 |
| 30 | 2 | 4.7 | 9 4 |
| 45 | 2 | 4.7 | 9.4 |
| 100 | 2 | 5.6 | 11.2 |
| 120 | 2 | 6.6 | 13.1 |
| 150 | 2 | 8.8 | 17.5 |
| 165 | 2 | 9.2 | 18.5 |
| 180 | 2 | 10.6 | 21.3 |
| 195 | 2 | 13.1 | 26.3 |
| 210 | 2 | 12.8 | 25.6 |
| 225 | 3 | 12.1 | 36.3 |
| 240 | 4 | 9.8 | 39.4 |
| 255 | 4 | 11.6 | 46.3 |
| 270 | 4 | 17.3 | 69.4 |
| 285 | 4 | 16.9 | 67.5 |
| 300 | 7 | 11.0 | 76.9 |
| 315 | 9 | 12.1 | 109.4 |

Dawson (1934) succeeded in isolating a rough variant from the pneumococcus, which was different from the type reported by Griffith. He showed that what Griffith had called the rough phase was in reality the smooth variant, whereas the phase previously considered the smooth variant became the mucoid or *M* variant. The mucoid phase has been recognized in a number of organisms, particularly in those members of the colon-typhoid-dysentery group.

A rough colony answers to the following description: The margin is very irregular; the surface is very flat, uneven, and granular; the organisms produce a granular sediment in broth and clump spontaneously in physiological salt solution.

A smooth colony may be described as follows: The margin is round and even; the surface is convex, smooth, and glistening; the organisms grow as a uniform turbidity in broth and produce a stable suspension in physiological salt solution.

***SR* Forms.**—An *SR* colony is not necessarily one that is intermediate between a smooth and a rough type. It means that the colony has an appearance that is intermediate between the *S* and the *R* forms. It arises from one form and proceeds into the other.

***M* Forms.**—An *M* culture form is characterized by the appearance of colonies having a pronounced moist, glistening, mucoid consistency. The colonies show a strong tendency to run together (Fig. 161). The cells are often capsulated. Both these characters have been observed in species in which such attributes were not believed to be present

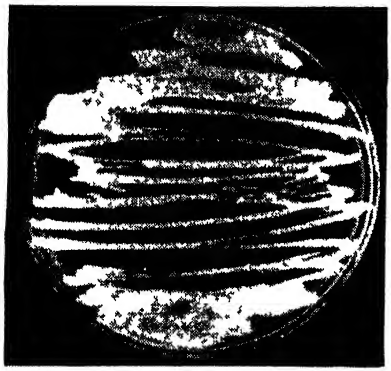


FIG. 161.—Mucoid culture of *Escherichia coli* from rat feces Variant obtained by incubating the plate at 15°C for 4 days

***D* Forms.**—Occasionally a pure culture of an organism, when streaked over the surface of a solid medium, may show the presence of two varieties of colonies designated as large and small colony forms. When separated into pure cultures, the two forms usually maintain their characteristic difference in size for many generations Pure cultures of each type, after many generations, may show the presence of some colonies of the other type The small colony type is referred to as the dwarf or *D* form These colonies are very small, sometimes scarcely visible, and measure about 1 mm. or less in diameter (Fig. 162). The organisms in both the large and the small colonies appear to be the same in morphology and physiology, differing only in colony size.

Youmans and Delves (1942) grew 17 strains of *Micrococcus pyogenes* var. *aureus* on media containing barium chloride and reported the production of numerous stable and unstable small colony variants within 2 to 6 days. Schnitzer, Camagni, and Buck (1943) reported the production of stable small colony variants from one strain of *M. pyogenes* var. *aureus* by the action of penicillin. Youmans, Williston, and Simon (1945) carried out similar experiments on 10 recently isolated hemolytic, pathogenic strains of *M. pyogenes* var. *aureus* and obtained small colony variants from 9 of the 10 cultures in from 2 to 9 days after an initial exposure to penicillin. All the small colony variants were of the unstable type. The results showed that penicillin was an effective agent for the production of small colony variants of *M. pyogenes* var. *aureus*.

***G* Forms.**—Sometimes the *D* forms have been confused with another minute colonial type designated as the *G* or gonidial form. The *G* colonies represent growth from filterable elements of the bacteria. In this respect, the *G* form differs from all the other variants that have been observed.

There appears to be considerable disagreement among bacteriologists on the question of the presence of filterable forms of bacterial species. Some have reported filterable stages in certain species; others have been unable to verify the results.

The filterable forms in cultures yielding positive results are related to the gonidial bodies present in cultures under certain environmental conditions In the absence of the *G* forms, negative filtration results will follow. Certain conditions must be fulfilled before it can be concluded that one is

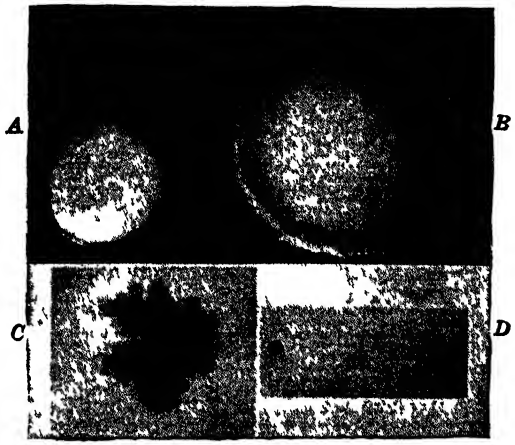



FIG 162 —The four main colony types of *Corynebacterium diphtheriae* *A*, smooth (*S*) colony grown 48 hr at 37°C and magnified 12 times, *B*, intermediate (*SR*) colony grown 48 hr at 37°C and magnified 12 times The two colonies were found growing side by side, *C*, rough (*R*) colony grown 48 hr at 37°C and magnified 18 8 times, *D*, dwarf (*D*) colony grown 48 hr at 37°C and magnified 18 8 times (*After Morton*)

dealing with filterable forms rather than with the normal cell types that have passed through large pores in the filters As Hadley stated,

It is probably only under exceptionally favorable conditions that genuine filterable forms can be demonstrated easily and with any degree of regularity. The original state of the culture material is the primary requisite. Because of this it would appear more important than is indicated by many recent works on filtration to possess, at the beginning of the experiments, some assurance that bacterial elements, whose morphology and size suggest their possible filtrability, are present before filtration in the material subjected to the test. Aside from this the next most important requisite is to utilize such mediums or conditions of cultivation as will ensure the upgrowth of the filterable elements, presumably the gonidia, regarding whose frequent dormancy there can be no question.

***M* to *S* to *R* Change.**—The smooth phase has been observed most frequently in the majority of bacterial species. This is followed by the rough phase. The mucoid variant has been observed the least of all.

Some believe that the different culture phases are, in most cases, at

least, easily convertible one into the other. There appears to be no evidence that any culture phase is stable in the true sense of the word, although it is true that some phases have not been reversed by the employment of in vitro methods. From the small number of observations at hand, it appears that the passage of a culture from one phase to another is not a haphazard transformation but follows a definite sequence of changes. The transition from *S* to *M* occurs more frequently than the change from *R* to *M*. The change from *S* to *M* is less commonly observed than the change from *M* to *S*. In arranging all the transformations reported in the literature, Hadley (1937) found that the sequence of *M* to *S* to *R* occurred more frequently than any of the others and appeared to be the most securely placed of all.

**Secondary Colonies.**—It has long been known that, under certain conditions, old or senescent colonies of many species of bacteria may again resume growth. This is generally referred to as the secondary growth phase. The secondary growth phase may be of short duration, resulting in the formation of minute protuberances or papillae, or it may be more prolonged, resulting in the formation of well-developed daughter colonies. Lewis (1933), in his studies on the dissociation of *Bacillus mycoides*, explained the appearance of secondary colonies by stating,

> The secondary phase of growth is due to depletion of preferred nutrients and subsequent utilization of unused substances by certain cells. Ability to attack unused nutrients is acquired through variation due to the specific stimulus exerted by the substance concerned. The substances found suitable for promoting secondary growth were sucrose and protein fractions of unknown identity contained in digested meat, casein, or gelatin.
>
> The subraces established from secondary colonies show enhanced capacity for utilization of the compound to which variation occurred and do not again produce secondary colonies in its presence. The variants are relatively stable.

Illustrations of secondary colonies of *B. mycoides* are shown in Fig. 163.

## RELATION OF DISSOCIATION TO CELL MORPHOLOGY

Earlier studies have shown that bacterial cells increase in length as they pass from the smooth to the rough phase. Tangled masses of filaments were usually observed among cultures of the rough variants. Stevens (1935) noted increasing cell size in *Clostridium perfringens* in passing from the mucoid to the smooth to the rough type. Dawson (1934) and others observed the same morphological picture in cultures of the pneumococcus. Dawson, Hobby, and Olmstead (1938), in a study of the variants of several types of streptococci, came to a similar conclusion (Figs. 164 and 165).

## RELATION OF COLONY FORM TO OTHER ATTRIBUTES

In earlier studies and before the mucoid phase was recognized as an entity, it was shown that the smooth variant carried greater virulence than

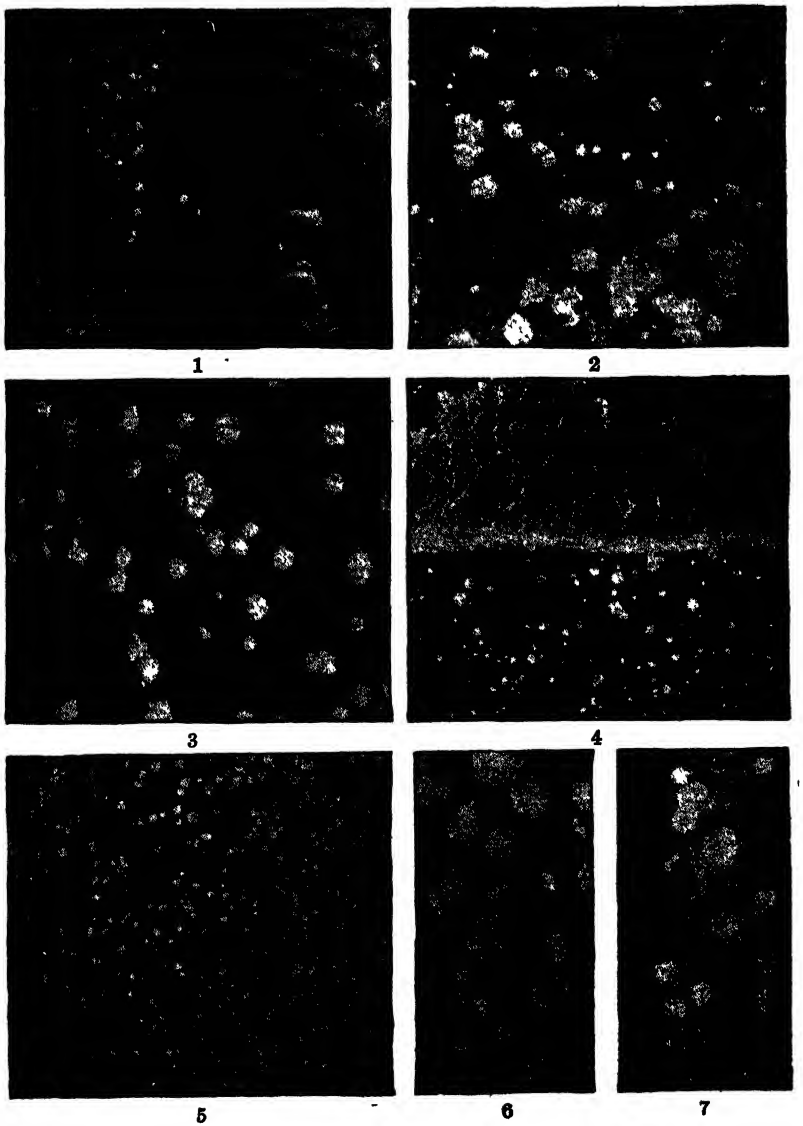



FIG. 163.—Secondary colonies of *Bacillus mycoides*. 1, portion of a giant colony of strain *B* on agar prepared from 14-day gelatin culture and photographed on the tenth day. 2, colonies of strain 421 at end of 14 days on agar containing 1 per cent peptone. 3, colonies of strain *A*, 60-day gelatin culture, on agar containing 0.5 per cent peptone and 0.3 per cent beef extract and photographed on the tenth day. 4, strain A.T.C. at end of 14 days on nutrient agar plus 2 per cent sucrose. Mixture of original and sucrose variant types. 5, strain A.T.C. on nutrient agar plus 2 per cent sucrose and photographed at the end of 21 days. 6, portion of same colony shown in (5). The original threads show only faintly at this age. 7, strain *A* at end of 30 days prepared as described under (3). The colonies are shown natural size in (4) and (5). All others are magnified 3.5 times. (*After Lewis.*)

the rough variant (which was usually avirulent) Results reported in the literature show that the above conclusions are essentially correct. Rough

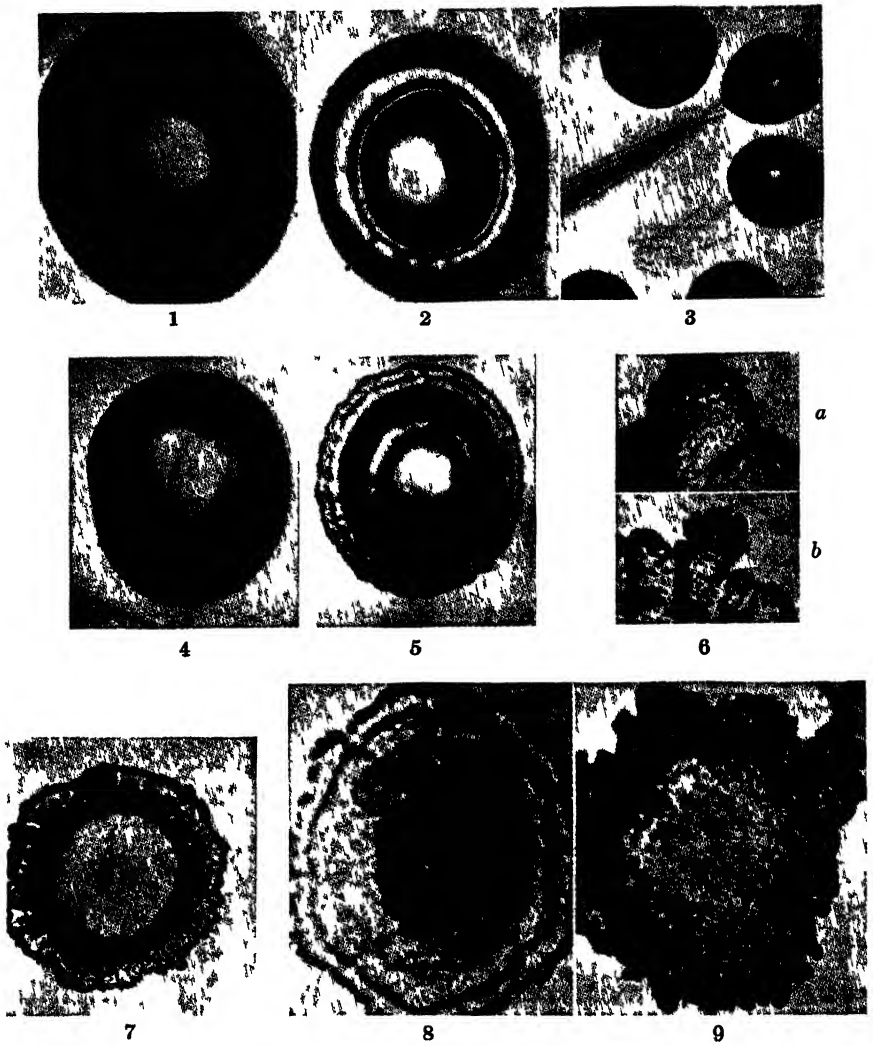



FIG 164.—*Streptococcus hemolyticus* 1, *M* colony, 18 hr, 37°C, 2, well-marked *S—M* variation 18 hrs, 37°C, 3, matt colonies, 18 hrs, 37°C, 4, *S* colony, two days, 37°C; 5, early *SR* colony four days 37°C, 6, *R* variation at margins of *SR* colonies, six days, 37°C, 7, *SR* colony approaching pure *R*, three days, 37°C, 8, *R* colony (possibly some *SR* elements at center), three days, 37°C, 9, *R* colony, eight days, 37°C (*After Dawson, Hobby, and Olmstead*)

cultures, as a rule, lack both virulence and toxigenicity Among those bacterial species in which the mucoid phase has been commonly recognized, this variant has been shown to carry maximum virulence and toxigenicity.

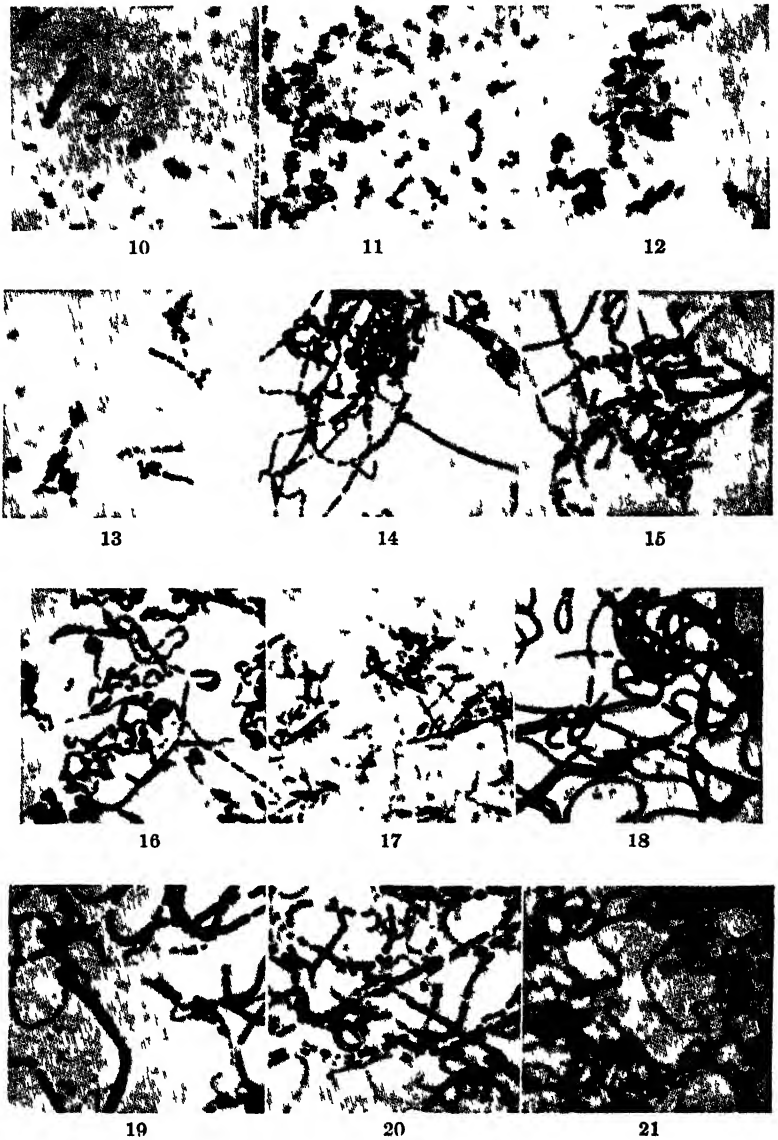



FIG 165.—*Streptococcus hemolyticus* 10, *M* organisms from 3-hr colony, 37°C., Hiss capsule stain, 11, *M* organisms from 15-hr colony, 37°C., Gram stain, 12, matt organisms from 15-hr colony, 37°C., Gram stain, 13, *S* organisms from 72-hr colony, 37°C., Gram stain, 14–17, successive stages in $S \rightarrow R$ variation, 37°C., Gram stain, 18, *R* organisms from 24-hr. colony, 37°C., Gram stain, 19–21, successive stages in $R \rightarrow S$ variation, 37°C., Gram stain. (*After Dawson, Hobby, and Olmstead.*)

Exceptions to this have been noted. Hemolysis (dissolution of red blood corpuscles by toxins secreted by some bacterial species) does not appear to be correlated with any of the variants.

Hadley (1927), in a review of the literature, showed that each culture phase was closely correlated with other morphological, cultural, and physiological characteristics, such as presence of capsules, morphology of the cell, immunological reactions, antigenic structure, virulence, motility, sensitiveness to bacteriophage, and resistance to phagocytosis. Since that time, reports have appeared suggesting that many exceptions exist, that these more recent observations support the general view that each characteristic of a species is subject to independent transmissibility. In a later communication, Hadley (1937) summarized these observations by stating,

> . . . although certain attributes of a bacterial species may be able to vary independently of the culture phase, these instances are so infrequent as hardly to affect the broad generalization that each culture phase, when existing in a relatively pure state, is closely related to a certain group of characters; and that, when one phase has become fully transformed into another, some of these attributes are lost while new ones are gained.

The characters that have been found more frequently to correlate with phase include cell morphology, cell grouping, motility, possession of specific carbohydrates, tendency to saprophytic existence, antigenic structure (including serological and immunological features), toxigenicity, and virulence. The characters that have been found not to correlate clearly with phase are chromogenesis, hemolysis, fermentations, and other physiological reactions.

For more information on the correlation of virulence with phase, see the report of Hadley and Wetzel (1943).

## FACTORS INCITING VARIATIONS

Variations may be classed as (1) nonhereditary and (2) hereditary. The former group includes those variations which result from changes in the environmental conditions. The latter group includes those variations which may be permanent or continue for a limited number of generations.

Most of the variations that have been observed are probably nonhereditary in character and result from changes in the environment. Variants may be easily observed by long-continued growth of an organism in a culture without transfer. Examination of smears prepared at definite intervals reveals the presence of forms that depart widely from the so-called normal type. The transfer of the variants from an old culture to a fresh tube of the same medium, followed by incubation, results in a reappearance of the "normal" cell form.

Revis (1911) noted that when cultures of *Escherichia coli* were treated

with malachite green, the organisms lost their ability to produce gas from fermentation of lactose, glucose, dulcitol, and mannitol. Acid formation, however, was not destroyed. The new physiological condition of the organism was found to be quite permanent, and all attempts to reproduce the power of gas formation failed. In other respects, the organisms appeared to be identical with those in the original cultures.

Penfold (1911) streaked a culture of *E. coli* over the surface of agar containing small amounts of sodium monochloracetate. He obtained two

FIG. 166.—1, Pneumococcus Type II (*R*) colonies; 2, pneumococcus Type II (*R*) variant grown on agar medium with the addition of active transforming principle isolated from Type III pneumococci. The smooth, glistening, mucoid colonies shown are characteristic of Type III pneumococcus and readily distinguishable from the small rough colonies of the parent *R* strain. (*After Avery, MacLeod, and McCarty.*)

types of colonies: (1) a normal type and (2) a dissociant type, which failed to produce gas in glucose, levulose, mannose, galactose, arabinose, xylose, lactose, dextrin, and salicin, but was still able to produce gas from the alcohols dulcitol, mannitol, and sorbitol. The variant retained the new characteristics for several generations. In a later communication, Penfold (1913) substituted monochlorhydrin for the sodium monochloracetate and found that *E. coli* threw off variants similar to those produced on the former medium.

Phenol agar (0.1 per cent) was found by Braun and Schaeffer (1919) to change the *H* or flagellar variant of *E. coli* to the *O* or nonmotile form. This change was only transient, the *H* form reappearing on transfer of the organism to the usual laboratory media.

Dawson and Sia (1931) grew *R* cells (unencapsulated) derived from Type II pneumococcus in a fluid medium containing *R* antiserum, and heat killed encapsulated *S* cells. They found that the *R* cells were trans-

formed into *S* Type III pneumococcus. The *R* forms had acquired the capsular structure and biological specificity of Type III pneumococcus.

Avery, MacLeod, and McCarty (1944), and McCarty and Avery (1946*a*, *b*) isolated a biologically active fraction from Type III pneumococci which, in exceedingly minute amounts, was capable of inducing the transformation of unencapsulated *R* variants of pneumococcus Type II into fully encapsulated cells of the same specific type as that of the heat-killed organisms from which the inducing material was recovered (Fig. 166). The active fraction consisted principally if not solely of a highly polymerized, viscous form of desoxyribonucleic acid.

The addition of chemicals (germicides) and dyes (bacteriostatic agents) to culture media frequently results in the appearance of variants that are sufficiently characteristic to be used in identifying some species (see page 424).

Three procedures are usually followed in securing pure cultures of variants: (1) growth of the organisms under environmental conditions inducing them to dissociate, (2) the use of selective culture media encouraging the appearance of variants, (3) macroscopic selection of the characteristic colonies appearing on the surface of solid media.

## DISSOCIATION AND CLASSIFICATION OF BACTERIA

The scheme that has been employed in the classification of bacteria is based on the monomorphic concept or fixity of bacterial species. It is now definitely established that bacteria are pleomorphic, that they may appear in more than one culture phase. If bacteria are classifiable, the question that naturally arises is which phase may be considered to be the normal form. Some believe that at the present time bacteria are not classifiable and will not be until bacteriologists learn more about what constitutes a bacterial species.

For further reading, see Emerson (1945), Gowen (1945), Luria (1947), Mellon (1942), Morgan and Beckwith (1939), and Parr and Robbins (1942).

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# CHAPTER XVIII

## ASSOCIATIONS OF BACTERIA

Organisms are rarely, if ever, found growing as pure species in their natural habitat. Mixed cultures of two or more species are the general rule. Because of this fact, it is sometimes erroneous to conclude from laboratory findings the exact changes that organisms produce in their natural environment.

Simple mixtures of two or more species may exist in which the organisms produce no effect on each other, but this is rarely true. Associations may exist (1) between different species of bacteria and (2) between bacteria and other classes of organisms, such as algae, protozoa, molds, and yeasts.

**Symbiosis.**—In many cases, growth and multiplication are more vigorous in friendly associations than with either species existing alone. Such a phenomenon is spoken of as symbiosis. This term may be defined as the living together of two or more species of organisms in friendly association for mutual benefit.

Certain soil bacteria of the genus *Rhizobium* are found growing in tumors or nodules produced on the roots of plants belonging chiefly to the family *Leguminosae*. These organisms utilize free atmospheric nitrogen and build it up into organic compounds. The plants are furnished available nitrogen by the bacteria, and the bacteria derive their nutrients from the plant sap. A perfect symbiotic relationship exists (see page 559).

Many examples of symbiosis reported in the literature are misnomers in that only one of the organisms in the association is apparently benefited. The favorable influence of an aerobe on the growth of an anaerobe may be mentioned. The aerobe reduces the oxygen tension and creates an environment suitable for the growth of the anaerobe. The anaerobe is benefited by the association while the aerobe either is not affected or is harmed. This should be regarded as an example of commensalism or of antibiosis rather than of symbiosis. True examples of bacteria growing in symbiosis with other species of bacteria where both are benefited by the association are rare in nature.

**Commensalism.**—An organism may be unable to grow in the presence of a certain substrate. If, however, another organism is present, capable of attacking the food material with the production of a compound or compounds utilizable by the first organism, growth will occur. Such an association is spoken of as commensalism. The term "commensalism" may be

defined as the living together of two species, one of which is benefited by the association while the other is apparently neither benefited nor harmed.

Waksman and Lomanitz (1925) showed that *Bacillus cereus* developed very rapidly in a synthetic medium containing casein as the only source of nitrogen. The casein was vigorously hydrolyzed with the production of amino acids and other split products. The hydrolysis took place more rapidly in the absence of a fermentable carbohydrate. *Pseudomonas*

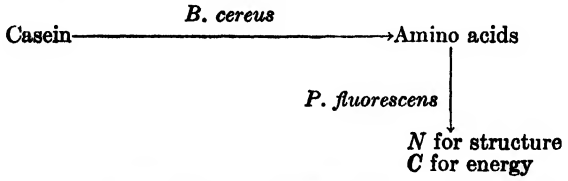



FIG. 167.—Action of *B. cereus* and *P. fluorescens* on casein and its split products.

*fluorescens*, on the other hand, was unable to attack and utilize the casein either for structure or for energy. However, the organism utilized amino acids with ease (Fig. 167).

*B. cereus* is a typical proteolytic organism capable of degrading native proteins to compounds having smaller molecular weights. *P. fluorescens* is nonproteolytic but can utilize free amino acids as a source of nitrogen for structure and carbon for energy.

When both organisms were inoculated into the above medium, the

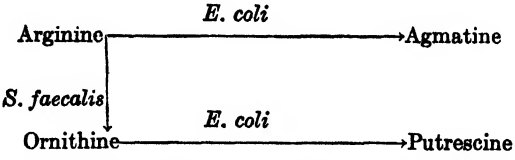



FIG. 168.—Action of *E. coli* and *S. faecalis* on arginine and ornithine.

changes that took place on the casein molecule were different from those produced by each organism acting separately. *B. cereus* first attacked the casein with the liberation of amino acids and other compounds. *P. fluorescens* decomposed the amino acids as they were formed. The result was that the culture soon showed more cells of *P. fluorescens* than of *B. cereus*. *P. fluorescens* was definitely benefited by the association while *B. cereus* was probably neither benefited nor harmed.

Gale (1940) reported that *E. coli* was capable of decarboxylating arginine to agmatine, and ornithine to putrescine, but was unable to hydrolyze arginine to ornithine. On the other hand, *Streptococcus faecalis* was capable of hydrolyzing arginine to ornithine but was incapable of decarboxylating arginine to agmatine, or ornithine to putrescine (Fig. 168).

In a medium containing arginine, neither *E. coli* nor *S. faecalis* acting

separately could produce putrescine. However, when both organisms were inoculated into the same medium, *S. faecalis* produced ornithine, which was then acted upon by *E. coli* to give putrescine. *E. coli* was benefited by the association while *S. faecalis* probably was not affected.

Another example of commensalism that is frequently employed in the laboratory is the cultivation of an anaerobe in the presence of a facultative aerobic organism. An appropriate solid medium is poured into a Petri dish, and the agar allowed to become firm. The anaerobic organism is streaked over the surface of one-half of the plate, and the facultative aerobe is streaked over the surface of the other half. The lid is sealed to the bottom half of the Petri dish by means of plasticine clay or similar material. The aerobe utilizes the free oxygen inside of the dish and eliminates carbon dioxide. The oxygen tension is soon reduced sufficiently to permit the growth of the anaerobe. The anaerobe is definitely benefited by the association while the aerobe is neither benefited nor injured. A liquid medium may also be used where a mixed culture is desired (Fig. 168*A*).

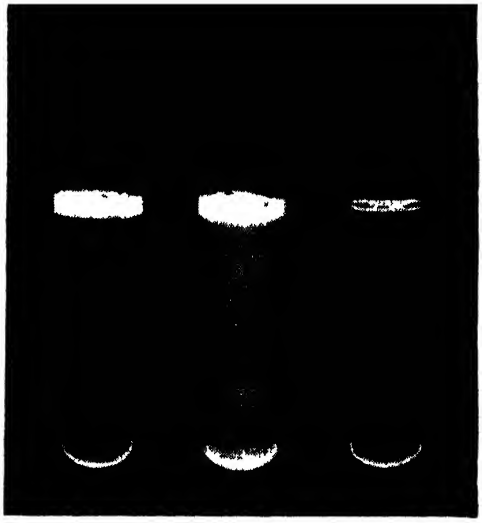


FIG. 168*A*.—Bacterial commensalism. Left, nutrient broth inoculated with *Bacillus subtilis* (note the heavy pellicle with absence of visible growth in the broth); right, nutrient broth inoculated with *Clostridium sporogenes* (the organism fails to grow); center, nutrient broth inoculated with a mixture of *B. subtilis* and *C. sporogenes* (the growth of *B. subtilis* creates conditions favorable to the growth of *C. sporogenes*).

**Synergism.**—This type of association is believed to be of common occurrence in nature. The term was first suggested by Holman and Meekison (1926). Synergism may be defined as the joint action of two organisms on a carbohydrate medium, resulting in the production of gas that is not formed by either organism when grown separately.

Sears and Putnam (1923) were probably the first to conduct systematic studies of synergism. They reported that many pairs of organisms were observed to produce gas from sugar media, which was not formed by either organism in pure culture. They explained the phenomenon by stating that one of the organisms of the pair was capable of forming acid while the other member produced the gas. The acid former degraded the carbohydrate and released a substance that was utilized by the second organism, resulting in the production of gas. The substance attacked by the gas-forming

member of the pair was not an end product of the action of the acid producer but an intermediate product of metabolism. Holman and Meekison confirmed the findings of Sears and Putnam and enlarged on their work. Their results of gas-forming pairs of organisms are given in Table 42. A typical experiment, using sucrose broth and the organisms *Micrococcus pyogenes* var. *aureus* and *Escherichia coli*, is shown in Fig. 169.

Castellani (1926) reported that *Salmonella typhosa* and *Proteus morganii* produced gas from some carbohydrates when grown in association but failed to do so when grown separately in pure cultures. Graham (1932) also worked with the same organisms and reported that the two species growing in association formed synergic gas from mannitol. *S. typhosa* produced a stable compound from mannitol which was degraded by *P. morganii* to gas. *S. typhosa* grown alone produced an intermediate compound which was found to be stable at 100°C. When a culture of *S. typhosa* was killed by heat and then inoculated with *P. morganii*, the intermediate compound was fermented with the liberation of gas. The gas was found to be the same as that produced from a mixture of the two organisms grown together.

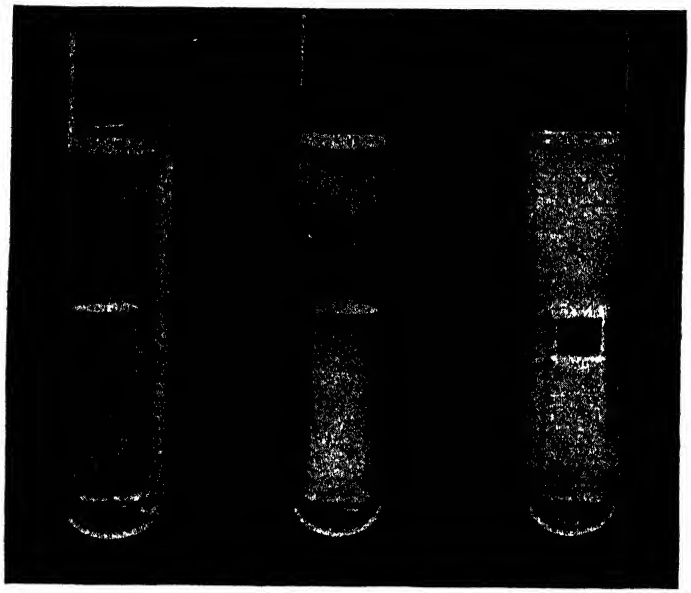


FIG. 169.—Bacterial synergism. Left, *Escherichia coli* grown in sucrose broth; center, *Micrococcus pyogenes* var. *aureus* grown in sucrose broth; right, a mixture of *E. coli* and *M. pyogenes* var. *aureus* in sucrose broth with gas formation.

Atkinson (1935), working with the same pair of organisms, reported synergic gas from mannitol and from xylose. The amount of gas produced was found to be greatly increased by growth of the organisms in the

presence of calcium carbonate, the function of which appeared to be a neutralizing action on the acids produced by *S. typhosa*, with the result that this organism remained in an actively growing condition for a longer period of time and continued to decompose the carbohydrate.

The phenomenon of synergism probably finds its greatest importance in the field of bacteriological water examinations. False positive presumptive tests in water analysis are sometimes caused by the associated activities of two or more species of organisms. The opposite effect might also be obtained; namely, the failure of a pair of gas-producing organisms to form gas when grown in association. Greer and Nyhan (1928) reported the production of gas by pairs of nongas-forming organisms. They concluded that synergic reactions in water examinations are not of common occurrence. The general tendency is for one member of a pair to inhibit or outgrow the other. This may be due to the elaboration of metabolic products by one organism detrimental to the other, to an increase in the hydrogen-ion concentration, to a higher growth rate by one of the members, etc. The presence or absence of gas does not necessarily mean that *E. coli* is present or absent in a water sample.

TABLE 42.—BACTERIAL PAIRS THAT PRODUCE GAS IN ASSOCIATION

| Carbohydrate | Organisms |
|---|---|
| Lactose | *Micrococcus pyogenes* var. *aureus* + *Salmonella schottmuelleri*<br>*Streptococcus faecalis* + *Salmonella schottmuelleri*<br>*Streptococcus faecalis* + *Salmonella choleraesuis*<br>*Micrococcus pyogenes* var. *aureus* + *Proteus vulgaris*<br>*Streptococcus faecalis* + *Proteus vulgaris*<br>*Streptococcus faecalis* + *Salmonella paratyphi* |
| Sucrose | *Micrococcus pyogenes* var. *aureus* + *Escherichia coli*<br>*Streptococcus faecalis* + *Escherichia coli*<br>*Streptococcus equinus* + *Salmonella schottmuelleri*<br>*Streptococcus equinus* + *Salmonella paratyphi*<br>*Micrococcus pyogenes* var. *aureus* + *Salmonella paratyphi* |
| Mannitol | *Micrococcus pyogenes* var. *aureus* + *Proteus vulgaris*<br>*Streptococcus faecalis* + *Proteus vulgaris*<br>*Streptococcus pyogenes* + *Proteus vulgaris*<br>*Shigella paradysenteriae* + *Proteus vulgaris*<br>*Salmonella typhosa* + *Proteus vulgaris* |

Atkinson and Wood (1938) stressed the importance of synergic pairs in producing false positive presumptive tests in water examinations. They isolated organisms growing in synergic association, one member being a nonlactose fermenter giving acid and gas from glucose, and the other giving acid but no gas from lactose. Two pairs of organisms were isolated, both of which contained a nonlactose fermenter of the *Proteus* group. In one

pair, the organism was combined with *Streptococcus faecalis*, and in the other pair with *Eberthella belfastiensis*. False positive presumptives due to the combined action of *E. belfastiensis* and a *Proteus* type were encountered most frequently.

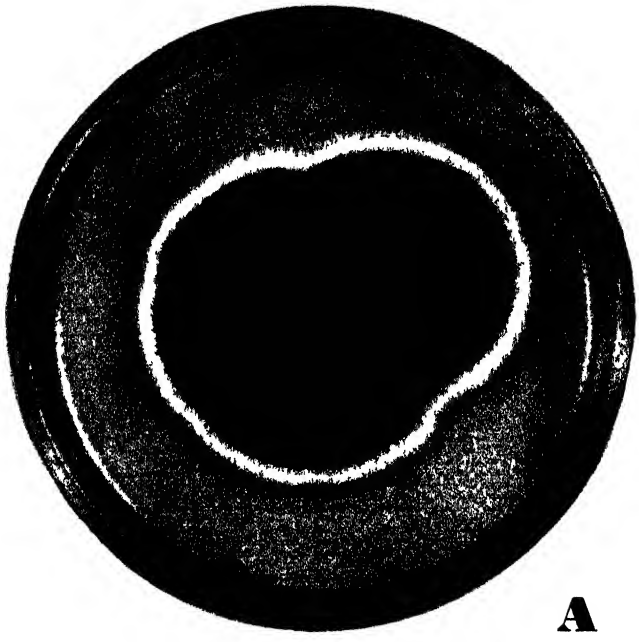
**Antibiosis.**—An organism protects itself against its enemies in various ways. It may produce metabolic waste products which change the conditions in the medium, such as pH, osmotic pressure, and surface tension, making the environment unfavorable to the growth of less tolerant organisms. It may excrete specific toxic substances which interfere with the metabolism of other organisms to such an extent that they are either killed or prevented from multiplying. This last type of activity is referred to as "antibiosis." Antibiosis may be defined as the living together of two organisms one of which is distinctly injurious to the other and which may result finally in the death of the latter.

The phenomenon of antibiosis is not a new discovery. As early as 1877, Pasteur noted that certain air-borne organisms were capable of inhibiting the growth of the anthrax bacillus. At about the same time, it was shown that pathogenic bacteria disappeared much more rapidly in untreated soil than in soil previously sterilized by heat. Since that time, many observations have been recorded of mixed cultures growing on agar plates in which one organism is distinctly antagonistic to another, resulting in the appearance of clear zones around colonies of the former.

Fleming (1929) obtained a mold contaminant on an agar plate culture of *Micrococcus pyogenes* var. *aureus* which produced a green pigment and prevented bacterial growth for some distance around it. He cultivated the organism in broth and found that a filtrate of the culture had the power, even when greatly diluted, to prevent the growth of a number of pathogenic bacteria. Since the mold proved to be a species of *Penicillium* (*P. notatum*), Fleming named the antibiotic "penicillin" (Fig. 170). Attempts to isolate and purify the antibiotic failed, and penicillin was almost forgotten by everyone except Fleming, who continued to experiment with cultures of the mold.

Dubos (1939) isolated a spore-bearing bacillus from the soil that was capable of destroying living Gram-positive cocci. Autolyzed cultures of the organism were capable of destroying living micrococci, pneumococci, and certain streptococci. The organism proved to be *Bacillus brevis*, a large Gram-positive, spore-producing rod similar to *B. subtilis*. He named the antibiotic "gramicidin." The addition of gramicidin to nutrient broth prevented the growth of Gram-positive cocci but failed to retard the multiplication of Gram-negative bacteria.

Meanwhile, a group of biochemists and bacteriologists at Oxford University, England, reexamined the possibilities of isolation and purification of penicillin. It was not until 1940 that they succeeded in isolating penicil-



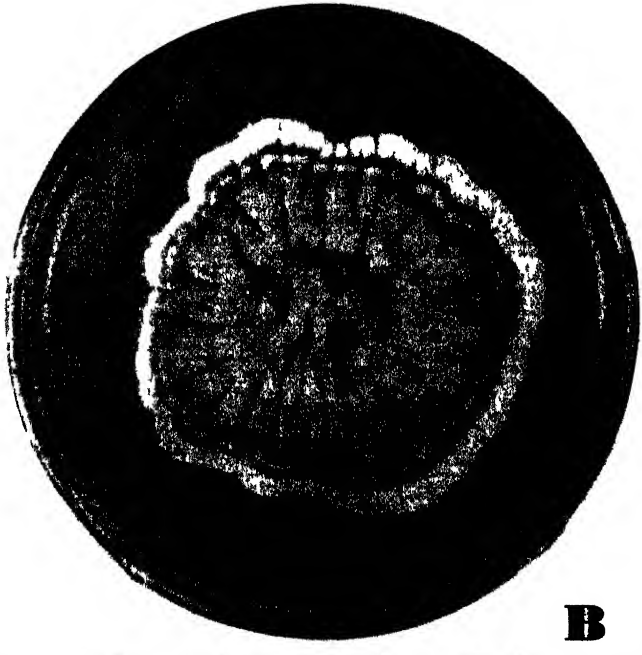




FIG. 170.—*A*, *Penicillium notatum*, descendent of Fleming's strain used in nearly all surface-culture production of penicillin; *B*, *Penicillium chrysogenum*, very high-yielding penicillin producer. (*Courtesy of Eli Lilly and Company.*)

lin in crude form and making it available for further study. The crude preparation was shown to be more effective against a number of diseases than the sulfonamide drugs. The antibiotic was found to be so nontoxic that amounts beyond the effective curative dose could be administered.

*Sources of Antibiotics.*—Many microscopic organisms are carried by air, any one of which might show antibiotic activity in appropriate media.

The soil is perhaps the richest source of microorganisms exhibiting antibiotic action. These include principally molds, bacteria, and actinomycetes. One of the properties possessed by these organisms is to excrete substances that inhibit or destroy the growth of their neighbors.

A simple method for isolating organisms exhibiting antibiotic activity is the following: A soil sample, rich in organic matter, is suspended in water and spread over the surface of a freshly prepared agar pour-plate culture of the organism for which an antagonist is wanted. If one is present in the soil sample, its colonies will appear surrounded by clear zones where the bacteria in the agar are either killed or prevented from multiplying.

*Methods of Cultivation.*—Apparently all antibiotic-producing organisms that have been studied must have air for normal metabolic activity. Three methods of cultivation are generally employed, all of them based on the introduction of a plentiful supply of oxygen into the environment: (1) shallow surface cultivation in which the organisms grow on the surface of liquid media and form a firm mat, (2) shallow submerged cultures in which the organisms are inoculated into thin layers of media, air reaching to the bottom of the cultures by diffusion, and (3) deep submerged cultures in which the media are mechanically agitated throughout the incubation period. This latter procedure produces a more abundant growth in a shorter period of time and requires less space and equipment.

*Measuring Antibiotic Activity.*—The method commonly employed for measuring antibiotic activity is known as the Oxford cylinder-plate or agar cup-plate test. In the Oxford cylinder method, agar is inoculated with the organism to be antagonized and poured into a Petri dish. When firm, porous clay cylinders, open at both ends, are placed on the surface of the agar and filled with dilutions of the antibiotic. Around the cylinders will appear clear zones where growth has been prevented by diffusion of the antibiotic from the cylinders into the agar (Fig. 171). In the agar cup-plate test, a plate containing inoculated agar is prepared as before. One or more disks are cut in the agar and removed from the plate. The cups are filled with dilutions of the antibiotic and the plate incubated at the appropriate temperature. After incubation, clear zones will appear around the cups where growth has been prevented by diffusion of the antibiotic into the agar.

Sometimes paper disks about 10 mm. in diameter are used instead of

porcelain cylinders. These are dipped in the dilutions of the antibiotic and placed on the surface of the inoculated agar (Fig. 172).

*Destruction of Antibiotics.*—Some organisms have been shown to be capable of rapidly destroying penicillin. This is due to the elaboration by these organisms of an enzyme which is generally referred to as a penicillinase. The enzyme has been shown to be present in *Escherichia coli* and in a number of air-borne bacteria. The term "penicillinase" would indicate

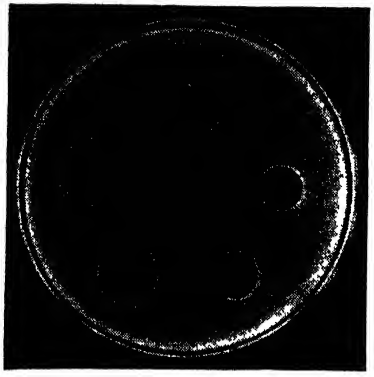


FIG. 171.—Measuring antibiotic activity by the Oxford cylinder-plate method. Melted agar was mixed with *Sarcina lutea* and poured into a Petri dish. Equal volumes of subtilin solutions ranging in concentrations from 1:1000 to 1:1,000,000 were pipetted into the cups. The largest zone was produced by the 1:1000 dilution of subtilin.

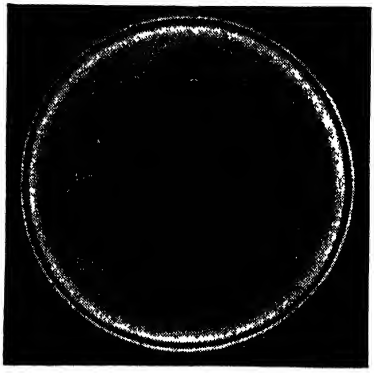


FIG. 172.—Measuring antibiotic activity by various methods. Melted agar was mixed with *Sarcina lutea* and poured into a Petri dish. A 1:1000 dilution of subtilin was used in all three tests. Top, agar cup-plate method; left, Oxford cylinder-plate method; right, paper disc-plate method.

that only one enzyme is capable of attacking penicillin, which is not true. Lawrence (1945*a*) showed that the enzymes papaine cysteine, bacterial amylase, clarase, and Takadiastase also inactivated penicillin. Although these enzymes have occasioned many difficulties in the production of penicillin, they have also found a field of usefulness in testing the sterility of therapeutic penicillin preparations. Penicillin is first destroyed by brief incubation with one of these enzymes, and the inactivated material is then cultured to test for the presence of any contaminating organisms.

*Importance of Antibiotics.*—Antibiotics have been shown to be elaborated by almost every group of living organisms. These include algae, lichens, flowering plants, and animals (lysozyme from egg white, tears, etc.). However, the most important antibiotics known today are all produced by the microorganisms classified as bacteria, actinomycetes, and molds.

The number of antibiotics produced by organisms is being constantly increased. At the present time, hundreds of them have been identified and

studied. Some have been shown to be produced by more than one organism. Some organisms are capable of elaborating two or more different antibiotics. For example, *Penicillium notatum* elaborates both penicillin and penatin; *Bacillus brevis* elaborates gramicidin and tyrocidin; *Aspergillus fumigatus* forms fumigacin, gliotoxin, and fumigatin. The more im-

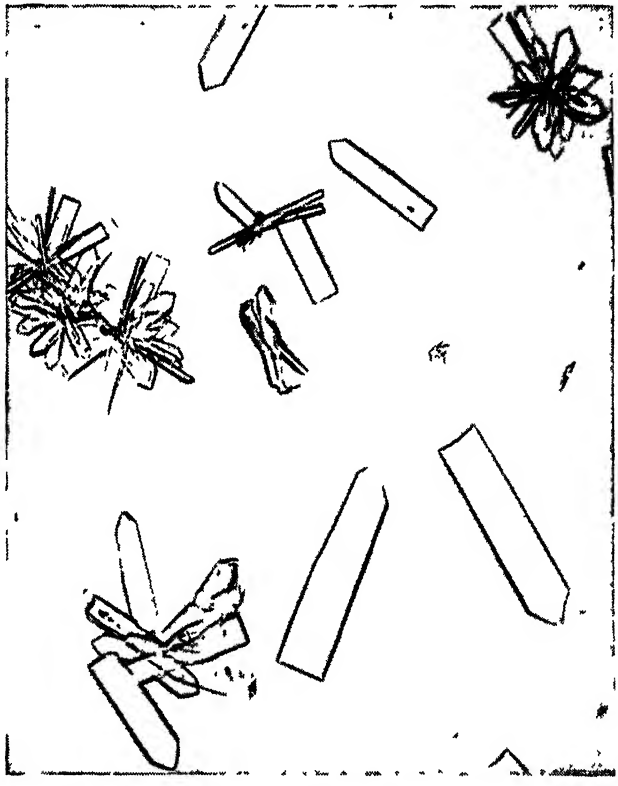


FIG. 173.—Sodium penicillin G crystals. (*Courtesy Squibb Institute for Medical Research.*)

portant antibiotics, together with some of their characteristics, are given in Table 43.

The field of antibiotics appears to offer unlimited possibilities in medicine. Powerful antibiotics, such as penicillin, have proved to be of such tremendous importance for the destruction of organisms, especially those capable of producing disease, that an ever-increasing search is being made for more and better ones. Since it is hard to believe that the best antibiotics have already been isolated, we may expect startling discoveries in the future.

For additional reading, see Bahn, Ackerman, and Carpenter (1945), Beadle, Mitchell, and Bonner (1945), Bruce, Dutcher, Johnson, and Miller

TABLE 43.—ANTIBIOTICS FROM A NUMBER OF MOLDS, BACTERIA, AND ACTINOMYCETES

| Organism | Antibiotic | Susceptible organisms | Properties |
|---|---|---|---|
| | | Molds | |
| *Penicillium notatum*.... | Penicillin (1929) (Fig. 173) | Gram-positive bacteria, gonococci, meningococci, spirochetes, actinomycetes, *C. diphtheriae* | Almost without toxicity: very active in vivo |
| *Penicillium citrinum* ... | Citrinin (1931) | Chiefly Gram-positive bacteria | Not active in vivo; toxic in relation to activity |
| *Gliocladium fimbriatum*, *Aspergillus fumigatus*. | Gliotoxin (1936) | Gram-positive bacteria, *Salmonella paratyphi*, *Shigella dysenteriae*, molds | Not active in vivo; very toxic |
| *Aspergillus fumigatus*... | Fumigatin (1938) | Chiefly Gram-positive bacteria | Very toxic |
| *Aspergillus clavatus*..... | Clavacin (1942) | Gram-positive bacteria; some molds | Active in vivo; very toxic |
| *Penicillium notatum*..... | (Penatin (1942) | Gram-positive and Gram-negative bacteria | Low toxicity; not active in vivo |
| *Penicillium aurantiovirens*..... | Puberulic acid (1942) | Gram-positive bacteria | |
| *Aspergillus fumigatus* mut. *Helvola*..... | Helvolic acid (1943) | Chiefly Gram-positive bacteria | Active in vivo; low acute toxicity; delayed liver damage |
| *Chaetomium cochliodes*... | Chaetomin (1943) | Gram-positive bacteria | Low toxicity; not active in vivo |
| | | Bacteria | |
| *Pseudomonas aeruginosa* | Pyocyanin | Gram-positive and Gram-negative bacteria; molds | Not active in vivo |
| *Bacillus brevis*..... | Tyrothricine | Gram-positive bacteria | Very toxic |
| *Bacillus brevis*..... | Gramicidin | Gram-positive bacteria | Local use only |
| *Bacillus brevis*..... | Tyrocidin | Gram-positive and Gram-negative bacteria; molds | Not active in vivo |
| *Bacillus subtilis*..... | Subtilin (1943) | Gram-positive bacteria, *Neisseria gonorrhoeae*, *Mycobacterium tuberculosis* | Very low toxicity; very active in vivo |
| *Bacillus brevis*..... | Gramicidin S (1944) | Gram-positive and Gram-negative bacteria | Local therapy |

TABEL 43.—(*Continued*)

Actinomyces

| | | | |
|---|---|---|---|
| *Actinomyces lavendulae* | Streptothricin (1942) | Gram-positive and Gram-negative bacteria; molds | Low acute toxicity; delayed lethal effect, cumulative |
| *Actinomyces griseus* | Streptomycin (1944) (Fig 171) | Gram-positive and Gram-negative bacteria, *Mycobacterium tuberculosis*, actinomycetes | Very low toxicity; active in vivo |

(1944), Burkholder and Evans (1945), Callow and Hart (1946), Cavallito, Bailey, Haskell, McCormick, and Warner (1945), de Beer and Sherwood (1945), Demerec (1945), Dutcher, Johnson, and Bruce (1944*a*,*b*), Foster

FIG. 174.—Streptomycin crystals. (*Courtesy Squibb Institute for Medical Research.*)

and Karow (1945), Geiger, Conn, and Waksman (1944), Gordon, Martin, and Synge (1943), Hobby (1944), Huddleson, Du Frain, Barrows, and Giefel (1944), Lawrence (1945*b*), Lee, Foley, and Epstein (1944), Lucas and Lewis (1944), Oxford (1942), Salle and Jann (1945, 1946*a*,*b*), Schatz and Waksman (1945), Waksman and Bugie (1944), Waksman, Reilly, and Schatz (1945), Youmans (1945), Youmans and McCarter (1945), and the monograph by Waksman (1945).

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# CHAPTER XIX

## BACTERIOLOGY OF AIR

Air is a mixture of gases composed approximately of 80 per cent nitrogen and 20 per cent oxygen. In addition, it contains about 0.04 per cent carbon dioxide and traces of other gases such as neon, argon, and helium. All samples of air contain some water in the form of vapor or mist. Almost any sample contains suspended matter consisting of dust, bacteria, yeasts, molds, pollen grains, etc. Unlike the gaseous content, the suspended matter is not uniform but shows considerable variation.

Air is not a natural environment for the growth and reproduction of microorganisms. It does not contain the necessary amount of moisture and kinds of nutrients in the form utilizable by bacteria and other microscopic organisms. Therefore, air does not possess a bacterial flora. Yet organisms are found in air, and their presence is of considerable importance economically and to public health.

**Bacteria in Air.**—Bacteria are introduced into the air by various forces. According to Chope and Smillie (1936), the principal source is from dust that contains dry vegetative cells and spores. These organisms are for the most part saprophytes, *i.e.*, forms that live on dead organic matter. They are of great importance to the canner, in the sugar refineries, in dairies, in the biological laboratories, etc. In short, they are the organisms responsible for contaminations from the air.

The species vary somewhat, depending upon the locality. However, certain forms are quite uniformly present. Molds and yeasts are quite commonly found in the air and in some localities even outnumber the bacteria. These organisms produce spores that are capable of resisting unfavorable conditions for long periods of time. The aerobic spore-forming bacilli from the soil are found quite frequently in the air. The best known member of this group is *Bacillus subtilis*. It is known as the hay bacillus and is probably the most common bacterial organism found in nature. Its natural habitat is in the soil and on vegetation. Since it is a spore-forming organism, it is very resistant to drying and other unfavorable environmental conditions. Sarcinae and micrococci are also found in air. The spherical, saprophytic, chromogenic organisms found in air usually belong to these two genera.

**Numbers of Bacteria in Air.**—The number of organisms present in air is dependent upon the activity in the environment and the amount of dust stirred up. An active environment shows a higher bacterial count

than a less active one. The numbers in dirty, untidy rooms are greater than in clean rooms. Also, the air of small, poorly ventilated rooms shows a higher count than that of larger rooms.

A rich, fertile, cultivated soil shows a higher viable count than a sandy, or clay, uncultivated soil. It follows that the air above the fertile, cultivated soil will contain more organisms than the air above the poor soil. Likewise, the air above a bare surface contains more organisms than the air above land covered with vegetation. This means that, where the earth is bare, the organisms can be blown more easily into the air, because the earth is not protected from air currents.

Wherever careful examinations have been made, microorganisms have been isolated from air over the ocean (ZoBell, 1942). However, marine air usually contains fewer organisms than that of continental or terrestrial origin. Because of differences in their salt requirements, it is possible to distinguish marine bacteria from terrestrial or fresh-water forms in 80 to 90 per cent of the cases. Most of the air-borne marine bacteria are Gram-negative, nonsporulating rods of the family *Bacteriaceae* with smaller numbers of *Pseudomonadaceae, Bacillaceae* and others. On the other hand, *Bacillaceae, Micrococcaceae,* and mold spores usually predominate in air of terrestrial origin.

Bacteria remain in air for varying periods of time, depending upon the speed of the air current, the size of particles on which they are attached, and the humidity of the air. Bacteria are slightly heavier than air and settle out slowly in a quiet atmosphere. A gentle air current is capable of keeping organisms in suspension almost indefinitely. This applies to organisms not attached to particles but existing in the free state. Bacteria attached to dust particles or in droplets of water settle out at a much faster rate.

A damp or humid atmosphere contains fewer organisms than a dry one owing to the fact that the organisms are carried down by the droplets of moisture. The air of a refrigerator is usually free from all organisms. Therefore, air during the dry summer months contains many more organisms than during the wet winter months. Gently expired air from the lungs is sterile. The moist passages of the upper respiratory tract remove the bacteria from the air. Cotton stoppers in pipettes are not necessary as far as contamination of the contents is concerned. They are inserted as a protection against aspirating infectious or other material into the mouth.

**Altitudes Attained by Microorganisms.**—Proctor (1935, 1938), in his studies on the microbiology of the upper air, found that organisms were able to attain considerable altitudes. To quote,

> The ability of these living microorganisms to attain altitudes of 20,000 ft. or more through the chance action of air currents is particularly significant as it

suggests the almost limitless possibilities of travel in a horizontal direction. The survival of such forms despite the many influences which are unfavorable to their existence is also significant in view of the length of time for which they may remain viable.

The presence of pollen at high altitudes also indicates the importance of air as a vehicle for the transmission of wind-borne pollens over wide areas.

The high dust counts obtained in comparison to the numbers of microorganisms is interesting in view of the various possible sources of dust, some of which, as from soil, might be also associated with high bacterial counts, while in other cases particles from smokestacks and industries might be sterile.

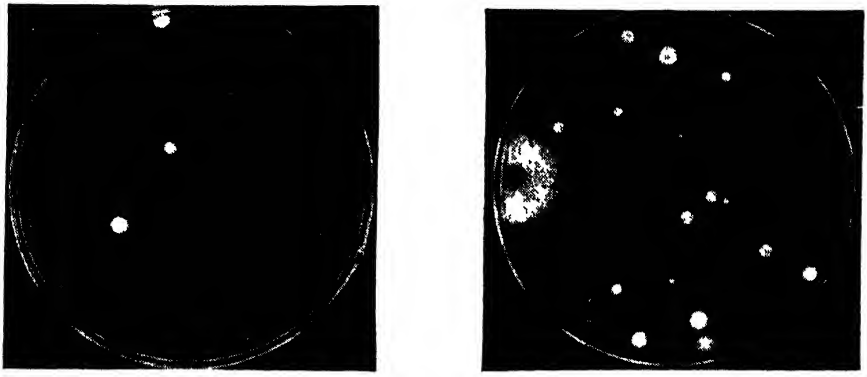

FIG. 175.—Bacteria in air. Left, colonies developing on an agar plate exposed to a quiet atmosphere (office) for 10 minutes; right, colonies developing on an agar plate exposed to an active atmosphere (laboratory) for the same period of time.

## METHODS EMPLOYED FOR ENUMERATION OF BACTERIA IN AIR

Koch (1881) employed solid media in plates and exposed them to air for varying periods of time. The plates were then incubated and the colonies counted.

This is one of the simplest procedures used for air examinations but is of no value from a quantitative standpoint. It does not indicate the number of organisms present in a known volume of air. However, the method does give relative results and is commonly employed for this purpose.

The results of plates exposed to different environments for the same period of time, then incubated at 37°C. for 24 hr., are shown in Fig. 175. It may be seen that the bacterial population increases as the activity of the atmosphere increases.

Petri (1888) filtered air through a tube containing sand that had been previously screened through a 100-mesh sieve. A definite volume of air was drawn through the tube to collect the organisms on the sand. Then the sand was shaken with sterile salt solution to suspend the bacteria, after which aliquot parts were pipetted into Petri dishes and mixed with

melted agar. Sedgwick (1888) recommended the use of sugar instead of sand. On being mixed with salt solution, the sugar dissolved, producing a suspension of bacteria without the presence of any insoluble sediment.

In 1909 the American Public Health Association officially adopted a modification of the sand method of Petri as the standard procedure for the bacterial analysis of air (Fig. 176). A layer of fine sand 1 cm. in thickness is supported within a glass tube, 70 mm. in length and 15 mm. in diameter, upon a perforated rubber stopper previously covered with a piece of bolting cloth. A tube 6 mm. in diameter and 40 mm. in length passes through the perforation in the stopper. This latter tube is attached to an aspira-

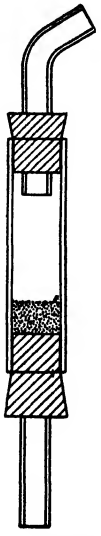

FIG. 176.—A modification of the Petri sand filter adopted by the American Public Health Association, 1909.

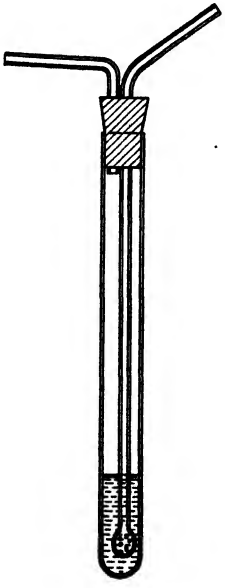

FIG. 177.—The Rettger aeroscope for collecting bacteria.

tor bottle. The upper end of the sand filter is closed with a cork stopper through which passes a tube of the same dimensions as the lower one. This tube is bent at an angle of 45° to prevent dust and bacteria from falling into the sand filter. After a known volume of air has been drawn through the filter, the sand is shaken into 10 cc. of sterile water, and aliquot portions plated out on nutrient agar.

Rettger (1910) attempted to simplify the method by drawing air through 5 cc. of salt solution (Fig. 177). The suspension was then mixed with an equal volume of double strength agar and plated. To quote,

The entire special apparatus consists of a glass tube with a small round bulb at one end. The bulb has eight or ten small perforations which serve the purpose

of allowing the air to pass through at a rapid rate and yet divide the gas to such an extent that every particle of it is brought into close contact with the filtering fluid. This glass tube or aeroscope is fitted into a small thick-walled test tube by means of a rubber stopper, which also bears besides the aeroscope a short glass tube bent at right angles. The upper end of the aeroscope is bent at an angle of about 45° in order to prevent bacteria and particles of dust from falling into the open end of the tube and still permit of the tube being drawn through the stopper without difficulty.

Ruehle (1915*a*,*b*) recommended a modification of the Petri sand method which was adopted in 1917 by the Committee on Standard Methods for

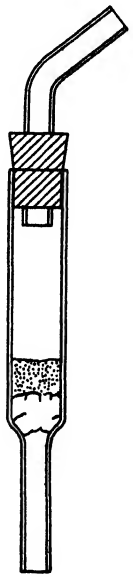

FIG. 178.—The Ruehle modification of the Petri sand filter adopted by the American Public Health Association, 1917.

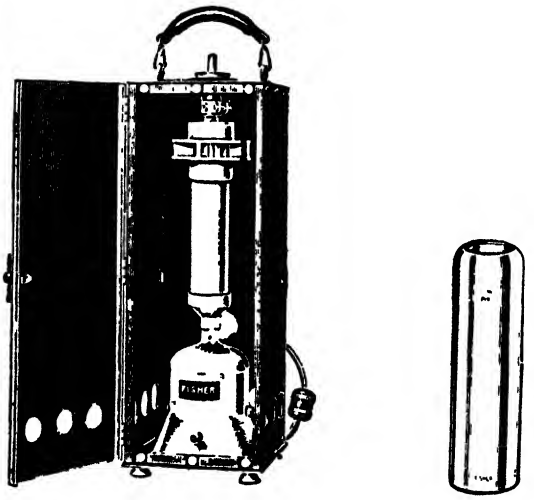


FIG. 179.—*A*, International Wells air centrifuge. *B*, air centrifuge tube. (*Courtesy Fisher Scientific Company.*)

the Examination of Air (Fig. 178). The modification differs from the standard tube in having the small lower tube fused into the large one, thus eliminating the rubber stopper and the bolting-cloth support. The layer of sand is supported by a plug of cotton resting on the shoulder at the junction of the small and large tubes.

Wells (1933) described an instrument for enumerating bacteria in air, utilizing the principles of the centrifuge for their separation (Fig. 179). The instrument operates in a manner similar to a milk clarifier, which separates heavier particles from a liquid. The rapid revolution of a glass cylinder about its vertical axis causes a current of air to enter through a

central tube and to escape along a thin layer of nutrient agar deposited on the walls of the cylinder. Bacteria and other microorganisms suspended in the air are deposited on the agar. After incubation has taken place, visible colonies appear where individual bacteria were precipitated and can be counted.

The author stated that four independent operations are combined in one compact instrument:

1. Air flow is created and regulated.
2. The amount of air is measured.
3. The bacteria are collected.
4. The bacteria grow and can be counted on the collection medium without separate plating.

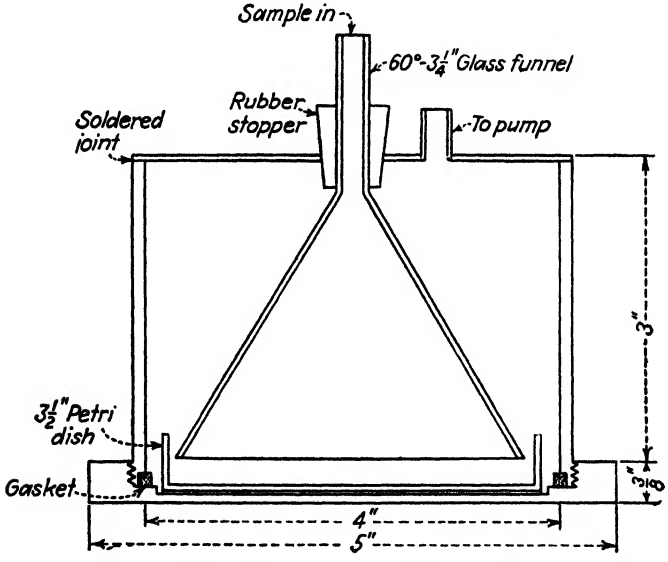


FIG. 180.—Funnel device used for sampling air-borne bacteria. (*After Hollaender and Dalla Valle.*)

The Wells centrifuge is simple to operate, quickly manipulated, and portable. All testing is carried out in one step. The apparatus has a wide range of application, since any type of solid medium may be used, depending upon the types of organisms to be cultivated.

Hollaender and Dalla Valle (1939) described a funnel device for sampling air-borne bacteria (Fig. 180). The sampling device consists of a brass container with a removable bottom. The container is fitted with an inverted 60° 3-in. glass funnel which sits approximately 1 cm. from the bottom of a standard-type Petri dish. The latter is placed in the lower portion of the container before use and is then screwed tightly against the

washer indicated in the figure. The inside of the funnel and the rim are swabbed with alcohol before use. The air sample passes through the funnel stem, and the air-borne organisms and dust are impinged upon the medium placed in the Petri dish. The air sample is drawn by means of an ordinary impinger pump in series with a flowmeter. A sampling rate of 1 cu. ft. per min. was found to be the most effective. Dalla Valle and Hollaender (1939) found their funnel device to be efficient and to compare favorably with the Wells centrifuge, giving slightly higher results when the bacterial population was low. The method is simple and portable, and all testing is carried out in one operation.

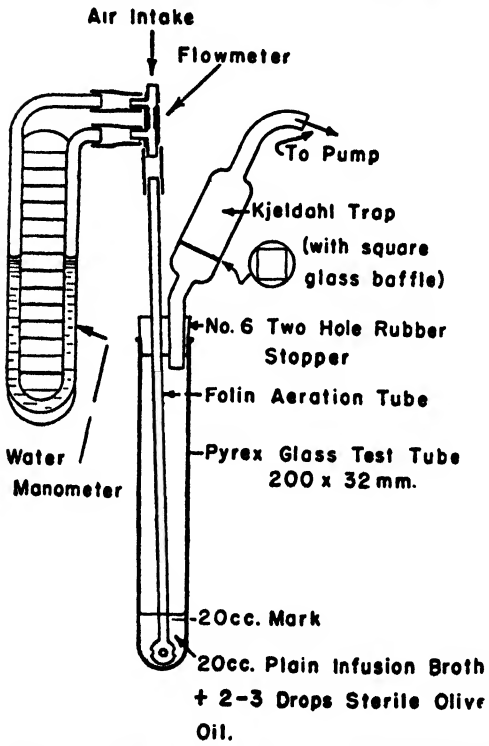


FIG. 181.—Device for the collection of air-borne bacteria. (*After Lemon.*)

Lemon (1943) has a simple device for the collection of organisms from a known volume of air (Fig. 181). The apparatus consists of a glass Folin aeration tube with a bulb at one end perforated by six holes. The tube is passed through a two-hole rubber stopper and the bulb centered near the bottom of the containing test tube. The Kjeldahl trap with square glass baffle is shortened at both ends for convenience, and a slight bend made in the intake, so that this may be inserted into the remaining hole of the stopper. A small flowmeter measures the rate of air flow entering the upper open end of the Folin tube. An air pump is attached to the exhaust end of the Kjeldahl trap.

The entire bubbler can be sterilized by autoclaving or by rinsing with 70 per cent alcohol and drying. Air drawn in at a rate of 25 to 30 liters per minute is dispersed through 20 cc. of broth containing two to three drops of olive oil to prevent foaming. Room-air samples generally require about 300 liters of air for satisfactory results.

For information on a comparative study of sampling devices for air-borne microorganisms, see du Buy, Hollaender, and Lackey (1945) and du Buy and Hollaender (1945).

## IMPORTANCE OF STATE OF SUSPENSION IN SANITARY AIR ANALYSIS

Organisms in air are seldom in a free state but usually attached to floating particles such as dust, saliva, or carbon. The state of suspension plays a very important role on the settling velocity of bacteria in air. It is of great importance to distinguish between ubiquitous saprophytic soil organisms raised as dust and those from body tissues introduced into the air during the processes of coughing, sneezing, talking, and singing. The former probably do not have any pathogenic significance; the latter do.

The state of suspension of bacteria introduced into the air under these conditions is different. Organisms in the free state are slightly heavier than air and settle out very slowly in a quiet atmosphere. A gentle current is capable of keeping them in suspension almost indefinitely. Dust particles laden with bacteria settle out rapidly and remain in a quiet atmosphere for a relatively short period of time. Droplets expelled into the air during coughing and sneezing do not necessarily fall immediately to the ground within a short distance from their source. As droplets decrease in size, the surface exposed to air resistance becomes relatively greater when compared to the weight or gravitational attraction toward the earth. This means that the droplets fall slower and slower as the size becomes smaller and smaller. This is in accordance with Stokes' law which states that the velocity is proportional to the surface area of the droplet or to the square of its diameter. Stokes' law may be expressed by the following equation:

$$V = \frac{2}{9} r^2 \frac{(s - l)g}{u}$$

where $V$ = velocity of settling
$r$ = radius of particle
$s$ = density of particle
$l$ = density of fluid
$g$ = acceleration of gravity
$u$ = viscosity of suspending fluid

If the physical constants of air are known, the equivalent diameter can be computed from the settling velocity. The number of particles of uniform size and distribution that settle on unit area in unit time will be equal to the product of the number per unit volume multiplied by falling velocity. The ratio of the number of particles settling on unit area divided by the number of particles per unit volume gives the settling velocity.

The rate of evaporation also depends upon surface area and becomes relatively more rapid as the size of the droplets becomes smaller. Some droplets are of such size that complete evaporation occurs in falling the height of a man. This droplet size has been estimated to be approximately 0.1 mm. in diameter. The residues of droplets of this size will float or

drift with the slightest air currents and become, in effect, a part of the atmosphere itself.

Air infections may then be said to occur by means of two types of droplets, depending upon their size. Droplet infection proper applies to droplets larger than 0.1 mm. in diameter, which rapidly settle out a short distance from their source before drying occurs. The other type of droplet may be called air-borne infection and applies to the dried residues of infected droplets (droplet nuclei) derived from droplets less than 0.1 mm. in diameter. The time they remain suspended in air depends upon the activity of the atmosphere. Droplet infection may become air-borne infection when large droplets evaporate in settling to the ground and then are lifted into the air as dust. It can be seen that droplet infection remains localized and concentrated whereas air-borne infection may be carried long distances and is dilute.

Jennison (1941, 1942), Jennison and Edgerton (1940), and Jennison and Turner (1941), by means of high-speed photography, showed that the distance to which the majority of respiratory droplets are actually expelled is not more than 2 or 3 ft., and often is less. However, droplets discharged during coughing and sneezing may move at a velocity as fast as 152 ft. per second. Such a velocity in dry air would result in nearly instantaneous evaporation, producing droplet nuclei. They showed also that the great majority of sneeze droplets, before appreciable evaporation occurred, measured 0.1 to 2 mm. in diameter.

For more information, see Wells, Winslow, and Robertson (1946).

### AIR-BORNE INFECTIONS AND THEIR CONTROL

Newer techniques developed during the past 15 years have caused an increased interest in the subject of air bacteriology. It was formerly supposed that air played an insignificant role in transferring infection from one person to another. This view is no longer tenable since it has been definitely established that air is capable of transmitting infections, especially those of the respiratory tract, and that infections transferred in this manner may be of frequent occurrence. This is especially true in closed spaces such as rooms, offices, theaters, and halls.

Wells (1935, 1938) in his researches on air-borne infections came to the following conclusions:

1. During coughing and sneezing, minute droplets containing microorganisms from infected surfaces may be ejected into the air.

2. Most of these droplets are sufficiently small to evaporate before they can settle to the ground, leaving suspended in the air minute residues.

3. These nuclei, in which the microorganisms remain viable for considerable periods, may drift in air currents like particles of cigarette smoke.

4. The air breathed commonly by the various persons congregated in a

room or other enclosed space can thereby transfer organisms from one person to another and plant them upon the susceptible tissues of the respiratory tract.

Pressman (1937) exposed evaporating dishes containing sterile saline solution for one week, in four tuberculosis sanitariums. The saline solution was centrifugated, the dust residue collected, digested, and stained or cultured. The results were overwhelmingly positive for the presence of the tubercle bacillus.

Wells and Lurie (1941) produced tuberculosis in rabbits by placing

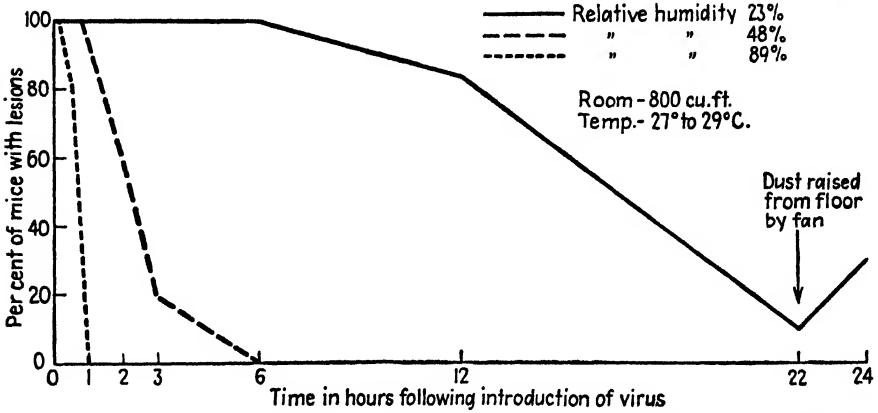


FIG. 182.—Persistence of influenza virus in air at different relative humidities. (*After Loosli, Lemon, Robertson, and Appel.*)

the animals in a chamber into which cultures of the organisms had been sprayed.

Wells and Henle (1941) and Loosli, Robertson, and Puck (1943) produced experimental influenza in mice by the air-borne route. The virus was atomized into a chamber containing the animals. An exposure period of only 2 min. to a highly diluted virus suspension resulted in death of all the animals. The virus was still capable of infecting mice 3 hr. after it had been sprayed into the chamber. In a later report, Loosli, Lemon, Robertson, and Appel (1943) found that room atmospheres of high humidity (80 to 90 per cent) into which virus suspension had been sprayed, were no longer infective after an hour. Atmospheres of 45 to 55 per cent relative humidity remained infective for 6 hr. and were lethal for mice up to 1 hr. and 10 min. At humidities of 17 to 24 per cent, similar amounts of virus continued to produce influenzal pneumonia in exposed mice for as long as 24 hr. After 12 hr. at this humidity, 40 per cent of the mice died and the remainder showed extensive pulmonary involvement. The results show that infections are more certain to occur in atmospheres of low rather than of high humidity (Fig. 182). In other words, the virus

particles remain infective for a longer period of time in a dry atmosphere than in a moist one. Similar results were reported by Edward, Elford, and Laidlaw (1943).

Buchbinder, Solowey, and Solotorovsky (1938*a*) found that *alpha* hemolytic streptococci were widely distributed in the air of enclosed places of congregation and of the open spaces of New York City. A large majority of these appeared to be of nasopharyngeal origin. The organisms were present most frequently and in greatest numbers in the air of school buildings. An apparent relationship was noted between the type of room and the degree of occupancy, and the numbers found. The largest counts were obtained from occupied assembly and classrooms. In another report (1938*b*), the same workers found that the causative agents of sore throats, scarlet fever, and certain respiratory diseases were relatively infrequent in air. They succeeded in isolating 52 strains, all but 5 from indoor locations.

Walter and Hucker (1942) found pathogenic β-streptococci to be relatively widespread in the floor sweepings of public places, particularly schools. Isolated streptococci were found to survive for more than 5 days when artificially inoculated into sterile, dry dust, and also to survive for more than 31 hr. when spread over the surface of floor boards and left at room temperature.

Hamburger, Green, and Hamburger (1945*a*,*b*) made nose and throat examinations of patients at two Army hospitals and reported that approximately two-thirds of those with streptococcal tonsillitis-pharyngitis or scarlet fever had positive nose cultures. In 95 per cent of the cases, β-hemolytic streptococci persisted longer in the throat than in the nose. Many more hemolytic streptococci were recovered from the air of wards housing patients with positive nose cultures than from wards where the patients' nose cultures were negative but throat cultures positive. Concomitant with the disappearance of organisms from the nose was a diminution of the numbers recovered from the environment. Carriers of β-hemolytic streptococci whose nose cultures were strongly positive represented a definitely more dangerous group than those in whom only the throat cultures were positive.

Other respiratory infections which have been shown to be transmitted by air include pneumonia (Commission on Acute Respiratory Diseases, 1945); hemolytic streptococcal infections (Wheeler and Jones, 1945); scarlet fever (Hodes, Schwentker, Chenoweth, and Peck, 1945); meningococcal meningitis (Phair and Schoenbach, 1945); mumps and chickenpox (Habel, 1945); and tuberculosis (Lurie, 1945).

**Dissemination of Organisms into Air During Laboratory Procedures.**—Infections have occurred among laboratory workers handling disease organisms, such as *Brucella abortus*, *B. melitensis*, and *Pasteurella tularensis*.

In most cases, the source of these infections has been obscure and has not been related to known accidents in handling cultures. Many laboratory techniques have been accepted with little consideration to the role they may play in the dissemination of organisms into the air.

Johansson and Ferris (1946), by means of high-speed photographic and

FIG. 183.—Blowing last drop from pipette. Pipette was allowed to drain, then last drop was blown out with moderate force. (*After Johansson and Ferris.*)

air-sampling techniques, showed that certain accepted bacteriological laboratory operations, such as pipetting, pouring, and vigorous agitation of dilution blanks, often produced bacterial contaminations of the surrounding air and environment (Fig. 183). Slightly more than one-half of the laboratory operations revealed droplet aerosols formed by blowing the last drop out of pipettes or removing the stoppers from dilution blanks that had been vigorously agitated. The dangers in handling some of the highly infectious agents with the commonly used laboratory equipment were apparent.

**Air Disinfection.**—Effective continuous disinfection of air of enclosed spaces has been practiced and found to be practicable. The methods

may be classed as physical (ultraviolet radiation) and chemical (germicidal vapors or aerosols).

*Effect of Hypochlorites.*—Edward and Lidwell (1943) showed that it was possible to obtain a 90 to 99 per cent or more destruction of suspended influenza virus in an atmosphere to which sufficient hypochlorous acid gas was added to produce a final concentration of 1:2,000,000. Similar results were obtained from the use of sodium hypochlorite. Masterman (1941) believed that the action of a hypochlorite depended upon the liberation of hypochlorous acid.

Challinor (1943) sprayed a 1 per cent solution of sodium hypochlorite in air and reported a marked reduction in the bacterial content. He suggested that the application of this simple, practical method of air disinfection of occupied places would reduce the spread of air-borne infections.

Elford and van den Ende (1945) reported that the effectiveness of hypochlorites and hypochlorous acid aerosols, in concentrations of 0.1 to 0.3 part per million HClO against air-borne organisms, depended upon the amount of moisture present. The degree of inactivation of streptococci and staphylococci sprayed as aerosols was found to be slight if the relative humidity fell below 50 per cent. If the relative humidity was maintained between 70 to 90 per cent, the organisms were very rapidly killed.

*Effect of Glycols.*—Robertson, Bigg, Puck, and Miller (1942) and Puck, Robertson, and Lemon (1943) atomized propylene glycol in air and reported that a concentration of 1 gm. of the vaporized compound in 2,000,000 to 4,000,000 cc. of air produced immediate and complete sterilization of air into which pneumococci, streptococci, staphylococci, *Hemophilus influenzae*, and other organisms as well as influenza virus had been sprayed. The killing process was found to be most effective at a temperature below 27°C. and an atmospheric relative humidity of 45 to 70 per cent.

Bigg (1943) believed glycols exert their bactericidal action by the chief physical property of glycol; namely, hygroscopicity. The glycol molecules dissolve in the film of moisture about each bacterial cell. When the glycol concentration becomes sufficiently great, moisture is drawn out of the cell, resulting in its death.

De Ome and the Personnel of U.S. Naval Laboratory Research Unit No. 1 (1944) found triethylene glycol to be approximately one hundred times more toxic to *Salmonella pullorum* than propylene glycol. The germicidal power of a given concentration of triethylene glycol vapor decreased as the temperature was raised from 28 to 37°C. and as the relative humidity deviated from approximately 45 per cent. At relative humidities of 15 and 80 per cent, triethylene glycol exhibited no appreciable germicidal power. Robertson, Puck, Lemon, and Loosli (1943) found triethylene glycol vapor to exert a pronounced toxic action against pneumococci, streptococci, and influenza virus.

Hamburger, Puck, and Robertson (1945), Puck, Hamburger, Robertson, and Hurst (1945), and Hamburger, Hurst, Robertson, and Puck (1945) determined the bactericidal effect of triethylene glycol vapor on air-borne hemolytic streptococci in hospital wards at relative humidities of 18 to 32 per cent and reported that the reduction in numbers was substantial, although less than when the relative humidity was maintained at 50 per cent. The concentration of triethylene glycol in air ranged from 0.0033 to 0.0084 mg. per liter of air.

The results indicate that glycol vapors are extremely toxic to bacteria and viruses and may be successfully used to control air-borne infections.

*Effect of Ultraviolet Irradiation.*—Ultraviolet rays of sunlight or of various types of lamps exert a pronounced destructive action on bacteria, viruses, and other microorganisms suspended in air. Radiant disinfection of air depends on type of infection, state of suspension, humidity of the atmosphere, volume of space, quality of the radiation, strength of ray, length of the ray, total exposure, uniformity of exposure, and air motion (Wells, 1942).

Low-pressure mercury-vapor lamps have been extensively used for this purpose. These lamps emit about 99 per cent of their lethal ultraviolet flux in a narrow spectral region near 2537 Å. (Luckiesh and Holladay, 1942*a*,*b*). However, the highly bactericidal range is between 2000 to 2950 Å. with a maximum at 2650 Å. (Hollaender and Oliphant, 1944).

Edward, Lush, and Bourdillon (1943) reported that at least 99 per cent and probably more of influenza and vaccinia viruses sprayed in air were killed in a few seconds by exposure to an ultraviolet lamp. Such lamps are particularly valuable in preventing the spread of virus infections of the upper respiratory tract. Others who emphasized the importance of ultraviolet rays include Hollaender (1943), Wells (1943*a*, 1944) and Hollaender, du Buy, Ingraham, and Wheeler (1944).

For more information, consult the reports of Challinor and Duguid (1944), Duguid and Challinor (1944), Giese (1945), Hollaender (1944), Lemon, Wise, and Hamburger (1944), Lurie (1947), Mudd (1945), Perkins (1945, 1947), Personnel of Naval Laboratory Research Unit No. 1 (1943), Robertson (1943, 1947), Wells (1941, 1943*b*, 1945), Wells and Wells (1943), and Wells and Zappasodi (1942).

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# CHAPTER XX

## BACTERIOLOGY OF WATER

Water receives its bacterial flora from air, soil, sewage, organic wastes, dead plants and animals, etc. This means that almost any organism may be found in water. Most of the bacteria soon die; a few are able to adapt themselves to the new environment. These organisms constitute the natural flora of water.

The great majority of the bacteria found in nature live on dead or decaying organic matter. They are called saprophytes (*sapro*, rotten, and *phyte*, plant) and belong to the so-called "metatrophic" group of organisms. Saprophytes are particularly rich in humus, a brown or black material formed by the partial decomposition of vegetable or animal matter.

Natural waters are commonly grouped into four well-marked classes: (1) atmospheric waters, (2) surface waters, (3) stored waters, and (4) ground waters.

**Atmospheric Waters.**—Rain and snow are included under the atmospheric waters. Sometimes these may contain considerable numbers of bacteria. After a snow or heavy rain, the atmosphere is washed nearly free of organisms so that many sterile plates, each inoculated with 1 cc. of water, may be obtained.

**Surface Waters.**—As soon as the raindrops and snowflakes touch the earth, they become quickly contaminated by the microorganisms in the soil. These are then known as surface waters. The extent of the contamination is dependent upon the numbers of organisms in the soil and, also, upon the kinds and quantities of food materials dissolved out of the soil by the water. The bacterial counts of surface waters are apt to show great variations. This is particularly true in the fall and spring, the seasons of heavy rains and melting snows. The washoff from the soil may upset the existing equilibrium in the surface water, resulting in considerable variation in the flora and bacterial count.

The first result of a mild rain is greatly to increase the bacterial contamination of a body of water. A prolonged rain exerts an opposite effect, owing to the fact that, after the main impurities have been removed from the upper layers of the soil, the subsequent rainfall acts merely as a diluent of the body of water. Rivers usually show their highest count during the rainy period.

**Stored Waters.**—The effect of storage is greatly to decrease the numbers of organisms in water. The forces that tend to produce bacterial self-purification now come into play. These are sedimentation, activities of other organisms, ultraviolet light, temperature, food supply, and perhaps osmotic effects.

Bacteria have a specific gravity slightly greater than that of distilled water, which means that they will slowly settle in a still body of water. However, the greatest factor responsible for the sedimentation of bacteria is their attachment to suspended particles. The suspended particles in settling mechanically remove the organisms from the upper layers of the water.

Protozoa present in waters play an important role in decreasing the number of bacteria. These microscopic animals easily engulf large quantities of dead or living bacteria. The protozoa remain alive, provided the water is well aerated. In the absence of sufficient dissolved oxygen, the protozoa gradually disappear.

Direct sunlight is very toxic to vegetative bacterial cells and even to spores if the action is sufficiently prolonged. Diffuse light is less effective as a sterilizing agent. In a water supply, the toxicity of ultraviolet rays is inversely proportional to its turbidity. This means that the light rays are practically without action in a turbid water. In a clear water, however, ultraviolet rays may be effective for a depth of from 1 to 2 meters.

Increasing temperatures exert a harmful effect upon the survival of some organisms in water, especially those producing disease. On the other hand, multiplication of certain soil and intestinal forms may actually occur when the temperature of the water is increased. Rector and Daube (1917), Savage and Wood (1917), Winslow and Cohen (1918), and Winslow and Falk (1923) all reported slight increases in the numbers of *E. coli* in various kinds of stored waters. More recently, Bigger (1937) showed that *E. coli* was capable of multiplying in water from various sources that had been autoclaved previous to inoculation, and then incubated at 37°C. In some instances, the numbers of organisms present were more than 10,000 times the original count. Cultures in autoclaved water kept at 22°C. showed higher counts and a greatly prolonged period of positive cultures as compared with those kept at 37°C. Raw waters also showed an increase in bacterial numbers when stored at 22 and 37°C., but not so great as when autoclaved water was used.

Increasing the food supply usually results in increasing the numbers of bacteria. On the other hand, certain toxic substances such as acids and bases produce marked reductions in the numbers of viable organisms. Various dissolved gases such as carbon dioxide and hydrogen also show a toxic effect. Environmental factors generally produce marked fluctuations in the bacterial counts. Apparently this is not due to any one factor but to a group of factors acting as a whole.

Obviously, all the factors that operate to decrease the numbers of bacteria in water will be more effective with an increase in time. This may be represented mathematically as follows:

$$\log \frac{N_1}{N_2} = kt$$

where $N_1$ = number of organisms at the beginning
$N_2$ = number of organisms at the end
$t$ = time
$k$ = a constant that varies with temperature and other environmental factors

**Ground Waters.**—Ground waters are, in general, relatively free from bacteria because of the filtering action of the earth through which the waters have penetrated. This filtering action removes not only most of the bacteria but also any suspended organic food particles. Deep wells contain usually fewer organisms than water from shallow wells, owing to the deeper layers of filtering material.

## QUANTITATIVE BACTERIOLOGICAL EXAMINATION OF WATER

The usual methods employed for the quantitative bacteriological examination of water give only a fraction of the total count. The nitrifying and strict autotrophic bacteria are cultivated on synthetic media composed of inorganic salts. They are unable to grow on the usual nonsynthetic culture mediums. The obligate anaerobic bacteria fail to multiply in the presence of air. Certain parasitic bacteria do not grow in the absence of rich animal fluids. Sulfate-reducing bacteria require the presence of sulfates for growth to occur. Cellulose-dissolving bacteria either do not grow or grow very poorly unless cellulose is present in the medium. The result is that most of the bacteria found in water escape detection. From twenty to seventy times more organisms have been enumerated by the direct or microscopic method as compared to the usual agar-plate procedure.

No great error is introduced by failure to obtain the total bacterial count of water. The sanitary bacteriologist is not interested in such organisms as the anaerobic spore formers, the nitrifying bacteria, the autotrophic forms, and the pathogenic organisms, but in a group of rapidly growing, rich food-loving bacteria found in sewage. Most of these organisms are members of the *Escherichia*, *Aerobacter*, and "intermediates" divisions of the colon group.

**Method.**—The method for the quantitative macroscopic bacterial count consists in placing a measured amount of the water sample in a Petri dish and mixing with sterile, melted agar. After the agar has solidified, the plate is incubated at a temperature of 37°C. for 24 hr. The

colonies are counted, and the count is expressed as the number of colonies per cubic centimeter of water that develop on the plate.

The sample of water is collected in a clean and sterile 100-cc. glass-stoppered or screw-cap bottle. The neck and top of the bottle are covered with a parchment paper cap and tied in place with a piece of string. The bottle is then sterilized in a hot-air sterilizer at a temperature of 170°C. for at least 1 hr. The purpose of the covering is to keep the neck and stopper of the bottle free from contaminating microorganisms.

In order to obtain a representative sample from a tap, the water should be allowed to run for at least 5 min. to remove any contaminating organisms present around the opening of the faucet. Also, changes in bacterial content are liable to occur in small pipes; some species tend to die, others to multiply. The bottle is grasped with the right hand. The stopper is removed with the left hand, holding it by the paper covering. After the sample is collected, the stopper with covering is carefully replaced to avoid contamination, and the paper tied in place with string. The fingers must not touch the inside of the neck or stopper, otherwise contamination of the contents may occur and lead to an erroneous result.

In sampling a still body of water, the cap is first removed with the left hand. The bottle is plunged mouth downward to a depth of about 1 ft., then inverted. When filled, it is removed and stoppered. If any current exists, the mouth of the bottle should be directed against it in order to avoid the introduction of bacteria from the fingers.

After a sample of water has been collected and stored, a rapid change in the bacterial content takes place. The numbers of organisms usually show marked increases. In some cases, the increase in numbers is gradual; in others, it is very rapid. The increase in numbers is due to a multiplication of the typical water bacilli. Disease and other organisms, whose natural habitat is the intestinal tract of man and animals, tend to die very rapidly.

An increase in bacterial numbers is greatly accelerated by an increase in temperature. Because of the rapid bacterial changes that may take place in bottled waters, even when stored at temperatures as low as 10°C., all samples should be examined as quickly as possible. "Standard Methods for the Examination of Water and Sewage" (1946), published by the American Public Health Association, recommends that the time allowed for storage or transportation of water samples and the beginning of the analysis should not be more than 6 hr. for impure waters and not more than 12 hr. for relatively pure waters. The samples during the period of storage should be kept at a temperature between 6 and 10°C.

The number of bacteria present in a sample should not exceed 300 per cubic centimeter. If the number is greater than this, dilutions should be prepared. Also, if more than 300 organisms per cubic centimeter are

present, many of them will fail to develop, owing to the inhibitory action of the waste products secreted by those organisms developing first.

Various factors influencing the numbers of colonies developing on agar plates include the composition and reaction of the medium, temperature and period of incubation, presence of an abundant supply of oxygen and moisture. Unless such factors are controlled, variable counts will be obtained.

## WATER-BORNE DISEASE

The most important bacterial diseases transmitted by water are dysentery, cholera, and typhoid. In this country, the most important of the three is typhoid. Since they are intestinal diseases, the causative agents are found in the intestinal contents. Therefore, the presence of sewage in a water supply means that one or more of these disease organisms might be present and that the water is potentially dangerous for human consumption.

Theoretically, it would be better to examine a water supply for the presence of disease organisms to determine its potability from a bacteriological standpoint. However, several difficulties enter into such a procedure. Chief among these are (1) the length of time that disease organisms remain viable in water and (2) the number encountered in a water supply.

**Disease Organisms Die very Rapidly in Water.**—It is doubtful if organisms causing the above diseases are able to survive beyond 1 or 2 weeks. Most of the organisms probably die in a few days. They may remain alive longer in water containing considerable organic matter, and in water that is cool. Infections caused by the presence of the intestinal disease organisms in water usually appear within a few days of each other, indicating that the bacteria were ingested at about the same time.

The numbers of disease organisms encountered in a water supply are usually exceedingly small. If only one person in a community is suffering from typhoid fever and the discharges from that individual are mixed with the sewage from all individuals, the high dilution would make it practically impossible to isolate the disease organisms from a convenient sample of water. If, on the other hand, many persons are suffering from typhoid fever, the concentration of bacilli in sewage might be high enough to make isolations relatively easy; but special culture media would be required for this purpose. For these reasons, attempts to isolate disease organisms directly from water supplies are rarely practiced.

**Salmonella typhosa-coliform Ratio.**—Kehr and Butterfield (1943) concluded, from the findings of others, that it is unlikely that a single individual would imbibe more than one typhoid organism, or at most only a few. They advanced the theory that a single typhoid organism is infective to a small percentage of the general population. They assumed that a drinking water showed a *Salmonella typhosa*-coliform ratio of 10 per

million which corresponded roughly to that found in the Thames and in London sewage. If the drinking water contained 500 coliform bacteria per 100 cc., then the chance of an individual obtaining one *S. typhosa* in a daily portion of 1 liter of water would be about 1 chance in 20. Two *S. typhosa* cells from a liter of water would presumably be imbibed by 1 in 400 persons and three by 1 in 8000 according to the laws of probability and assuming uniform distribution of bacteria. The presence of 500 coliform organisms per 100 cc. of drinking water, in the absence of any knowledge concerning the concentration of *S. typhosa*, would probably give rise to outbreaks of gastroenteritis followed by a few cases of typhoid fever in the majority of instances.

These studies of Kehr and Butterfield of the available data in the literature, emphasize the basic value of the coliform test as an indicator of the possible presence of pathogens, and indicate that a very real danger may exist when coliform bacteria, in even moderately high concentrations, are present.

**Presence of Escherichia coli in Sewage.**—*E. coli* was first isolated by Escherich (1885) from the feces of an infant. It was shown later to be a normal inhabitant of the intestinal tracts of man and animals. Since it is present in the discharges from all individuals with rare exceptions (Parr, 1938), tests for the potability of a water supply are generally based on the presence or absence of this organism. Its presence in water does not mean necessarily that disease organisms are present but that they might be present. In other words, all sewage-polluted waters are potentially dangerous.

The procedures employed for the bacteriological examination of water do not give necessarily positive tests for *E. coli* alone but for other closely related organisms that possess little or no sanitary significance. These closely related organisms are generally placed with *E. coli* under the "colon group." The two most important members of the group are *E. coli* and *Aerobacter aerogenes* (Figs. 184, 185). The colon group includes all aerobic and facultative anaerobic short, Gram-negative, nonspore forming rods, producing acid and gas from lactose and other carbohydrates. The division of the colon group into a number of subgroups is discussed on page 488.

### PRESUMPTIVE TEST

The first step in water examinations is known as the presumptive test, which consists in placing graduated amounts of water in a series of lactose fermentation tubes, each containing at least twice as much medium as water. It is the usual practice to employ five fermentation tubes each containing 10 cc. of water, one tube containing 1 cc. of water, and another with 0.1 cc. of water. The tubes are incubated at 37°C. for 48 hr.

The formation of 10 per cent or more of gas in 24 hr. at 37°C. constitutes a positive presumptive test (Fig. 186). It is presumptive evidence

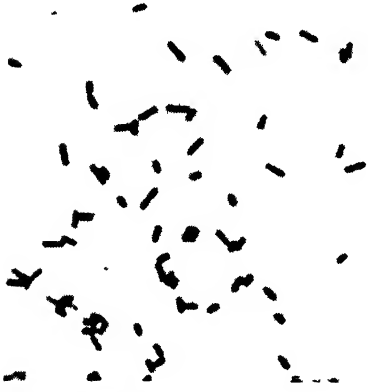


FIG. 184.—*Escherichia coli*, stained with 1:1000 crystal violet.

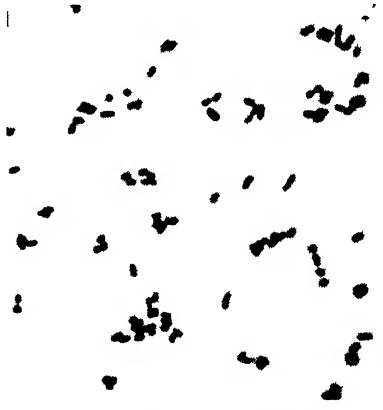


FIG. 185.—*Aerobacter aerogenes*, stained with 1:1000 crystal violet.

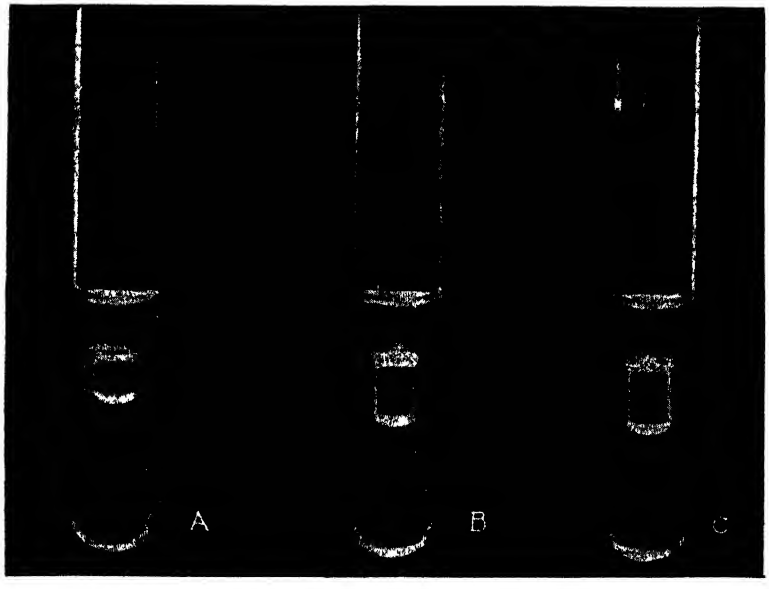



FIG. 186.—Lactose broth fermentation tubes. *A*, 1 cc. water sample; *B*, 0.5-cc. water sample; *C*, 0.1-cc. water sample. All tubes show fermentation of the lactose with the production of acid and gas.

for the presence of members of the colon group. The presence of less than 10 per cent of gas in 48 hr. constitutes a doubtful presumptive test and requires further examination. The absence of gas formation after an

incubation period of 48 hr. constitutes a negative presumptive test and no further tests need be performed. The water may be considered satisfactory from a bacteriological standpoint.

**False Positive Presumptive Tests.**—A positive presumptive test does not necessarily mean that members of the colon group are present. In most cases it is true, but there are exceptions. False positive presumptive tests are caused by (1) the presence of other organisms capable of fermenting lactose with the production of acid and gas, and (2) bacterial associations or synergism.

A number of other organisms have been encountered in water capable of fermenting lactose with the production of acid and gas. These include *Clostridium perfringens*, *Bacillus aerosporus*, some members of the genus *Klebsiella*, Houston's leather bacillus, several species of *Erwinia*, and some species of *Serratia*.

Positive presumptive tests are frequently caused by a type of bacterial association known as synergism. Bacterial synergism may be defined as the joint action of two organisms on a carbohydrate, resulting in the production of gas that is not formed by either organism when grown separately. This is discussed in greater detail on page 443.

**Elimination of False Presumptive Tests.**—Probably the most important procedure employed for the elimination of false positive presumptive tests is to incorporate a very small amount of a suitable triphenylmethane dye in the lactose broth medium. In most cases synergism is caused by a Gram-positive and a Gram-negative organism growing together. A concentration of dye just sufficient to prevent the growth of Gram-positive organisms will have no effect on the growth of the Gram-negative bacteria. This will result in the elimination of a synergistic reaction. False positive presumptive tests caused by the presence of gas-forming, Gram-positive aerobes and anaerobes will also be eliminated by this procedure.

## CONFIRMED TEST

The lactose broth fermentation tubes showing a positive or doubtful presumptive test are utilized in the following procedures: It is customary to employ the tube showing at least 10 per cent of gas from the smallest amount of water tested. For example, if all the tubes show the presence of acid and at least 10 per cent of gas, the test is confirmed only from the tube containing 0.1 cc. of water.

A loopful of the culture is streaked over the surface of an Endo or an eosin methylene blue (E.M.B.) agar plate. The plate is incubated at 37°C. and examined at the end of 24 or 48 hr., depending upon the reaction obtained.

If typical colonies appear on the plate, the test is considered positive. If no typical colonies appear in 24 hr., the plate should be reincubated for

another 24 hr. The absence of typical colonies at the end of 48 hr does not mean necessarily that the test is negative for members of the colon group, since some strains form atypical colonies. Regardless of whether

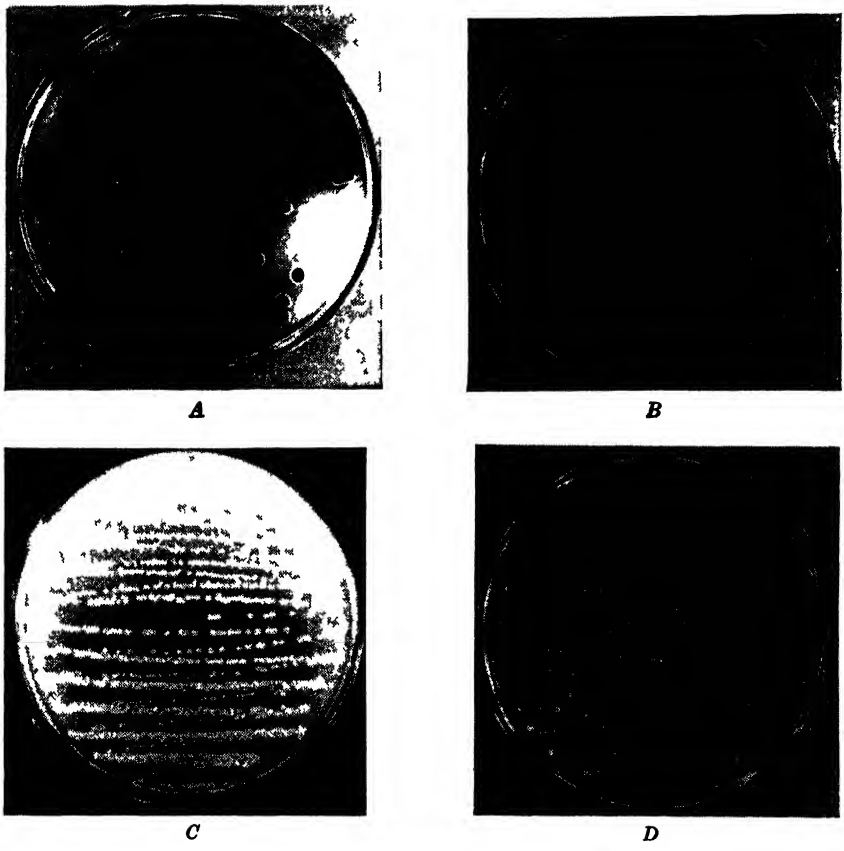



FIG 187.—*Escherichia coli*, streaked on *A*, Endo agar, *B*, eosin methylene blue agar. The colonies have dark centers and a metallic sheen *Aerobacter aerogenes*, streaked on *C*, Endo agar, *D*, eosin methylene blue agar The colonies do not have dark centers or a metallic sheen

typical or atypical colonies appear, it is necessary to continue with the test as described under Completed Test, page 483.

The presence of typical colonies on Endo or E.M.B. agar plates after 24 hr. constitutes a positive, confirmed test (Fig. 187). If no typical colonies appear at the end of 24 or 48 hr., the plates are retained for the completed test.

**Eosin Methylene Blue Agar Medium.**—This medium is prepared by adding definite quantities of the two dyes eosin and methylene blue to melted lactose agar and pouring about 15 cc. into each Petri dish.

When typical strains of *E. coli* are streaked over the surface of this medium and the plates incubated at 37°C. for 24 hr., the colonies show dark centers and possess a characteristic greenish metallic sheen. On the other hand, typical colonies of *Aerobacter aerogenes* show brown centers and rarely possess a metallic sheen. Their colonial characteristics, according to Levine (1918, 1921), are given in Table 44.

TABLE 44

| | *E. coli* (1) | *A. aerogenes* (2) |
|---|---|---|
| Size | Well-isolated colonies are 2 to 3 mm. in diameter | Well-isolated colonies are larger than *E. coli;* usually 4 to 6 mm. or more in diameter |
| Confluence | Neighboring colonies show little tendency to run together | Neighboring colonies run together quickly |
| Elevation | Colonies slightly raised; surface flat or slightly concave, rarely convex | Colonies considerably raised and markedly convex; occasionally the center drops precipitately |
| Appearance by transmitted light. | Dark almost black centers, which extend more than three-fourths across the diameter of the colony; internal structure of central dark portion difficult to discern | Centers deep brown; not as dark as *E. coli*, and smaller in proportion to the rest of the colony. Striated internal structure often observed in young colonies |
| Appearance by reflected light. | Colonies dark, button-like, often concentrically ringed with a greenish metallic sheen | Much lighter than *E. coli*, metallic sheen not observed except occasionally in depressed center when such is present |

1. Two other types of *E. coli* colonies have been occasionally encountered. One resembles the type described except that there is no metallic sheen, the colonies being wine-colored. The other type of colony is somewhat larger (4 mm.), grows effusely, and has a marked crenated or irregular edge, the central portion showing very distinct metallic sheen. These two varieties constitute about 2 or 3 per cent of the colonies observed.

2. A small type of *A. aerogenes* colony, about the size of the *E. coli* colony, which shows no tendency to coalesce has been occasionally encountered.

Wynne, Rode, and Hayward (1942) made a study of the mechanism of the selective action of eosin methylene blue agar medium. They found that the color of colon forms on this medium depended upon two factors: (1) the reaction of eosin (an acid dye) with methylene blue (a basic dye) to form a dye compound of either acidic or neutral nature and (2) the production, by lactose-fermenting colonies, of a sufficiently low pH so that this dye compound was taken up by individual cells of a colony. Non-lactose-fermenting organisms were not colored because the compound was not taken up in alkaline solution.

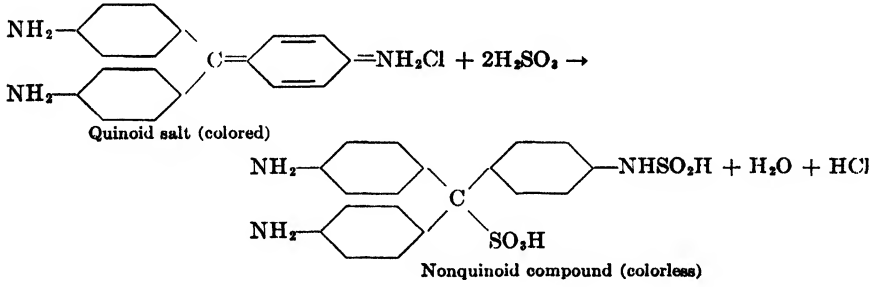
The medium is relatively stable. Prepared plates have been kept in the refrigerator for a week or longer before use and found satisfactory. The plates should not be exposed unnecessarily to the light, otherwise toxic substances might be formed in the medium (see page 161).

**Endo Agar Medium.**—This medium is prepared by adding basic fuchsin, previously decolorized with sodium sulfite, to melted lactose agar and pouring about 15 cc. into each Petri dish.

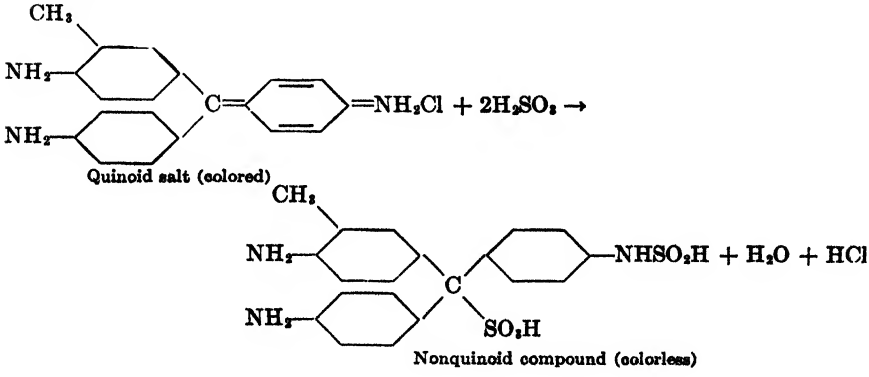
When typical *E. coli* is streaked over the surface of this medium, the red color of the dye is restored and metallic gold-like colonies appear. If the fuchsin-sulfite solution is added after 24, 48, and 72 hr. of incubation, no reaction takes place. The substance responsible for the reaction is detected only when the bacteria are grown in the presence of the sulfite. It is believed that Endo agar acts as a trapping agent for acetaldehyde, which is the compound responsible for the characteristic reaction. Acetaldehyde is an intermediate compound in the fermentation of lactose.

The basic fuchsin of commerce consists largely of a mixture of pararosaniline and rosaniline hydrochlorides. On the addition of a sulfite, the compounds are decolorized. According to Margolena and Hansen (1933), the dyes react with sulfurous acid as follows:

Pararosaniline hydrochloride:



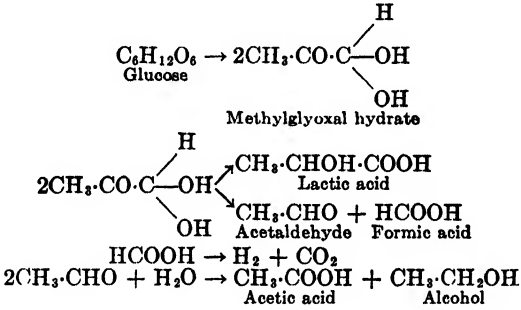

Rosaniline hydrochloride:



When an aldehyde, such as formaldehyde, is added to the decolorized compound, it reacts with the sulfite or sulfurous acid to form an addition compound. The dye is then released from the combination, resulting in the restoration of the red color. Margolena and Hansen showed that the restored dye was different chemically from the original compound, being more purplish in color. The process appears to be essentially more complicated than just a removal of the sulfite and a liberation of the basic fuchsin dye.

The results obtained by Neuberg and Nord (1919), in their studies on the fermentation of glucose by *E. coli*, may be offered as additional evidence in support of the aldehyde hypothesis. They found that acetaldehyde accumulated without question when sodium sulfite was added and that none was found when the sulfite was omitted.

Their reactions are as follows:




In summary, it may be stated that the restoration of the red color is due to the production of acetaldehyde, which is capable of forming an addition product with sulfites. The metallic gold-like sheen imparted to the colonies is due to the precipitation of the liberated dye by the organic acids (such as lactic acid). The restored dye is not the same as the original fuchsin but possesses a more purplish color.

**Liquid Confirmatory Media.**—One or more of several liquid media may be employed for the confirmed test in place of the Endo or E.M.B. agar plates. These are brilliant green lactose bile broth; crystal violet broth; fuchsin lactose broth; and formate ricinoleate broth. The liquid media are all equivalent for the confirmed test but, since waters vary considerably in their microflora, one medium may give results superior to the others. Therefore, a selection of any one of them should be based upon the correlation of the confirmed tests thus obtained with a series of completed tests.

All the media are liquid, contain lactose, and are dispensed in test tubes with inverted vials. Their composition is such that they are sup-

posed either to eliminate entirely or reduce to a minimum the growth of organisms not members of the colon group.

The test is made by transferring a loopful of culture from the lactose broth fermentation tube showing a positive, or doubtful, presumptive test, to a tube of one of the liquid confirmatory media. The formation and presence of gas in any amount within 48 hr. at 37°C. constitute a positive, confirmed test.

The Committee on Standard Methods conducted an exhaustive study in an attempt to determine the comparative, practical utility of these four liquid confirmatory media when employed in routine water analysis. The results of the tests were reported by McCrady (1937). Twenty-one laboratories situated in various parts of the United States and Canada collaborated in the study. The results obtained indicated that brilliant green lactose bile broth was the most generally satisfactory medium of those tested. Also, the results compared very favorably with those obtained from the standard methods completed test.

### COMPLETED TEST

The purpose of the completed test is to determine (1) if the colonies appearing on an Endo or an E.M.B. agar plate are again capable of fermenting lactose with the production of acid and gas and (2) if the organisms transferred to an agar slant show the morphological and tinctorial picture of members of the colon group.

At least one typical colony or, if no typical colonies are present, at least two atypical colonies considered likely to be members of the colon group are each transferred to a lactose fermentation tube and to an agar slant. The fermentation tube is incubated at 37°C. and examined at the end of 24 and 48 hr. The agar slant is incubated at 37°C. for 24 hr. and then examined microscopically by the Gram technique. The formation of gas in any amount in the fermentation tube within 48 hr. and the presence of Gram-negative, nonspore-forming rods on the agar slant shall be considered a positive, completed test for members of the colon group. The absence of gas or failure to show the presence of rods answering to the above description in a gas-forming culture shall constitute a negative, completed test.

The above procedures make no distinction between so-called fecal and nonfecal types. The American Public Health Association feels that any attempt to evaluate a drinking water on the basis of a distinction between the above two types is unwarranted. However, the procedures that follow make an attempt to distinguish between fecal *E. coli* and nonfecal *A. aerogenes*. The tests are employed in many laboratories but are not to be regarded as official in any sense of the word.

## DIFFERENTIATION OF FECAL FROM NONFECAL MEMBERS OF THE COLON GROUP

A strong correlation exists between the normal habitat of the *Escherichia* and *Aerobacter* divisions of the colon group and their biochemical activities. *Escherichia* are typical intestinal parasites of man and animals, whereas *Aerogenes* are only occasionally present in the intestinal contents but are consistently present in soil and on grains. Considering the normal habitat of *Aerobacter*, it is not surprising that these organisms are occasionally present in feces for relatively short periods of time, after which they cannot be isolated from the individual. Because of their occasional occurrence in feces, the terms "fecal" and "nonfecal" *Aerobacter* were coined, on the assumption that certain strains were intestinal parasites. No such parasitic strains have ever been reported; consequently, all attempts to distinguish fecal from nonfecal strains of *Aerobacter* have failed (Stuart, 1941).

When members of the colon group are inoculated into lactose broth fermentation tubes, some cultures produce more gas than others. A differentiation of the organisms reveals the fact that typical strains of *E. coli* rarely produce more than 25 per cent of gas in the inverted vial, whereas typical strains of *A. aerogenes* produce from 75 to 100 per cent of gas. This indicates that there are distinct differences in the carbohydrate metabolism of the two subgroups.

Rogers, Clark, and Davis (1914), Rogers, Clark, and Evans (1914, 1915), and Rogers, Clark, and Lubs (1918) demonstrated that typical strains of *E. coli* produce carbon dioxide and hydrogen in approximately equal amounts and that typical members of the *A. aerogenes* division produce about twice as much carbon dioxide as hydrogen. The former are known as the low-ratio organisms ($CO_2/H_2 = 1$) and the latter as the high-ratio fermenters ($CO_2/H_2 = 2$).

The high-ratio organisms (*A. aerogenes*) are only occasionally found in the intestinal contents of man and animals (about 6 per cent). They are normally present in the soil and on grains. For this reason, very little sanitary significance is attached to their presence in a water supply. On the other hand, the low-ratio organisms (*E. coli*) are rarely found on grains and in the soil but constitute one of the predominating organisms found in the intestinal contents of man and animals. They are only occasionally found in localities not showing recent fecal pollutions.

It is generally stated that members of the *A. aerogenes* division of the colon group are considerably more viable in a water supply than are members of the *E. coli* division. It is true that there is some difference, but this is not so great as was formerly supposed. Parr (1937) inoculated typical *E. coli* into sterile tap water containing bits of string and found

that the organisms were still viable after 2 years, 4 months, and 4 days. Several other experiments gave essentially the same type of results. He concluded,

*Escherichia coli* and *Aerobacter aerogenes* remain viable in a variety of environments for long periods of time. The difference in viability of coli and aerogenes when studied outside the body is not as great as has been supposed. When both organisms coexist in an environment, certainly in feces, aerogenes survives longest.

**Voges-Proskauer Test.**—This reaction was first observed by Voges and Proskauer in connection with their studies on the organisms of the hemorrhagic septicemia group. They found that the addition of potassium hydroxide to a culture of the organisms under observation resulted in the development of a pink color, if the tube was allowed to stand at room temperature for 24 hr. or longer.

The chemistry of the reaction was worked out by Harden and Walpole (1906), Harden (1906), and Harden and Norris (1912*a*,*b*). They found that distinct differences existed in the carbohydrate metabolism of typical *E. coli* and *A. aerogenes*. The fermentation of glucose by the two organisms yielded the products shown in Table 45.

TABLE 45

| Product | Per cent by weight of glucose fermented | |
|---|---|---|
| | *A. aerogenes* | *E. coli* |
| Alcohol | 17.10 | 12.85 |
| Acetic acid | 5.10 | 18.84 |
| Succinic acid | 2.40 | 5.20 |
| Formic acid | 1.00 | 0.00 |
| Lactic acid | 5.50 | 31.90 |
| Carbon dioxide | 38.00 | 18.10 |
| Total | 69.10 | 86.89 |
| Ratio, $CO_2/H_2$ | 2.40 | 0.83 |

The figures show that 87 per cent of the carbon is accounted for in the case of *E. coli*, but only 69 per cent in cultures of *A. aerogenes*. Harden and Walpole found that the discrepancy was due to the formation by *A. aerogenes* of 2:3-butylene glycol ($CH_3{\cdot}CHOH{\cdot}CHOH{\cdot}CH_3$) and acetylmethylcarbinol ($CH_3{\cdot}CO{\cdot}CHOH{\cdot}CH_3$) but not by *E. coli*. The acetylmethylcarbinol in the presence of potassium hydroxide and air is further oxidized to diacetyl ($CH_3{\cdot}CO{\cdot}OC{\cdot}CH_3$) which, in the presence of peptone, gives an eosin-like color. The constituent of peptone responsible for the eosin-like color is the guanidine nucleus [$NH{:}C(NH_2)NH{\cdot}R$] in the amino acid arginine.

The Voges-Proskauer test appears to possess considerable sanitary

significance because it distinguishes to a high degree between typical fecal (low-ratio) and typical nonfecal (high-ratio) members of the colon group.

The presence of acetylmethylcarbinol (acetoin) in bacterial cultures may be easily detected by the method of Coblentz (1943) which is a combination of the procedures employed by Barritt (1936) and O'Meara (1931). The organism being studied is inoculated heavily into a tube of glucose broth and incubated at 30°C. for 6 hr. Then 0.6 cc. of $\alpha$-naphthol (5 gm. of $\alpha$-naphthol in 100 cc. of 95 per cent ethyl alcohol) is added, followed by 0.2 cc. of 40 per cent KOH to which has been added 0.3 per cent creatine. The tube is shaken vigorously for about 1 min. A positive reaction is indicated by the appearance of an intense pink to rose color.

**Methyl Red Test.**—Michaelis and Marcora (1912) noted that cultures of *E. coli* fermented lactose with the production of acids until a pH of about 5.0 was reached. This acidity was sufficient to prevent further growth of the organisms. They concluded that the final hydrogen-ion concentration of cultures of *E. coli* was a physiological constant. This same principle applies to any fermentative organism.

Clark (1915) and Clark and Lubs (1915) confirmed the work of Michaelis and Marcora. They stated that the metabolism of members of the colon group can be so controlled that the hydrogen-ion concentration of cultures of one subgroup can be made to diverge widely from those of the other subgroup. From a given amount of sugar, *E. coli* will produce more acid than *A. aerogenes*. This is due to the fact that *E. coli* does not produce 2:3-butylene glycol and acetylmethylcarbinol, whereas *A. aerogenes* does. The amount of fermentable carbohydrate that is just sufficient for *E. coli* to produce its maximum acidity is inadequate for *A. aerogenes* to produce its limiting hydrogen-ion concentration. The *E. coli* organisms will be stopped in their growth, whereas *A. aerogenes* will exhaust the sugar and, being insufficient for them to produce their limiting hydrogen-ion concentration, will then attack the nitrogenous constituents of the medium for both structure and energy. The reaction of the medium becomes progressively more alkaline.

The amount of buffer present greatly influences the final hydrogen-ion concentration attained by an organism when grown in the presence of a fermentable substance. The greater the buffer content, the smaller will be the final hydrogen-ion concentration (higher pH). This means that, as fermentation is prolonged, metabolic products other than acids accumulate to produce an increased toxic effect on the organisms.

The medium used for the test contains 0.5 per cent glucose sufficiently buffered with dibasic potassium phosphate and peptone to give a limiting hydrogen-ion concentration of about pH5.0 when inoculated with typical *E. coli*. The final hydrogen-ion concentration of cultures of typical *A. aerogenes* will be at a much higher pH. The methyl red indicator used in

the test is turned red by cultures of *E. coli* and orange or yellow by *A. aerogenes*.

**Uric Acid Test.**—The Voges-Proskauer and methyl red tests are based on a difference in the carbohydrate metabolism of the members of the colon group. The tests do not consider changes that take place by action of the organisms on the nitrogenous constituents of the medium.

Koser (1918) observed that cultures of typical *A. aerogenes* were capable of utilizing uric acid when added to a synthetic medium as the only source of nitrogen. Cultures of typical *E. coli* were unable to attack the compound. The result was that *A. aerogenes* multiplied and grew luxuriantly and *E. coli* failed to develop. The results correlated almost 100 per cent with the methyl red and Voges-Proskauer tests.

Later, Chen and Rettger (1920) found that xanthine could be substituted for uric acid with the same result. They obtained almost 100 per cent correlation with the methyl red and Voges-Proskauer tests. Xanthine yields uric acid on oxidation. The formulas are given on page 291.

**Sodium Citrate Test.**—Koser (1923, 1924), in his studies on the utilization of the salts of organic acids by members of the colon group, found that the organisms could be separated into two subgroups on the basis of their action on sodium or potassium citrate.

Typical fecal *E. coli* were unable to utilize sodium or potassium citrate when added to a synthetic medium as the only source of carbon; *A. aerogenes*, or the strictly soil types, utilized sodium citrate readily. A synthetic medium containing citrate as the only source of carbon is employed in the test.

Koser showed that the test for citrate utilization correlated more closely with the source of the organisms than did any of the other differential tests. Parr (1938) isolated a coliform organism that failed to show growth at first on citrate agar but produced a few colonies after 3 or 4 days. In a check of over 5000 strains isolated from fresh and stored fecal specimens, this same result occurred only 29 times, indicating that the phenomenon is not of common occurrence.

LeGare (1944) showed that the addition of riboflavin to Koser's citrate medium stimulated growth of *E. coli* but not of *A. aerogenes*. Greatest growth occurred in tubes containing 2 micrograms of riboflavin per cubic centimeter of medium. It would appear that *A. aerogenes* is able to synthesize this vitamin, thus permitting growth, whereas *E. coli* is unable to do so, with the result that no growth occurs unless the accessory substance is added to the medium.

**Eijkman Test.**—Eijkman (1904) found that cultures of *Escherichia* produced acid and gas from glucose broth at 46°C., whereas *Aerobacter* failed to do so.

Recent improvements in the method have renewed interest in the test.

Stuart, Zimmerman, Baker, and Rustigian (1942) found that *Aerobacter* and "intermediates" seldom produced gas from lactose at 45.5°C., whereas *Escherichia* seldom failed to do so. In their Eijkman characteristics, the intermediates were much more closely related to *Aerobacter* than to *Escherichia.*

Taylor (1945) on the basis of his results concluded that 97 per cent of cultures of typical or type I *E. coli* (page 489) examined fermented lactose with the production of acid and gas between 40 and 44°C. The number was not appreciably reduced at 45°C. but was markedly reduced at 46°C. On the other hand, 15 per cent of cultures of typical or type I *A. aerogenes* were found to be positive at 44°C. but only 2 per cent at 45°C. The results are shown in Table 46.

TABLE 46.—PRODUCTION OF ACID AND GAS IN LACTOSE BROTH BY VARIOUS TYPES OF COLIFORM BACTERIA AT DIFFERENT TEMPERATURES

| Type | No. of cultures tested | Percentage of cultures forming acid and gas at | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 37°C. | 40°C. | 42°C. | 43°C. | 44°C. | 45°C. | 46°C. |
| *Bact. coli*, type I..... | 96 | 100 | 97 | 97 | 97 | 97 | 93 | 52 |
| *Bact. coli*, type II..... | 50 | 100 | 64 | 36 | 32 | 28 | | |
| Intermediate type I... | 78 | 100 | 78 | 56 | 23 | 0 | | |
| Intermediate type II.. | 53 | 100 | 55 | 4 | 2 | 0 | | |
| *Bact. aerogenes*, type I.. | 80 | 100 | 83 | 53 | 35 | 15 | 2 | |
| (or *Bact. cloacae*) | 52 | 100 | | | | 15 | | |
| *Bact. aerogenes*, type II | 25 | 100 | 80 | 12 | 0 | 0 | | |

The production of gas from carbohydrates at the optimum temperature by the different genera of the family *Enterobacteriaceae* (1. *Escherichia*, 2. *Aerobacter*, 3. *Klebsiella*, 4. *Erwinia*, 5. *Serratia*, 6. *Proteus*, 7. *Salmonella*, 8. *Shigella*) tends to increase from *Serratia* through *Erwinia* to *Aerobacter*, then to decrease through *Escherichia*, *Proteus*, *Salmonella* to *Shigella*. A similar condition appears to be true of the Eijkman relationships of these genera.

## RELATIONSHIPS OF COLIFORM ORGANISMS

Studies of recent years have emphasized the complexity of the colon group. The general practice followed in classifying members of the colon group is to designate as *Escherichia* those strains which are indole and methyl red positive, do not produce acetylmethylcarbinol, and are incapable of utilizing sodium citrate as the only source of carbon. Strains classified as *Aerobacter* are indole and methyl red negative, produce acetylmethylcarbinol, and are capable of utilizing citrate as the only source of carbon. Parr (1936) coined the mnemonic IMViC to designate these four reactions. The term IMViC is one of convenience to designate *I*

for indole, *M* for methyl red, *V* for Voges-Proskauer, and *C* for citrate. The letter *i* between the *V* and *C* is added solely for euphony. According to this system, the symbol for typical *E. coli* is "++−−" and for typical *A. aerogenes* "−−++." Using these four characters as a system of classification, Parr (1938*a*) showed that 16 different organisms are possible (Table 47). The most commonly occurring types are −+−−, ++−−, −+−+, and −−++.

TABLE 47.—IMViC CHARACTERS OF COLIFORM ORGANISMS FROM FRESH FECES

| Indole | Methyl red | Voges-Proskauer | Sodium citrate | Type |
|---|---|---|---|---|
| + | + | − | − | Typical *E. coli* |
| − | + | − | − | Atypical *E. coli* |
| + | − | − | − | Atypical *E. coli* |
| + | + | + | − | Intermediate |
| + | + | − | + | Intermediate |
| − | + | − | + | Intermediate |
| + | − | + | − | Intermediate |
| + | − | − | + | Intermediate |
| − | + | + | − | Intermediate |
| + | + | + | + | Intermediate |
| + | − | + | + | Intermediate |
| − | + | + | + | Intermediate |
| − | − | − | + | Atypical *A. aerogenes* |
| − | − | + | − | Atypical *A. aerogenes* |
| − | − | + | + | Typical *A. aerogenes* |
| − | − | − | − | |

According to Parr (1938*a*), coliform intermediates may be defined as

those organisms of the group which have one or more coli and one or more aerogenes characteristics and some, principally the typical fecal form, produce hydrogen sulfide. Essential *E. coli* characteristics are the positive indol and methyl red reactions. Those most characteristic for *A. aerogenes* are the positive Voges-Proskauer and citrate utilization reactions.

Changes in the IMViC characteristics produce changes in the colonial appearance of the organisms. Many types of colonies have been recognized, ranging from flat, round, smooth, glistening colonies typical of *E. coli* to raised, markedly convex, confluent, mucoid colonies typical of *A. aerogenes*.

Parr concluded that

there is great variability in the so-called normal coliform flora and in the flora of the same individual from day to day. Stool specimens may contain no coliform organisms at all and citrate utilizing forms may be present to the exclusion of the colon bacillus. No hope is offered for short cuts or simplifications in the bacteriology of water and foods through the domination of a typical intestinal form.

The complexity of the coliform group is emphasized. It seems impossible to translate the work of the past into terms used today, making necessary the duplication of much work. Doubt is expressed as to the primary etiology of atypical, coliform organisms and it is suggested that the ecological point of view should receive more emphasis in intestinal bacteriology.

Stuart, Griffin, and Baker (1938) and Vaughn and Levine (1942) also recognized the complexity of the colon group and suggested that it be divided into three subgroups: *Aerobacter*, intermediates, and *Escherichia* on the basis of their biochemical reactions.

**Pigmented Coliform Bacteria.**—In connection with the maintenance of a collection of coliform cultures stored in the refrigerator at 0 to 5°C., Gililland and Vaughn (1943) noted that 12 of them displayed a yellowish color. Three additional pigmented cultures were obtained from other sources making a total of 15. Twelve of these were found to be *Escherichia* and three to be *Aerobacter*. Incubation at relatively low temperatures was necessary for pigment production by 12 of the cultures. These cultures produced pigment at 19°C., but not at 30 to 37°C. However, three of the cultures were able to produce pigment at 37°C. The anaerobic glucose metabolism of typical nonpigmented strains did not differ significantly from that of the pigmented forms.

**Slow Lactose-fermenting Organisms of the Colon Group.**—Parr (1938*c*) stated that the flora of fresh, fecal specimens may consist of coliform bacteria (*Escherichia*, intermediates, *Aerobacter*), enterococci (*Streptococcus*), obligate, spore-forming anaerobes (*Clostridium*), nonspore-forming anaerobes (*Bacteroides*), *Micrococcus*, *Lactobacillus*, thermophilic organisms (*Streptococcus*), aerobic spore formers (*Bacillus*), *Pseudomonas*, *Proteus*, *Alcaligenes*, members of the Friedländer group (*Klebsiella*), molds, yeasts, algae, and spirochaetes.

Parr showed that the flora of an individual differs from time to time even though the person is in normal health and the diet relatively stable. Occasionally, coliform bacteria could not be detected; at other times, coliform organisms (*Aerobacter*, intermediates), but not *E. coli*, were present. It can be seen that fresh fecal specimens may be encountered that can pollute water without detection by the bacteriological procedures now in use. Fortunately, such exceptions are rare and offer very little difficulty in sanitary interpretations of bacteriological results.

The bacterial flora of long-stored feces is different from that of fresh feces. Jordan (1926) found that, shortly after feces left the body, increase in bacterial numbers took place, followed later by a decrease. This occurred at all temperatures but was most rapid at 37°C. He found that increase in numbers was not due to *E. coli* but to other organisms. *E. coli* was eventually eliminated in stored feces. Parr reported that about

14 per cent of all fresh fecal specimens contained only *E. coli*. He concluded that, when the original fresh specimens contained only *E. coli*, no amount of storage brought about the presence of any other type of coliform organism. When the original fecal specimen contained both citrate-utilizing, coliform organisms and *E. coli*, the citrate utilizers (*A. aerogenes*, intermediates, etc.) multiplied at a faster rate than the *E. coli*, eventually replacing them. At the same time, there was an increase in strains that fermented lactose slowly or with acid production only.

Several investigators have reported the transformation of late, lactose-fermenting organisms of the colon group into rapidly fermenting strains by frequent subculture in lactose broth or nutrient broth. The IMViC characteristics showed that the organisms belonged to either the *Escherichia* or *Aerobacter* subgroups. Late lactose fermenters have been isolated from water supplies and from feces of both healthy individuals and those ill with diarrhea.

Hershey and Bronfenbrenner (1936) found that a rapid lactose fermenter could be changed to a nonlactose-fermenting form by inoculation into a sodium succinate medium. They suggested that late, lactose-fermenting organisms may not be identical with other lactose-fermenting members of the colon group. Stokes, Weaver, and Scherago (1938) reported that they were able to convert late, lactose fermenters into rapid fermenting forms and back again to late fermenters. They concluded that the strains studied were members of the *Escherichia* and *Aerobacter* divisions of the colon group.

Dulaney and Smith (1939) examined 400 stool specimens and found that 20 per cent contained slow lactose fermenters. They showed that in most cases slow lactose fermenters required from 2 to 5 days to produce acid and gas in fermentation tubes. Daily transfers to lactose broth accelerated the rate at which the sugar was fermented. They also isolated slow lactose fermenters from urine, from blood cultures, and from cases of diarrhea.

McCrady (1939) submitted a questionnaire to about 30 laboratory workers and found that the majority of them favored inclusion of the slow lactose fermenters in the coliform estimate in judging the sanitary quality of a water supply. He concluded,

> Slow lactose fermenters are found, although in small proportion, in fresh feces, in greater proportion in stored feces, and occasionally in discharges from cases of gastrointestinal disturbance; the presence of these organisms even in natural waters, therefore, cannot always be dismissed as of negligible sanitary significance. Furthermore, since other organisms contained in the sample may reduce the amount of gas produced in lactose broth by typical coliform organisms, the volume of gas produced cannot be accepted as a sure indication of the type of organism present.

It is evident, therefore, that the great majority of the laboratory workers are opposed to excluding slow lactose fermenters from the coliform group of organisms. In view of the various facts and arguments that may be adduced to support their position, it is suggested that, until sufficient evidence to the contrary is presented, water laboratories are well advised to include, in their routine coliform estimates, all coliform organisms confirmed from primary lactose broth.

Stuart, Mickle, and Borman (1940) suggested a classification for the slow, lactose-fermenting organisms, placing all strains under the general name of "aberrant coliforms." Their conclusions concerning the importance of slow fermenters were essentially the same as those of McCrady.

Summarizing, it may be stated that at one time the general tendency was to regard late lactose fermenters as attenuated forms of colon organisms, indicating an old pollution and having no sanitary significance. At the present time, available evidence seems to point to the fact that the slow fermenters are forms that have departed from the normal types but should be regarded as possessing considerable sanitary significance.

**Paracolon Bacteria.**—The paracolon bacteria occupy a position intermediate between the coliform organisms (*Escherichia*—intermediates—*Aerobacter*) and the *Salmonella*. They have some of the cultural characteristics of the coliforms and some of the pathogenicity of the *Salmonella*. Topley and Wilson (1938), Stuart and Rustigian (1943), and Stuart, Wheeler, Rustigian, and Zimmerman (1943) found that certain of the species were pathogenic for man, producing a gastroenteritis; others were under suspicion; and some were nonpathogenic. The paracolons, like the coliform bacteria, can be divided into three sections: paracolon *Aerobacter*, paracolon intermediates, and paracolon *Escherichia*, according to their IMViC reactions. A frequent property of the paracolons is that they either do not ferment lactose or attack it very slowly. For this reason, plate colonies on differential media are often mistaken for pathogenic nonlactose fermenters. The organisms are frequently the cause of food poisoning and have been mistaken for members of the *Salmonella*.

For more information, consult Griffin and Stuart (1940), Levine (1943), McBee and Speck (1943), McCrady (1943), Mallmann and Darby (1941), Parr (1937, 1938*b*, 1939), Perry and Hajna (1944), Prescott, Winslow, and McCrady (1946), Stahly and Werkman (1942), Stuart, Baker, Zimmerman, Brown, and Stone (1940), Stuart, Rustigian, Zimmerman, and Corrigan (1943), Stuart, Wheeler, and Griffin (1938), Wallick and Stuart (1943) and Wheeler, Stuart, Rustigian, and Borman (1943).

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# CHAPTER XXI

# BACTERIOLOGY OF MILK AND MILK PRODUCTS

## MILK

Milk is considered the most satisfactory single food preparation elaborated by nature. It contains protein, fat, carbohydrate, inorganic salts, and vitamins.

According to the U.S. Public Health Service (1939),

> Milk is defined to be the lacteal secretion obtained by the complete milking of one or more healthy cows, excluding that obtained within 15 days before and 5 days after calving, or such longer period as may be necessary to render the milk practically colostrum free; which contains not less than 8 per cent of milk solids not fat, and not less than 3¼ per cent of milk fat.

The constituents of milk may be placed into three groups on the basis of their solubilities: (1) Some of the constituents are in true solution, (2) some partly in solution and partly in suspension or colloidal solution, and (3) some are present entirely in colloidal solution (see page 362).

**Color of Milk.**—The color of milk is due largely to the presence of carotene. Carotene exists in at least three isomeric forms: $\alpha$-carotene, $\beta$-carotene, and $\gamma$-carotene. Another pigment closely related to carotene is cryptoxanthin, which occurs in yellow corn. All these pigments are precursors of vitamin A. One molecule of $\beta$-carotene is capable of yielding two molecules of vitamin A; one molecule of each of the others yields only one molecule of vitamin A.

Carotene is found in hay, grass, green leaves, some fruits, carrots, etc. The carotene content of cow's milk is dependent upon the carotene content of the ration. Not all the carotene of the ration is converted into vitamin A. When cows consume carotene-containing foods, some of the pigment is converted into vitamin A and some is found unchanged in the milk. Vitamin A is colorless whereas carotene is yellow.

Milk also contains ascorbic acid and riboflavin. Holmes and Jones (1945) exposed bottled milk to sunshine and found that the ascorbic acid content was destroyed after 30 min. and the riboflavin almost completely destroyed after 2 hr. The results indicated that milk should not be allowed to stand in strong sunlight for any appreciable length of time.

## NORMAL SOURING OF MILK

**Reaction of Fresh Milk.**—Milk when freshly drawn may show considerable variation in reaction. As a general rule, the pH is slightly acid,

ranging from about 6.3 to 7.2 with an average at about 6.75. The pH fluctuates at different stages of the milking operation. The fore milk is usually the lowest in acidity, the middle milk the highest, and the strippings between the two.

**Changes in the Reaction of Milk.**—On standing, unsterilized milk rapidly ferments with the production chiefly of lactic acid from the lactose of the medium. The first stage is believed to be a hydrolysis of the lactose to one molecule of glucose and one of galactose. In the second stage, the hexoses are fermented to lactic acid.

It is generally stated that acidity in milk is first detected by taste when the pH drops to about 6.0. As the acid concentration continues to increase, it eventually causes a precipitation of the casein. This is said to occur when the pH reaches 4.78 to 4.64. Boiling produces a curdling of milk at a much higher pH (lower acidity). The acidity continues to rise until the concentration is sufficient to prevent growth of the bacteria producing the fermentation. The lactic acid produced in the milk prevents the growth of most types likely to be present and thus acts as a preservative.

Molds and yeasts are capable of growing in soured milk. They utilize some of the acid and produce a corresponding decrease in the acidity. Conditions now become favorable for the rapid decomposition of the milk proteins by the growth of putrefactive bacteria. As a rule, several weeks are required for putrefactive changes to occur. The utilization of the acid occurs at a faster rate if the milk is placed in shallow well-aerated layers. This is the general cycle of changes that occur in raw milk when allowed to stand at ordinary temperature.

**Streptococcus lactis.**—The organism responsible for the normal souring of milk is *Streptococcus lactis*. Several varieties of the organism are recognized, which show differences in the flavor produced, character of the fermented milk, rate of acid formation, rate of litmus reduction, and in other ways. Hammer and Baker (1926), Stark and Sherman (1935), and others suggested a number of varieties of the organism: (1) *S. lactis* var. *maltigenes*, (2) *S. lactis* var. *hollandicus*, (3) *S. lactis* var. *anoxyphilus*, and (4) *S. lactis* var. *tardus*. Other varieties include (5) *S. amylolactis*, (6) *S. raffinolactis*, and (7) *S. saccharolactis* (Orla-Jensen and Hansen, 1932). Further proof of the similarities of the different varieties or strains of *S. lactis* was furnished by Sherman, Smiley, and Niven (1940). They produced species-specific grouping of sera and found that such sera gave good precipitation reactions with the extracts of all strains of *S. lactis* tested.

*S. lactis* does not occur in the udders of cows. This was shown by Rogers and Dahlberg (1914). Stark and Sherman (1935) succeeded in isolating *S. lactis* repeatedly from certain plants. They suggested that plants may represent the natural habitat of the organism and that it would

seem likely that surviving strains would sometimes be found in the feces of animals. The organism can be obtained from the coat of the cow. Since it is normally present in cow dung, it is believed that this is the agent responsible for the contamination of milk.

*S. lactis* is Gram-positive and may appear in pairs, in short chains, or in long chains (Fig. 188). The organism is spherical but sometimes appears slightly elongated in the direction of a chain. The organism produces dextrolactic acid. No gas is formed. Occasional strains have been noted that fail to ferment lactose. In broth cultures, the final pH falls between 4.0 and 4.5. The organism will not grow at pH9.5 but will grow at 9.2. It grows at 10°C., or lower, and at 40°C. but not at 45°C. The optimum temperature is about 30°C. Litmus is completely reduced (decolorized) before the milk is curdled.

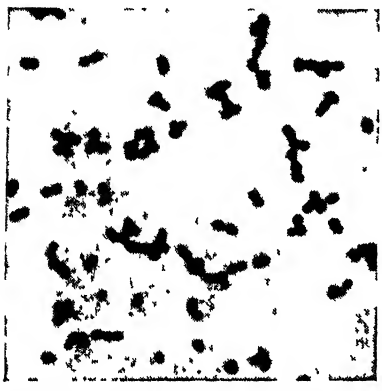


FIG. 188.—*Streptococcus lactis*, the cause of normal souring of milk.

### QUANTITATIVE EXAMINATION OF MILK

Normal udders of cows are probably never sterile. Organisms are present in abundance in freshly drawn milk. The first milk drawn shows the highest count, the middle milk shows a smaller count, and the strippings the least of all. The comparatively high count of the fore milk is due to the washing out of the easily removable organisms present in the milk passages. The numbers washed out become less and less during the milking process. It is the general practice to discard the first portions of milk containing the highest bacterial counts. It has been shown that the rejection of the fore milk decreases the bacterial count an average of about 4 per cent. However, most of the organisms found in milk are chiefly those that gain entrance during the operations of milking and handling. It would seem, therefore, that the contamination of milk by bacteria is largely preventable.

Two methods are generally used for making a quantitative bacteriological examination of milk: (1) the agar plate method and (2) the direct microscopic method. In addition, two other tests are employed where rapid results are required: (3) the methylene blue (reductase) test and (4) the resazurin test.

**Agar Plate Method.**—This method consists in preparing a series of dilutions of the milk, pipetting 1-cc. amounts into a series of Petri dishes, mixing with agar previously melted and cooled to 45°C., and incubating the plates at 37°C. for 48 hr. The factors that influence plate counts include temperature of incubation, period of incubation, medium used for

plating, etc. Unless a standard procedure is followed, the results obtained cannot be compared with those from other laboratories. Results reported by Abele (1939) and others indicate that an incubation temperature of 32°C. gives higher plate counts than one of 37°C.

*Disadvantages of the Plate Method.*—Disadvantages of the method are numerous. Pathogenic organisms are usually not detected. If the organisms grow, they cannot be distinguished from nonpathogenic species by appearance. The number of colonies appearing on agar plates do not represent all the organisms in milk. Many of the organisms fail to develop on an agar medium. Anaerobic organisms do not find conditions favorable for growth. This means that no single medium is capable of giving growth of all viable organisms likely to be found in milk. Also, a temperature of 37°C. is not favorable for the growth of all organisms. Shaking the sample does not break up all the clumps or groups of bacteria. Chains of streptococci usually remain intact and record as only one colony. The colony counts represent only a fraction of the total bacterial content of milk. Because of the long incubation period, the milk is usually consumed before information on the number of bacteria present is obtained. Therefore, agar plate counts should be regarded as estimates rather than as exact numbers.

FIG. 189.—Roll-tube culture showing colonies (*After Prouty, Bendixen, and Swenson.*)

Prouty, Bendixen, and Swenson (1944) described a roll-tube technique, instead of the use of Petri dishes, for enumerating the bacterial population of milk (Fig. 189). To quote,

> The tubes have an over-all length of 153 mm. and an inside diameter of 19 mm. A constriction in the tube about 2 to 3 cm. from the top prevents the medium from wetting the cotton plug when the tube is rapidly rotating in a horizontal position. For making counts the tubes are filled with 7 cc. of an agar medium, containing preferably 2.0 per cent agar to give the desired consistency to the medium. The tubes are plugged and sterilized, and just before use are tempered in a water bath to 45°C., inoculated with the milk, and after careful mixing of the contents rotated at a speed (about 2,000 r.p.m.) sufficient to deposit the agar in a layer of uniform thickness against the inside wall where it congeals after a short time. The tubes are then incubated in a nearly horizontal position with the bottom of the tubes slanting slightly downward to carry down any small amount of moisture that may collect and which may cause the development of spreaders over

the agar surface. . . . After incubating, the counting of the colonies may be facilitated by using a lens attached to a metal cylinder which is slipped over the agar roll-tube.

Advantages of the method:

1. Tubes subject to less breakage, require less space, and more easily handled than Petri dishes.
2. Tubes may be filled with agar and kept on hand in the refrigerator ready for instant use.
3. Less incubator space required.
4. Less danger of contamination than plates.

Disadvantages of the method:

1. Method requires special motor-driven apparatus for rotating the tubes.
2. Incidence of spreading colonies apt to be greater than with Petri dish cultures.
3. Colonies more difficult to count than on Petri dishes.

**Direct Microscopic Method.**—The official method consists in spreading 0.01 cc. milk over an area of 1 sq. cm. on a glass slide, allowing the film to dry, removing the fat, staining the film, and examining under the microscope.

Mallmann, Bryan, and Baten (1944) simplified the method by employing a 4-mm. loop (outside diameter) instead of a pipette. The contents of the loop smeared over an area of $4 \times 8$ mm. gave approximately the same density of milk as 0.01 mm. spread over an area of 1 sq. cm. The slide was then immersed in xylol to remove fat and stained by a special alcoholic solution of methylene blue acidified with hydrochloric acid (Mallmann and Churchill, 1942). The use of a Wratten M filter No. E-22 between the light source and the object was found to give a sharper contrast of the organism to its background and revealed poorly stained organisms that were invisible with a blue filter or even natural light.

*Advantages of the Microscopic Method.*—The method possesses a number of advantages over the plate procedure. Results can be obtained quickly, usually in about 15 or 20 min. Since less work is required, more samples can be examined by this method than by the plate procedure. The amount of equipment necessary is much less than by the plate method. The slides can be preserved as a permanent record and examined whenever occasion arises, whereas the plates must be examined and discarded. Some idea of the morphological types can be obtained from slide preparations. This is frequently of great value in determining the cause of the bacterial count. Microscopic examination reveals the presence of leucocytes and other body cells in milk. An excessive number of leucocytes indicates a diseased condition of the udder. The slide method gives a better quantitative determination than the agar plate method.

*Disadvantages of the Microscopic Method.*—An important disadvantage of the slide method is that it cannot be used on pasteurized milk. Dead

cells are not easily distinguished from living ones. However, it does give important information as to the number of organisms present before pasteurization.

Another disadvantage is that, unless the milk sample contains a high count, the microscopic method may be the source of considerable error. A large factor is used for converting the number of bacteria per field to the number per cubic centimeter of milk. Significant errors in the average number of organisms per field are not likely to be of great importance when bacteria are numerous in milk. However, in low-count milk a considerable error may be introduced. This is especially true where many fields may be seen that show no bacteria and some fields that may show a cluster or chain of organisms so that the variation per field is great.

**Comparison of Counts by the Two Methods.**—The microscopic method gives much higher counts than the agar plate method. The differences between the counts by the microscopic and plate methods are considerably greater on samples showing low bacterial counts than on those showing high counts. The organisms in low-count milk represent external contaminants that fail to develop on agar, whereas those organisms in high-count milk are forms that have developed in the milk. Also, low-count milk usually shows a greater percentage of the organisms in clumps than does high-count milk. It is generally stated that the ratio of the microscopic count to the plate count is, on the average, about 4:1. Therefore, this ratio is generally used to compare the results of one method in terms of the other, although this is not necessarily true.

For an interesting discussion of the merits of the agar plate and direct microscopic methods, see Brew and Breed (1945).

**Reduction of Methylene Blue (Reductase Test).**—Methylene blue acts like a respiratory pigment when introduced into bacterial cultures. It accepts hydrogen to produce an intracellular oxidation under anaerobic conditions. At the same time, the dye becomes decolorized to the leuco compound. The decolorization of the dye is the result of the consumption of the dissolved oxygen by the growing bacteria. An aeration of the culture results in the loss of hydrogen followed by the restoration of the blue color. Therefore, the methylene blue reduction time will depend upon the oxygen-consuming power of the organisms present (see page 312).

Methylene blue is of value in making a rapid survey of the quality of raw milk. The rate of decolorization depends upon the number of organisms present. The test can be employed to determine, in a rough way, the bacterial population of a milk sample. The procedure is quickly and easily carried out and with a minimum of expense. It is particularly valuable in making rapid inspections of large numbers of samples to determine if the milk received by companies answers the requirements prescribed by law.

The test is expressed as the period of time required for the color of methylene blue to disappear when incubated at 37°C. Under some conditions, the blue color does not disappear uniformly. In such cases, the end point is taken as the time required for the milk to show no blue color after it is mixed.

There is not always good agreement between the methylene blue reduction time and the agar plate count because (1) some organisms fail to grow on nutrient agar, (2) a clump of organisms records as only one colony whereas the rate of decolorization is due to the combined effect of each member of the mass, (3) the rate of decolorization of the dye is not the same for all organisms, and (4) the test becomes less accurate as the reduction time is increased, freshly drawn milk requiring at least 10 hr. to decolorize methylene blue. *Streptococcus lactis* reduces methylene blue more vigorously than any of the other organisms likely to be found in milk.

Morris (1943) reported that milk samples frequently contained coliform organisms which reduced methylene blue very slowly at 37°C. This slow reduction was found to be due to two factors present in raw milk, both of which were destroyed by heating to 70°C. for 1 hr. These factors were (1) a specific bactericidal substance and (2) a growth-inhibiting factor.

The classification of milk on the basis of the methylene blue reduction time is as follows:

*Class* 1.—Excellent milk, not decolorized in 8 hr.
*Class* 2.—Good milk, decolorized in less than 8 hr., but not less than 6 hr.
*Class* 3.—Fair milk, decolorized in less than 6 hr., but not less than 2 hr.
*Class* 4.—Poor milk, decolorized in less than 2 hr.

For more information, consult Abele (1945).

**Resazurin Test.**—This test was first introduced by Pesch and Simmert (1929). The original test has been modified and provisionally adopted by the Ministry of Agriculture (England) as a rapid method of determining the quality of milk.

Resazurin (diazoresorcinol) is an indicator having a pH range between 3.8 and 6.5. At pH6.5 and above, the indicator is purple; at pH3.8 and below, it is orange. The dye is capable of being reduced by bacteria. Because of this fact, it may be used to estimate the number of bacteria in milk. The greater the number of bacteria present in the milk, the quicker it will be reduced. The test is quickly performed and with a minimum of expense. It is particularly valuable in detecting infections of the udders of cows (mastitis).

The test is performed as follows: The milk sample is thoroughly mixed and 10 cc. pipetted into a $\frac{5}{8} \times 6$-in. test tube. One cubic centimeter of 0.005 per cent (1:20,000) resazurin solution is added to the milk, and the tube inverted twice slowly to mix well. The tube is placed in a 37.5°C.

water bath and examined every 15 min. up to an hour. The color of the milk should be compared with a series of standard disks prepared for the purpose. If the color of the milk remains blue or lilac (disk 6 or 5) for 1 hr., the milk is normal in bacterial content. If mauve or mauve-pink (disk less than 5), the bacterial content is high and the milk should be regarded as abnormal. If a disk number less than 3 is obtained in ½ hr. the milk is grossly abnormal. If the resazurin is completely reduced (disk 0) in 1 hr. or less, the milk is very high in pus cells and is probably teeming with mastitis organisms.

For more information, see Barkworth, Davis, Egdell, Rowlands and Watson (1943), Davis (1943*a*,*b*,*c*), Davis and Newland (1943), Davis, Newland, and Knuckey (1943), Davis and Watson (1943), and Erskine, Fischer, Smith, and Davis (1943).

## GRADING OF MILK

The numbers of organisms permissible in different grades of milk vary considerably, depending upon standards set up by local public health authorities.

The highest grade of milk is known as certified milk, which is safeguarded at every step in its production, collection, and distribution. It is produced according to rules and regulations formulated by medical milk commissions established in a number of localities in the United States. The rules and regulations deal with such matters as the cleanliness of the barnyard and dairy buildings, quality of the water supply, sterilization of all utensils used in handling the milk, the periodic examination of the cows for the presence of tuberculosis and other diseases, and the examination of the milkers and others concerned with the handling of the milk. Persons suffering from contagious diseases or carriers of such organisms are not permitted to be employed in certified milk dairies.

Milk collected under conditions not so carefully controlled is graded as *A*, *B*, or *C*. The ratings are based upon the bacterial count of milk and also upon the hygienic conditions under which it was produced. The standards of the various grades reported here are those set up by the U.S. Public Health Service Milk Ordinance (1939). They are as follows:

Raw Milk:

**1. Certified.**—This must conform to standards set up by American Association of Medical Milk Commissions. These vary for different localities, but the usual standard is that the count must not go above 10,000 organisms per cubic centimeter. All milk having a count in excess of this number must be placed in one of the following grades:

**2. Grade A.**—The average bacterial plate count must not exceed 50,000 per cubic centimeter or the average direct microscopic count must not exceed 50,000 per cubic centimeter if clumps are counted or 200,000 per cubic centimeter if individual organisms are counted, or the average reduction time must not be less than 8 hr.: *Provided*, that,

if it is to be pasteurized, the corresponding limits shall be 200,000, 200,000, 800,000 per cubic centimer, and 6 hr. respectively.

**3. Grade B.**—This is raw milk which violates the bacterial standard and/or the abortion testing requirement for grade *A* raw milk, but which conforms with all other requirements for grade *A* raw milk and has an average bacterial plate count not exceeding 1,000,000 per cubic centimeter, or an average direct microscope count not exceeding 1,000,000 per cubic centimeter, if clumps are counted or 4,000,000 per cubic centimeter, if individual organisms are counted, or an average reduction time of not less than 3½ hr.

**4. Grade C.**—This is raw milk that violates any of the requirements for grade *B* raw milk.

Pasteurized Milk:

**1. Certified Milk—Pasteurized.**—This is certified milk—raw milk that has been pasteurized, cooled, and bottled in a milk plant conforming with the requirements for grade *A* pasteurized milk.

**2. Grade A.**—This is grade *A* raw milk that has been pasteurized, cooled, and bottled in a milk plant, and the average bacterial plate count must not exceed 30,000 per cubic centimeter after pasteurization and until delivery.

**3. Grade B.**—This is pasteurized milk which violates the bacterial standard for grade *A* pasteurized milk but which conforms with all other requirements for grade *A* pasteurized milk, has been made from raw milk of not less than grade *B* quality, and the average bacterial plate count must not exceed 50,000 per cubic centimeter after pasteurization and before delivery.

**4. Grade C.**—This is pasteurized milk that violates any of the requirements for grade *B* pasteurized milk.

Samples for bacteriological examinations are collected by inspectors or other officials. At least 10 cc. of well-agitated milk or cream is collected and placed in a sterile sample bottle, which should be of such size that only about two-thirds of it is filled. This provides sufficient air space for vigorous agitation to ensure a suspension of organisms of uniform turbidity before plating the milk. The sample must be kept below 50°F. (10°C.) until plated. The average bacterial plate count is expressed as the logarithmic average of the plate counts of the last four consecutive samples taken on separate days.

**Why Milk Is Graded.**—According to the U.S. Public Health Service,

It is widely accepted that the bacterial count of milk is an index of the sanitary quality of milk. A high count does not necessarily mean that disease organisms are present, and a low count does not necessarily mean that disease organisms are absent; but a high bacterial count does mean that the milk has either come from diseased udders, has been milked or handled under undesirable conditions, or has been kept warm enough to permit bacterial growth. This means, in the first two cases, that the chances of infection have been increased, and, in the last case, that any infection which has reached the milk has been permitted to grow to more dangerous proportions. In general, therefore, a high count means a greater likelihood of disease transmission.

On the other hand, a wrong interpretation of the significance of low bacterial counts should be avoided, since low-count milk may be secured from tuberculous

cows, may have been handled by typhoid carriers, and may even have been handled under moderately unclean conditions.

The above constitutes the public-health reason for grading milk partly on the basis of the bacterial count or the reductase test.

## INFLUENCE OF TEMPERATURE UPON THE KEEPING QUALITY OF MILK

The number of organisms in milk at the outset depends upon the degree of care exercised in its production and collection. After collection, the numbers increase rapidly unless milk is stored at low temperatures. The temperature at which it is stored determines to a large extent the bacterial count and the microflora of milk.

**Germicidal Property of Milk.**—Freshly drawn milk contains substances that are capable of exerting a bactericidal action. These substances are destroyed by heat, but the temperature required varies for different organisms.

Morris (1945) heated raw milk to temperatures of 52 and 53°C. for 30 min., then inoculated the samples with a young culture of coliform organisms. Plate counts were made immediately after inoculation and after holding the milk for 4 hr. at 37°C. The results are given in Table 48. From the results, it would appear that the bactericidal substances are completely destroyed by heating the milk at 53°C. for 30 min. and that this destruction is critical to within 1°C.

TABLE 48.—EFFECT OF HEAT ON THE GERMICIDAL PROPERTY OF MILK

| Temperature to which milk was heated for 30 min. before inoculation | Count per cc. immediately | Count per cc. after 4 hr. at 37°C. |
|---|---|---|
| Culture No. 1: | | |
| 52°C. | 1,328,000 | 1000 |
| 53°C. | 848,000 | 40,000,000 |
| Culture No. 2: | | |
| 52°C. | 316,000 | 31,000 |
| 53°C. | 640,000 | 28,000,000 |

For many organisms, at least, low-temperature pasteurization produces very little, if any, destructive action on the bactericidal property of milk. In the flash or high-temperature method, this property may be largely destroyed.

It may be concluded that bacteria increase at a more rapid rate in strongly heated milk than in raw milk or milk heated at low temperatures.

**Milk Held in Frozen Condition.**—If milk is held below the freezing point (−0.55°C.), no multiplication of organisms occurs. Since the milk is frozen throughout, there is no chance for the organisms to obtain their

nutrients. Milk treated in this manner shows a decrease in bacterial numbers. If the milk is slowly frozen, there is a gradual precipitation of casein and an immediate destruction of the fat emulsion. When such milk is brought back to room temperature, especially if it has been frozen for some time, it does not regain its normal consistency.

**Milk Held Just above Freezing.**—If raw milk, or milk pasteurized at temperatures below 70°C., is kept at 0 to 5°C. for 24 hr., the plate count decreases. After a lapse of about 1 week, there is an increase over the original plate count of the milk. At the same time, the number of organisms capable of liquefying gelatin increases. This continues until enormous numbers are present. Some of the organisms are acid formers, others are neutral types, still others are strongly proteolytic forms.

This is followed by protein decomposition and putrefaction of the casein. In this condition, toxic waste products may be present in the milk, rendering it not only unfit but dangerous for human consumption. Milk and cream are generally stored at 0°C., but the period at which it can be kept at this temperature should not be over 10 days, for the above reasons.

The bacterial flora of milk kept at different temperatures is as follows:

**0 to 5°C.**—The fluorescent bacteria predominate. These include *Pseudomonas fluorescens, P. schuylkilliensis,* and *Bacillus fluorescens.*

**5 to 10°C.**—Fluorescent bacteria (see above), *Proteus vulgaris,* micrococci such as *Micrococcus caseolyticus, M. flavus, M. conglomeratus, M. roseus, M. freudenreichii, M. epidermidis, M. candicans, M. viscosus,* alkali-producing organisms including *Alcaligenes viscosus, A. marshallii,* and *A. albus.*

**10 to 15°C.**—Streptococci, such as *Streptococcus agalactiae, S. lactis, S. cremoris, S. faecalis, S. liquefaciens, S. acidominimus,* and *Aerobacter aerogenes.*

**15 to 30°C.**—Streptococci, especially *S. lactis.*

**30 to 40°C.**—*Escherichia coli, Aerobacter aerogenes,* the lactic acid-forming rods such as *Lactobacillus caucasicus, L. lactis, L. helveticus, L. bulgaricus, L. thermophilus, L. casei, L. plantarum, L. leichmannii, L. brevis, L. fermenti,* and a few streptococci.

**40 to 50°C.**—Lactic acid-producing rods, including *L. caucasicus, L. lactis, L. helveticus, L. bulgaricus, L. thermophilus,* and *S. faecalis, S. thermophilus,* and yeasts.

The smaller the initial plate count, the greater will be the time required to sour the milk. As has already been shown, the fluorescent bacteria found in the soil are able to multiply at a temperature as low as 0°C. If milk is to be kept for any length of time, it should be frozen; at somewhat higher temperatures, organisms of the *Proteus* group develop, with the result that putrefactive products accumulate in the milk.

Coagulation seldom takes place in milk stored below 10°C. Above this temperature, a coagulum forms in a few days owing to the combined action of rennin and acid-producing organisms. At a temperature of 20°C., the bacterial flora is composed of about 90 per cent streptococci. This results in a rapid coagulation of the milk. The acidity produced is

sufficient to inhibit the growth of most other species of organisms likely to be present. Above 20°C., rod-shaped bacteria predominate, which are capable of producing still higher concentrations of lactic acid. Also, this is the most favorable temperature range for the growth of the butyric acid-producing anaerobes.

The aerobic organisms grow best near the surface of milk where there is an abundant supply of dissolved oxygen. The organisms predominating near the bottom include the anaerobes and *S. lactis*. This means that the spontaneous curdling of milk usually starts at the bottom.

## COLIFORM ORGANISMS IN MILK

Isolated instances are on record where coliform organisms have been recovered from milk taken directly from the udder. However, this is unusual; for all practical purposes, normal milk as it comes from the udder of the cow is entirely free from such bacteria.

The presence of *E. coli* in milk usually represents contamination caused by manure. Since *A. acrogenes* is found in water, soil, on hay, grains, and other food crops consumed by cows, its presence in milk does not necessarily indicate fecal contamination. As milk leaves the farm, it almost always contains members of the coliform group regardless of the care observed in its production. Under careful conditions, raw milk usually contains less than 100 coliform organisms per cubic centimeter. Under careless conditions of production, the number may run as high as 2000 per cubic centimeter. It may be seen that, regardless of the conditions of production, milk and cream always contain coliform organisms in varying numbers.

As has already been seen (Chap. XX), the examination of water for the presence of *E. coli* is a standard procedure for the determination of the healthfulness of a water supply. On the other hand, it is a controversial subject as to whether the *E. coli* test should be used for the determination of the healthfulness of dairy products. The reason for this is that certain strains of *E. coli* have been shown to resist the pasteurization process. The organisms develop rapidly after pasteurization unless the milk is held at a very low temperature. Therefore, the presence of some coliform organisms under ordinary market conditions does not necessarily indicate that the milk was improperly pasteurized. However, the presence of a large number immediately after pasteurization indicates (1) that the milk was not heated to 63°C. for 30 min., or (2) that it became contaminated by the handler or the equipment. Under these conditions, the *E. coli* test should be valuable for control work.

The methods followed for the identification of the gas-forming organisms in milk are similar to those employed for the bacteriological examination of water (page 473).

## ROPY OR SLIMY MILK

A number of organisms are capable of producing a condition in milk known as "ropiness." The milk becomes ropy or slimy and may be pulled out into long threads. Sometimes the change is very slight; sometimes the ropy consistence may be so pronounced that the milk can be drawn out into threads 3 ft. or more in length.

Several organisms are capable of producing this condition in milk. Probably the most important species is *Alcaligenes viscosus*. This organism produces its maximum amount of ropiness at a temperature of 18 to 20°C. The ropy condition is due to the formation of capsular material by the organisms. The slime is a carbohydrate gum, although some protein as well as carbohydrate appears to be necessary for its formation. A microscopic examination of the milk reveals the presence of individual cells embedded in a mass of capsular material.

*A. viscosus* is a small, short rod, sometimes almost spherical in shape. The cells are usually Gram-negative, although occasionally they may appear Gram-positive. The organism produces both a pellicle and ropiness in milk. The pellicle is the result of the aerobic character of the organisms. This explains why the ropiness is often noted in the cream layer only. The organism produces an alkaline reaction with no coagulation of the casein.

Some members of the *Escherichia-Acrobacter* group have been responsible for the ropy condition of milk. Most strains are unable to do so, but occasionally a strong capsule producer is encountered. *A. aerogenes* is probably of more frequent occurrence than *E. coli*.

Another organism producing ropiness in milk is *Micrococcus cremoris-viscosi*. This organism produces an acid coagulation of milk followed by a peptonization and the appearance of slimy material. It grows best at a temperature of about 30°C.

Organisms causing ropiness in milk are commonly found in pools, wells, and streams. Water from such sources contaminates the dairy utensils and equipment with the organisms. When once present, it is of utmost importance to remove the organisms as quickly as possible, otherwise great economic losses may result. All utensils and equipment coming in contact with the milk should be sterilized. The stable should be thoroughly cleaned and disinfected. The flanks of the cows should be wiped with a cloth wetted with an appropriate disinfectant. The organisms causing ropiness are generally destroyed in one of the pasteurization processes, but outbreaks sometimes occur in pasteurized milk. These are due to contamination from the plant equipment after the pasteurization process.

Slime-producing lactic acid organisms have been used in the manufacture of cheese, but such practice has been largely discontinued, owing to

the fact that it is difficult to separate the whey from the cheese. Also, the presence of the organisms in cream results in a poor yield of butter. The property of producing slime appears to be lost by growing the organisms at higher temperatures. Conversely, some organisms that ordinarily do not produce slime can be made to do so if cultivated at lower temperatures.

## CLOSTRIDIUM PERFRINGENS IN MILK

*Clostridium perfringens* (*welchii*) is an anaerobic, spore-forming organism of widespread occurrence in nature. The organism is present in the intestinal tract of man and animals, in the soil, in fish, mollusks, milk, cheese, water, etc. It occurs abundantly in the soil. The organism is generally considered the most important etiological factor in gas gangrene.

The organisms are short, thick rods, occurring singly, in pairs, and less frequently in short chains. The rods are nonmotile, capsulated, and Gram-positive. The spores are large, oval, and central or subterminal.

It is a strongly saccharolytic organism capable of fermenting all the common sugars with the production of acid and large amounts of gas. The organism produces a characteristic "stormy" fermentation of milk (Fig. 190). The curd becomes torn to shreds by the vigorous fermentation and evolution of gas. In this condition, the milk proteins are not attacked.

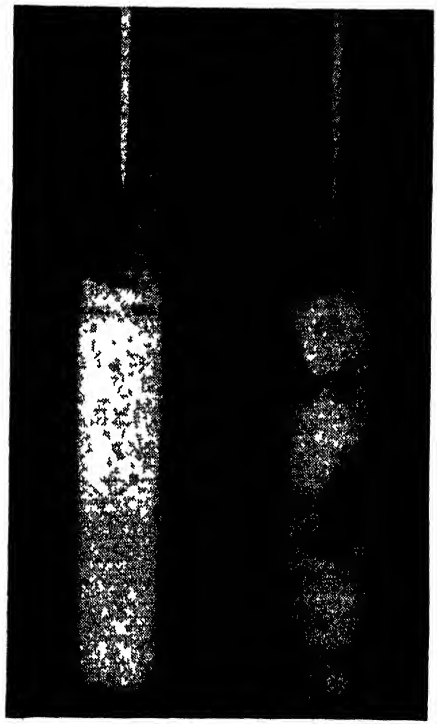


FIG. 190.—Stormy fermentation of milk. Left, fermentation of a sample of milk free from *Clostridium perfringens;* right, fermentation of a sample of milk containing *C. perfringens.* The vaspar seal is pushed up by the gas pressure and the casein is curdled.

Since the organisms are present in the intestinal contents of cows, the presence of *C. perfringens* in milk usually indicates a manurial contamination.

## COLORED MILK

Several organisms have been isolated that are capable of changing the color of milk. These changes occur only occasionally and are of minor importance economically.

Many organisms isolated from milk produce colored colonies, but these are not to be confused with those organisms which are capable of elaborating brilliant colors in milk. Chromogenic organisms are usually strongly aerobic. This means that growth and pigment formation are observed first in the surface layer of milk.

**Blue Milk.**—Blue milk is caused by the growth of the pigmented organism *Pseudomonas syncyanea.* This is a fluorescent organism, capable of producing a gray color in neutral or alkaline milk. In the presence of acid, the gray color is changed to blue. Therefore, acid-producing organisms, such as *Streptococcus lactis,* must be present to change the gray color to blue. The organism grows best at a temperature of about 25°C.

**Red Milk.**—The growth of *Serratia marcescens* produces a red color in milk. It is a small coccobacillus occurring singly and occasionally in short chains. The cells are motile and Gram-negative. The organisms produce an acid reaction in milk with the formation of a soft coagulum. Since pigment production is best in the presence of an abundance of oxygen, the red color appears first on the surface of milk. The organism grows best at a temperature of about 25°C.

**Yellow Milk.**—*Flavobacterium synxanthum* produces a canary yellow pigment when grown in milk. The organism produces a slow fermentation of the lactose, resulting in a coagulation of the casein. Since the slow fermentation fails to exhibit a protein-sparing action, the casein is attacked and peptonized. The alkaline products of peptonization are sufficient to neutralize the primary acidity to give an alkaline, ropy milk. The organism grows best at a temperature of about 30°C.

## MILK-BORNE INFECTION

Milk is an excellent culture medium for a great variety of organisms. Pathogenic as well as saprophytic organisms not only remain viable for considerable periods but are capable of multiplying in milk. For this reason, it is difficult to obtain pure milk and keep it pure. The prevention of milk-borne disease is one of the most important problems of public health.

Pathogenic organisms of both bovine and human origin have been isolated from milk. Many serious epidemics have been caused by the consumption of such products before this fact was clearly recognized. This is to be expected when one takes into consideration the enormous quantities of milk and its products that are consumed daily. Even today, epidemics are spread through milk, but they are of rare occurrence compared to the number reported during the early years of public health.

The abnormal changes that occur in milk are usually easily detected by appearance, taste, and smell. However, the presence of disease organisms cannot be shown by such a procedure. Milk containing disease bacteria looks normal in appearance and gives no warning to the consumer. The

disease organisms present in milk may be derived from (1) diseased cows or (2) persons collecting and handling milk.

**Diseases of Bovine Origin.**—The disease organisms of bovine origin found in milk include (1) *Mycobacterium tuberculosis* var. *bovis*, (2) *Streptococcus agalactiae*, (3) *Brucella abortus*, and (4) the virus of foot-and-mouth disease.

Tuberculosis is common among dairy cows. The organism of bovine tuberculosis is very similar in appearance to the species causing the disease in humans. Adults are probably not susceptible to infection by the organism causing bovine tuberculosis, but children, especially those under five years of age, may become infected by drinking raw milk from tuberculous cows. If the udders of cows are infected with the organisms, contamination of milk cannot be avoided. If cows are suffering from tuberculosis of the lungs, the sputum is swallowed, instead of being expectorated, with the result that the organisms appear in the feces. Since most milk contains some excreta, it is likely to show the presence of such organisms. It is doubtful if the organism multiplies in milk, but it can live in milk and may retain its virulence for a considerable period of time.

The disease known as mastitis refers to an inflammation of the udder and may be produced by a number of organisms (Table 49). However, the organism most commonly associated with the disease is *S. agalactiae*, which is found in the udders of nearly all milch cows. Frequently, it remains latent and does not produce an inflammation. Sooner or later, it may start an active infection resulting in an inflammation (Breed, 1943). If the mastitis is severe, pus and blood may appear in the milk. Milk containing appreciable numbers of the organisms and blood cells must be regarded as unfit for human consumption. All the organisms associated with mastitis are killed by pasteurization.

Another disease organism frequently found in cow's milk is *Brucella abortus*, which produces contagious abortion in cows. The organism may produce the same effect in mares, sheep, rabbits, and guinea pigs. Organisms of a similar nature are *B. melitensis* from goats and *B. suis* from hogs. *B. melitensis* may also infect cows and be excreted in the milk. *B. suis* produces abortion in swine and frequently attacks horses, dogs, cows, monkeys, and laboratory animals.

All three of the organisms are pathogenic for man, producing the disease known as Malta fever, so named because it has been prevalent for centuries on the island of Malta in the Mediterranean where humans become infected by drinking contaminated goat's milk. The disease is now generally referred to as undulant fever or brucellosis (after Bruce, who first isolated the organisms from the spleen in fatal cases of Malta fever).

Undulant fever may be contracted by drinking raw milk and, less frequently, certified milk. Pasteurized milk should be safe since the organisms

TABLE 49.—MICROORGANISMS THAT PRODUCE INFLAMMATION OF THE BOVINE UDDER (MASTITIS) *

| Name of Organism | Prevalence in Cattle |
|---|---|
| *Streptococcus agalactiae* | Latent to active infection present in 50 to 95 per cent of bovine udders; distribution in other mammals little known |
| *Streptococcus pyogenes* | Rare, normally derived directly from some human source |
| *Micrococcus pyogenes* var. *aureus* and *M. pyogenes* var. *albus* | Commonly found in udder and on skin of cattle. On occasion may cause inflammation and formation of pus in udder |
| *Aerobacter aerogenes* and *Escherichia coli* | Usually found in udder but infections rare |
| *Mycobacterium tuberculosis* | Practically eradicated in United States; still common in Europe and other parts of world |
| *Actinomyces bovis* and *A. lignieresi* | Found in cattle with lumpy jaw. Infects other organs, including the udder |
| *Brucella abortus*, *B. suis*, and *B. melitensis* | Bovine organism widespread; porcine variety common in hog-raising areas; caprine variety found in North America, in New Mexico, and Southwestern United States |
| *Corynebacterium pyogenes* | Common cause of pus formation in cattle and not infrequently found in udder |

* Adapted from Breed (1943, 1944).

are destroyed in the heat process. Because of this fact, many public-health authorities believe that all milk should be pasteurized before it reaches the consumer.

Foot-and-mouth disease is a highly contagious virus disease of domesticated animals. It produces fever, digestive disturbances, and a vesicular eruption on the mucous membranes of the mouth and on the skin between the toes. The vesicles may be present also on the udder and teats of the cow. The virus produces a high death rate among cattle. In man, the death rate is low, owing to the fact that the disease runs a mild course.

The presence of the virus in infected cattle may be demonstrated in milk, saliva, urine, and feces. The infection may be transmitted by feeding and drinking troughs, stalls, cattle cars, etc. The disease is stamped out by slaughtering herds showing the presence of any infected animals.

**Diseases of Human Origin.**—Some of the diseases of human origin that have been disseminated by milk are (1) typhoid fever, (2) scarlet fever, (3) diphtheria, (4) septic sore throat, (5) infantile diarrhea, and (6) infantile paralysis. The organisms may be transferred to milk by contaminated hands of the workers, by droplets expelled during coughing, sneezing, and talking, by moistening the hands with saliva during wet milking, and in other ways.

Many typhoid epidemics that have occurred in recent years have been

traced to the consumption of contaminated milk. Further investigation usually revealed the fact that only one dairy supplying the milk was responsible for the spread of the infection. The organisms were introduced into the milk by a typhoid carrier or an unrecognized case of typhoid fever among the workers at the dairy. The isolation of this individual resulted in a disappearance of new typhoid cases in the community. Typhoid epidemics have been traced not only to milk but to a lesser extent to ice cream, cheese, and butter.

Scarlet fever and septic sore throat are probably both caused by *Streptococcus pyogenes*, a pus-producing organism. Epidemics have been caused by the consumption of milk containing this organism. The milk may become contaminated by handlers or by infected udders of cows. Usually a milker suffering from scarlet fever or sore throat infects the udders with the organisms by means of contaminated hands. The organisms rapidly multiply in the milk in the udders. Abscesses form in the udders from which the milk becomes heavily contaminated. The contaminated milk may produce septic sore throat or scarlet fever in persons who consume the raw milk.

## PASTEURIZATION OF MILK

The destruction of all organisms in milk is called "sterilization." The high temperature required to achieve this purpose usually results in the milk having a cooked flavor. Such milk possesses two serious objections: (1) the cooked flavor is not so pleasant as that of raw milk, and (2) heating to a high temperature may result in a decrease in the vitamin content. These objections are largely overcome by heating milk to temperatures lower than that required to sterilize completely but sufficiently high to destroy all disease organisms.

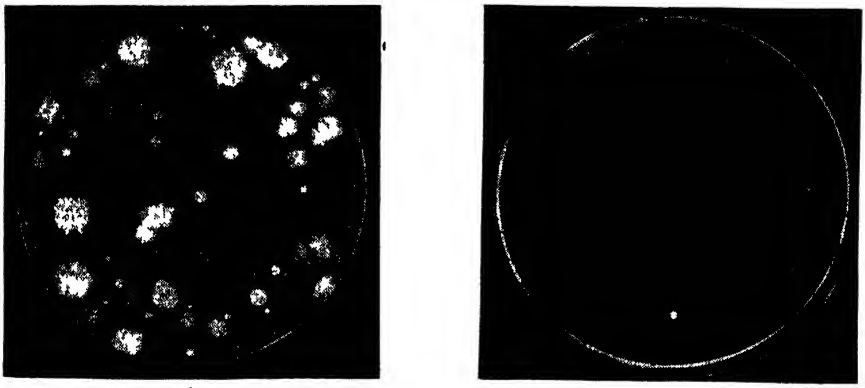
The destruction of disease organisms in milk without resorting to complete sterilization is called "pasteurization." Pasteurization refers to the process of heating every particle of milk or milk products to at least 143°F. (62°C.), and holding at such temperature for at least 30 min., or to at least 160°F. (71°C.), and holding at such temperature for at least 15 sec., in approved and properly operated equipment.

During pasteurization, the milk must be kept in constant agitation to prevent the formation of a scum on the surface. The scum acts as a protective layer around many of the bacteria by preventing the penetration of heat. The reaction of milk should be as nearly neutral as possible. If it is acid, a coagulation of the casein may occur during the heating process.

Gibson and Abdel-Malek (1940, 1941) and Abdel-Malek (1943) found that the microflora of certified milk consisted almost entirely of *Streptococcus* (*S. lactis*, *S. agalactiae*), *Micrococcus*, and *Microbacterium*, with an occasional sample showing *Pseudomonas fluorescens*. After pasteurization,

the microflora consisted only of *Microbacterium* and, in certain samples, a smaller number of *Micrococcus*. The *Streptococcus* was destroyed. In milk produced without care, the principal organisms that survived the treatment were *Streptococcus*, *Microbacterium*, and *Micrococcus*, in the order named. Organisms of other groups were of little importance in the processed milk unless introduced into the plant. The occurrence of large numbers of *Microbacterium* in pasteurized milk has been overlooked in the past, owing largely to the fact that these organisms do not grow on agar plates incubated at 37°C.

The pasteurization process reduces the bacterial count from 90 to 100



*A* *B*

FIG. 191.—Pasteurization of milk. *A*, 1 cc. of a 1:10 dilution of milk before heating; *B*, 1 cc. of a 1:10 dilution after heating.

per cent (Fig. 191), depending upon the kinds and numbers present at the time of heating.

**Phosphatase Test.**—The phosphatase test is employed to determine the efficiency of the pasteurization process. The test is based on the property of the heat-labile enzyme phosphatase to liberate phenol from phenyl phosphoric ester which, in turn, is measured quantitatively by adding 2,6-dibromoquinonechloroimide (BQC) to form an indophenol blue. Any blue color developed in the test tube is extracted with butyl alcohol and compared with a series of permanent standards.

The enzyme is always present in raw milk. When milk is heated to a temperature of 143°F. for 30 min., 96 per cent of the phosphatase is destroyed; heating above 145°F. (63°C.) for 30 min. ensures complete inactivation.

The quantitative determination of the enzyme reveals either faulty pasteurization or the subsequent addition of raw milk. The amount of phosphatase present may be easily and quickly measured colorimetrically. Disease organisms likely to be present in milk are killed at a temperature

lower than that required to destroy the enzyme. Therefore, a heat-treatment adequate to destroy the phosphatase should ensure a milk that is free from the common pathogenic bacteria.

For more information on milk, see Eckles, Combs, and Macy (1943), Guthrie (1943), Hammer (1938), Prescott and Dunn (1940), and Prescott and Proctor (1937).

## BUTTER

Butter is composed of milk fat, water, casein, lactose (milk sugar), and salt. The water content varies from 10 to 16 per cent by weight. The amount of casein and lactose present depends upon the extent to which butter is washed during the process of manufacture. From 1 to 3 per cent of salt is usually added, which is completely dissolved in the water. Since the salt does not dissolve in the butter fat, the liquid portion of butter consists of a 10 to 30 per cent solution of salt.

Butter was originally prepared by churning fresh, sweet cream, either raw or pasteurized, to separate the fat globules from the other constituents. This method is no longer practiced to any extent because it necessitates churning daily while the milk or cream is still fresh. It is the practice now to allow the milk or cream to sour first, after which the butter fat may be more easily separated from the casein.

The cream may be soured naturally or by the addition of a culture of lactic acid and other organisms known as a starter. The advantages of first souring the cream are (1) the yield of butter is increased, owing to a better separation of the casein and fat and (2) the aroma and flavor may be greatly improved.

If fresh, sweet cream is used, the butter will have a mild flavor, consisting chiefly of the natural flavor of milk fat. If the cream is carelessly handled and not cooled to a sufficiently low temperature, the organisms will multiply at a very rapid rate. The resulting aroma and flavor will depend largely on the organisms predominating in the cream. Since the acidity produced is not likely to be sufficient to suppress the growth of the putrefactive organisms, the flavor and aroma might be undesirable.

**Butter Cultures.**—Butter cultures consist of a mixture of two types or organisms: (1) those producing a high acidity (lactic acid) and (2) those imparting the characteristic aroma and flavor to butter.

*Lactic Acid Type.*—The lactic acid type generally consists of *Streptococcus lactis*, the organism responsible for the normal souring of milk. Another organism of this type is *S. cremoris*. These organisms produce relatively large amounts of lactic acid from the lactose of milk, together with small amounts of secondary products. Growth of the organisms in milk does not result in a product having a butter culture flavor. However, the compounds formed by the lactic acid organisms greatly influence the

action of the flavor bacteria, resulting in a product having a more pronounced aroma and flavor.

The lactic acid organisms produce from 0.7 to 1.0 per cent lactic acid in milk, with a maximum of about 1.2 per cent. These percentages correspond to a pH range of about 4.3 to 5.0.

*S. cremoris* is generally larger than *S. lactis*. It forms long chains in milk. The cells are Gram-positive. *S. cremoris* ferments the lactose of milk with the production chiefly of lactic acid. The optimum growth temperature is about 30°C. Its thermal death point is 65 to 70°C. in 10 min. The organism grows in a 2 per cent but not in a 4 per cent salt solution. It grows poorly on artificial media. *S. cremoris* has been isolated from raw milk and milk products. It is commonly employed in commercial starters in the manufacture of butter and cheese.

The characteristics of *S. lactis* are given on page 497.

*Aroma and Flavor Type.*—The organisms responsible for the aroma and flavor of butter consist of a mixture of *Leuconostoc dextranicum* and *L. citrovorum*. They produce very little, if any, lactic acid but are capable of attacking the citric acid of milk with the formation of acetic acid, possibly some formic and propionic acids, carbon dioxide, acetylmethylcarbinol ($CH_3 \cdot CO \cdot CHOH \cdot CH_3$) and diacetyl ($CH_3 \cdot CO \cdot OC \cdot CH_3$). Diacetyl in high dilution suggests the odor of butter. Acetylmethylcarbinol in pure form is odorless; in the impure state, it gives off an odor not unlike that of diacetyl. Cultures having a satisfactory aroma and flavor contain relatively large amounts of these two compounds, whereas those cultures not having a satisfactory flavor contain deficient amounts of the above compounds.

The aroma and flavor of butter is dependent upon the citric acid content of milk. Milk normally contains about 0.2 per cent citric acid. The addition of more citric acid to milk results in a great increase in the concentration of acetylmethylcarbinol and diacetyl with a corresponding increase in flavor.

*L. dextranicum* occurs as spheres, and in pairs and short chains. The cells are Gram-positive. The organisms grow best at 21 to 25°C. *L. dextranicum* is found on plant materials and in milk products. It is frequently employed in dairy starters.

*L. citrovorum* is similar to *L. dextranicum*. It grows best at 20 to 25°C. The first flavor compound produced is believed to be acetylmethylcarbinol. This is oxidized to diacetyl. Under conditions of high acidity, acetylmethylcarbinol and diacetyl accumulate in the milk. Under conditions of low acidity and suitable temperature, some acetylmethylcarbinol is reduced to 2:3-butylene glycol ($CH_3 \cdot CHOH \cdot CHOH \cdot CH_3$). The citric acid fermentation may be represented as shown in Fig. 192.

Elliker (1945) showed that the loss of aroma of butter, butter sub-

stitutes, and other food products was due to the action of microorganisms on the diacetyl content. Members of the genus *Pseudomonas* were found to be active in destroying diacetyl with accompanying loss of flavor. The studies emphasized the importance of keeping butter and other products containing diacetyl free from contaminating organisms.

*Preparation and Use of Butter Culture.*—The butter culture is prepared by pasteurizing milk at a temperature of 70 to 85°C. for 30 min., then

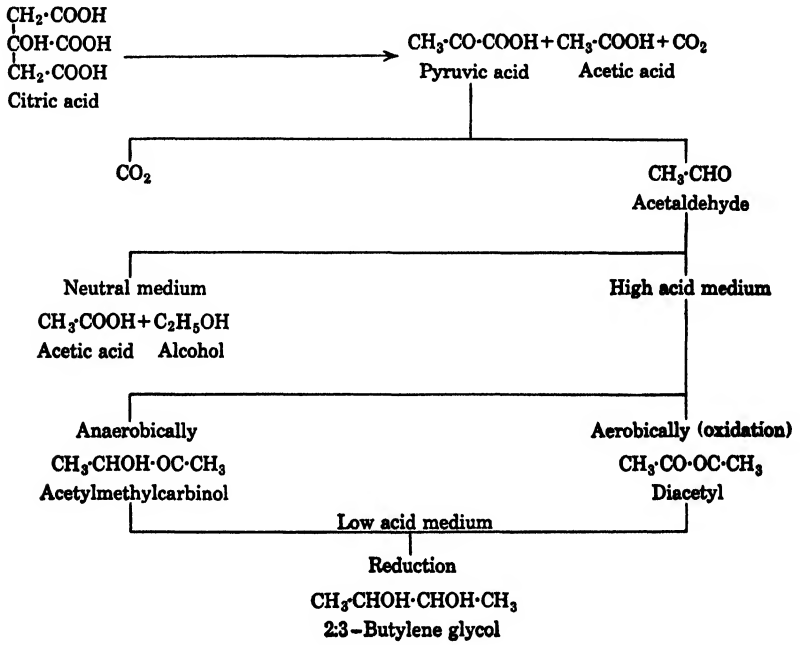



FIG. 192.—The citric acid fermentation.

inoculating with the desired organisms. After the starter is prepared, it should be handled with great care to prevent entrance of organisms likely to produce undesirable changes. Butter cultures are commonly ripened at a temperature of 21.1 to 22.2°C. for maximum development of aroma and flavor. The flavor compounds are not produced in significant amounts until the butter culture has an acidity of about 0.8 per cent. Usually from 0.25 to 1 per cent butter culture is added to cream that is to be ripened for the preparation of butter.

**Undesirable Changes in Butter.**—Butter prepared from sweet, unpasteurized cream contains the same microflora as the cream from which it was prepared. Also, the bacterial changes that take place during storage are the same as those which occur in milk kept under the same conditions. Butter prepared from cream previously pasteurized at high temperatures,

and then inoculated, generally contains only those organisms that were added to promote ripening. Some molds and yeasts may be present that result from air contamination. Since molds are strongly aerobic, they grow only on the surface of butter.

Undesirable changes that take place after butter has been manufactured are due largely to the activities of microorganisms. Many of the organisms responsible for producing defects in butter are present as a result of contamination after its manufacture. Therefore, the same precautions used in handling milk and cream apply in the handling of butter. The extent of recontamination is roughly an indication of the care exercised in handling the butter.

*Rancidity of Butter.*—The first stage in the appearance of rancidity is a hydrolysis of the glycerides, comprising the fat, to glycerol and fatty acids. Many organisms, both aerobic and anaerobic, have been shown to be capable of producing the reaction.

*Fishiness in Butter.*—Fishiness in butter is due to high acidity and to the decomposition of lecithin, resulting in the formation of trimethylamine. Lecithin is composed of fatty acids, glycerol, phosphoric acid, and choline (see page 273). Trimethylamine originates from the base choline. The organism *Proteus ichthyosmius*, first isolated from a can of evaporated milk having a fishy odor, is capable of producing the same effect when inoculated into fresh milk. The organism is a Gram-negative, motile rod, occurring as single cells. It does not ferment lactose. The optimum temperature for growth is 20°C.

*Tallowiness in Butter.*—A tallow-like odor is due largely to oxidation. This may result from the action of the ultraviolet rays of sunlight or of the oxidases naturally present in cream. It has been said that certain mold enzymes are capable of producing a similar effect. The changes may be prevented by high-temperature pasteurization, which results in a destruction of the oxidizing enzymes.

**Quantitative Bacteriological Examination of Butter.**—Butter is not a favorable medium for the growth of most bacteria. Multiplication usually occurs only in the small droplets of water containing the dissolved salt and possibly some lactose and casein. Since the high salt content makes this an unfavorable medium, butter never shows bacterial counts as high as those obtained from milk. The count is highest in freshly prepared butter and becomes less and less as the butter ages.

The bacterial flora of the surface of butter differs from that of the interior, owing to contamination from the air and differences in the oxygen tensions. For these reasons, it is difficult to obtain a representative sample for examination. A sample is usually obtained by removing a cylinder of butter from a cake by means of a sterile sampler. The butter is melted in a water bath at a temperature not to exceed 40°C. and dilutions pre-

pared in sterile water blanks, previously heated to the same temperature.

For more information on butter, see Babel and Hammer (1944), Hales (1945), Hammer and Babel (1943), Hedrick and Hammer (1942), and Hoecker and Hammer (1944, 1945).

## ICE CREAM

Ice cream is a frozen dairy product composed of cream, sugar, gelatin, and flavoring. Sometimes condensed milk is also added. Unlike the preparation of butter and cheese, bacteria play no part in the process of manufacture.

The bacterial content of ice cream depends largely upon (1) the number present in the cream at the time of preparation and (2) the number of organisms present in the various ingredients employed in its manufacture. It has been shown that some bacteria actually multiply in ice cream kept in cold storage. The numbers decrease at first, then show a gradual increase after a period of about a month.

The lactic acid organisms, *i.e.*, those which are responsible for the souring of cream, fail to multiply at low temperatures. An increase in numbers indicates the presence of other species, such as the putrefactive types. These organisms may possibly produce objectionable metabolic waste products. The presence of pathogenic organisms in ice cream is usually the result of using contaminated cream in its manufacture.

## CHEESE

According to the standards of the Food and Drug Administration of the U.S. Department of Agriculture, cheese may be defined as

> . . . the product made from the separated curd obtained by coagulating the casein of milk, skimmed milk, or milk enriched with cream. The coagulation is accomplished by means of rennet or other suitable enzyme, lactic fermentation, or by a combination of the two. The curd may be modified by heat, pressure, ripening ferments, special molds, or suitable seasoning.

The solid curd is molded into various shapes according to the variety of cheese being manufactured. Freshly prepared and molded curd is known as green cheese. In order that it be made satisfactory for consumption, it must be set aside to ripen. Certain conditions, such as temperature and moisture, are carefully controlled during the ripening process. The cheese changes considerably during this stage. The insoluble casein is rendered soluble, and the digestibility is greatly improved. The consistency changes, resulting in a softer product. Also, the flavor characteristic of the finished product develops during the ripening period.

Two general processes are used for the preparation of curd: One is due

to the action of enzymes; the other is associated with the growth of organisms. The latter is indirectly also an enzymatic process. The cheeses in the first group are known as rennet curd cheeses; those in the second group, as acid curd cheeses.

As has already been said, the changes that take place during ripening are largely enzymatic. However, this does not explain all the changes that occur during the long aging period. The flavors that make their appearance during the latter period of the ripening process are not the result of enzymatic action but of the associated activities of bacteria, yeasts, and molds. The enzymes improve the consistency and digestibility of cheeses but play no part in improving the flavors. The flavors and characteristics of the various cheeses depend upon the kinds and numbers of organisms present.

**Hard Cheeses.**—Hard cheeses are prepared from curd subjected to heavy pressure to remove as much of the whey as possible. This gives a very hard, tough curd, which does not become softened to any extent during the ripening period. Examples of hard cheeses are American (Cheddar) cheese, Swiss cheese, Cheshire cheese, and Edam cheese. Since the curd is very compact and tough, the ripening stage requires a considerable period of time to produce a satisfactory product. Enzymatic and bacterial changes proceed simultaneously.

Several phases may be recognized during the ripening period. In the first phase, the lactic acid bacteria multiply more rapidly than the other species present, resulting in a predominance of these organisms. This continues for several days, after which the numbers show a gradual decrease. At the end of the ripening period, the number of lactic acid bacteria may be smaller than at the beginning of the process.

**Desirable Organisms.**—Many organisms are responsible for the aromas, flavors, and characteristics of the various types of cheeses. Apparently each type has its own characteristic flora. Some of the organisms that have been isolated from cheeses are (1) *Streptococcus lactis* (page 497), (2) *S. cremoris* (page 515), (3) *S. thermophilus*, Gram-positive spheres, occurring singly and in short chains, optimum temperature 50°C., ferment lactose with the production chiefly of lactic acid, (4) *Leuconostoc citrovorum* (page 516), (5) *L. dextranicum* (page 516), (6) *Lactobacillus casei*, nonmotile, Gram-positive rods, ferment lactose with the formation chiefly of lactic acid, optimum temperature 30°C., (7) *L. lactis*, Gram-positive rods, occurring singly, in pairs, and chains, produce lactic acid, optimum temperature 40°C., (8) *L. bulgaricus*, large, nonmotile, Gram-positive rods, appearing singly and in chains, ferment lactose vigorously, optimum temperature 45 to 50°C., commonly employed as a starter in the manufacture of butter and cheese, (9) *L. helveticus*, large, nonmotile, Gram-positive rods, occurring singly and in chains, ferment lactose largely to lactic acid, optimum temperature 40 to 42°C., commonly employed as a starter in the manufacture

of butter and cheese, (10) *L. plantarum*, large, nonmotile, Gram-positive rods, occurring singly and in short chains, produce lactic acid from lactose, optimum temperature 30°C., (11) *Propionibacterium shermanii*, small spherical cells, mostly in pairs and short chains, nonmotile, anaerobic, Gram-positive, produce lactic acid from lactose.

**Undesirable Organisms.**—The presence of undesirable organisms is responsible for numerous types of faulty cheeses. The milk becomes contaminated through carelessness in collecting and handling. Considerable losses are experienced at times by cheese manufacturers. For this reason, it is generally advisable to use milk previously pasteurized and then inoculated with the desired organism or organisms rather than to start with unpasteurized or raw milk. Some cheese faults affect the taste, others are concerned with the appearance of the finished product. One of the most common faults is swollen or blown cheese, due to fermentation of lactose with the production of acid and gas. The gas bubbles cause the cheese to swell until it may actually burst. Unpleasant flavors are produced by the organisms. Coliform organisms are usually involved.

The presence of putrefactive organisms may be responsible for putrid odors and flavors. The bacteria proliferate and become active when the acidity of the cheese is reduced during the ripening period. The presence of chromogenic organisms may be responsible for discolorations in cheese. This may be due also to various chemical reactions with metals, such as copper and iron, from utensils used in handling the raw materials. Blue spots in Edam cheese are said to be due to *Bacillus cyaneofuscus*. The organisms die before the cheese has fully ripened. Red and brown spots in Emmentaler cheese are caused by the growth of chromogenic propionic acid bacteria. *Lactobacillus brevis* var. *rudensis* is responsible for the appearance of rusty spots in Cheddar cheese. Since it is particularly prevalent in the spring, the organism is believed to originate from green grass. Surface discolorations are produced by many molds such as *Penicillium casei*, *Cladosporium herbarum*, *Monilia niger*, and *Oöspora crustacea*. Red and yellow torulae (false yeasts) play some part in the process.

**Soft Cheeses.**—Soft cheeses are prepared by allowing the whey to drain from the curd without the application of pressure. Cheeses prepared in this manner contain more moisture than hard curd cheeses and result in a much softer finished product. The first stage in the preparation of soft cheeses is a fermentation of the lactose by means of lactic acid bacteria with the formation of an acid curd. Subsequent changes depend upon the control of certain well-defined conditions.

In the Camembert types, the surface of the cheese is kept dry. This furnishes an excellent opportunity for the growth of certain molds such as *P. camemberti* and *Oidium lactis*. Enzymes secreted by *P. camemberti* act upon the surface of the cheese to produce a slow liquefaction of the casein. The enzymes gradually penetrate the product until the whole of

the curd is affected. The result is the formation of a soft, creamy mass at the completion of the ripening period. The characteristic flavor is due probably to the growth of *O. lactis*.

In cheeses of the Roquefort type, the blue-green mold *P. roqueforti* and related species are inoculated into the curd. Since the organisms are aerobic, holes are punched in the curd to facilitate development of the mold throughout the cheese. The enzymes elaborated by the organisms soften the casein, and certain metabolic products produce the characteristic aromas and flavors.

Cheeses of the Limburger type are produced by keeping the surface wet to prevent the growth of molds and to stimulate the growth of bacteria. The ripening process involves the decomposition of casein by enzymatic and bacterial action.

For information on the pH of cheese and its relation to quality, see the report by Irvine (1944).

## FERMENTED MILK

Milk is probably the most important article of food among many pastoral tribes of Europe and Asia. Because of the primitive sanitary conditions under which the people live, the milk is usually fermented before it is consumed. This is especially true during the warm seasons of the year.

The consumption of soured milk preparations is widespread because of their supposedly therapeutic value. The fact that they appear under various names does not mean necessarily that each product is fermented with a different organism. The names identify the country or region in which they are produced. Many of these preparations result from the combined action of two or more organisms. They furnish excellent illustrations of associations.

**Yoghurt.**—The soured milk preparation of Bulgaria and Turkey is known as yoghurt. This is usually prepared from camel's or mare's milk. The fermentation is produced by acid-forming organisms of the *Lactobacillus bulgaricus* type. Sometimes yeasts are also present and produce a small amount of alcohol and carbon dioxide. The organisms produce from 1.5 to 2.5 per cent acid calculated as lactic acid.

**Matzoon.**—This is the soured milk preparation of Armenia and is similar to yoghurt in flavor and microflora.

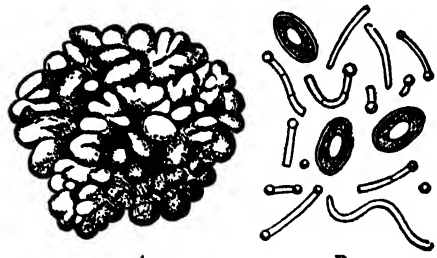
**Gioddu.**—Gioddu is the fermented milk preparation prepared on the island of Sardinia. It contains the same organisms as Bulgarian yoghurt and Armenian matzoon.

**Leben.**—The Egyptian drink known as leben is prepared by the action of lactic acid-producing bacteria and yeasts on cow's, goat's, or buffalo's milk. The bacteria hydrolyze the lactose to glucose and galactose, after

which some of the sugar is fermented by the yeasts to alcohol and carbon dioxide, and some attacked by the bacteria with the production of lactic acid.

**Kumiss.**—Kumiss is a Russian product prepared by the fermentation of mare's or cow's milk by yeasts, lactobacilli, and lactic streptococci. The yeasts produce alcohol and carbon dioxide, and the bacteria produce lactic acid.

**Kefir.**—Kefir is prepared by inoculating milk with kefir grains. These grains resemble minute cauliflowers and are composed of *Saccharomyces kefir*, *Lactobacillus casei*, and streptococci. The yeast produces alcohol and carbon dioxide; the bacteria produce lactic acid (Fig. 193).



*A* *B*

FIG. 193.—*A*, Kefir grain. *B*, yeasts and bacterial cells present in the grains. (*After Freudenreich; from Lipman's "Bacteria in Relation to Country Life," The Macmillan Company, New York.*)

**Curds.**—The fermented milk preparation of Ceylon is usually referred to as curds. It is manufactured from cow's or buffalo's milk. The milk is boiled, cooled, and, while still warm, inoculated with a piece of curd from a previous lot. The milk is allowed to ferment for at least 36 hr. before it is eaten. The organisms responsible for the reaction include yeasts, *Streptococcus lactis*, and a Gram-negative *Lactobacillus*. In most samples, the yeast and *S. lactis* predominate. The preparation is similar to that of kumiss and kefir.

**Bulgarian and Acidophilus Milk.**—Yoghurt is consumed in large quantities as an article of diet by the people of Bulgaria. Metchnikoff (1908) noted that centenarians were more numerous in Bulgaria, in proportion to population, than in other countries. He believed that the increase in the life span was due to the ingestion of large quantities of soured milk, produced by the action of the rod-shaped Gram-positive organism *L. bulgaricus* (see page 520). Because of this fact, Metchnikoff advocated the consumption of Bulgarian milk for the prolongation of life.

According to Metchnikoff, growth of *L. bulgaricus* in the intestinal tract produced a high percentage of lactic acid, which was capable of inhibiting the growth of the putrefactive bacteria. Disorders that were supposed to be associated with autointoxication (absorption of putrefactive metabolic waste products from the intestinal tract) would be prevented.

*L. bulgaricus* is not a normal inhabitant of the intestinal tract of man and, therefore, does not become acclimated to the new environment. It was shown that the consumption of Bulgarian milk stimulated the growth of *L. acidophilus*, an organism normally present in the intestinal tract of adults.

Moro (1900) first isolated *L. acidophilus* from the feces of breast-fed infants. It is present in the intestinal contents of adults. On a mixed diet, the numbers are small. If the diet is supplemented with large quantities of milk or carbohydrates, such as lactose or dextrin, the numbers are greatly increased. The organisms ferment the carbohydrate with the production of a high concentration of lactic acid (about 3 per cent) which is sufficient to inhibit the growth of the putrefactive types (*Escherichia coli*, etc.). In the absence of a high carbohydrate diet, the flora again becomes predominantly putrefactive in character. The numbers are also increased by the ingestion of milk fermented by *L. acidophilus*, especially when taken with lactose or dextrin to increase the fermentable constituents in the intestinal tract.

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# CHAPTER XXII

## BACTERIOLOGY OF FOOD

Food may be preserved indefinitely if kept free from organisms, or if the organisms are prevented from multiplying. Decomposition and spoilage of food are the result of the activities of living organisms, particularly those grouped with the bacteria, the yeasts, and the molds.

Different kinds of organisms produce different types of changes in food. The decomposition of foods rich in carbohydrates results usually in various types of fermentations. The action of organisms on high-protein foods results in putrefactions. The products of the former are usually harmless, whereas those of the latter are objectionable and even dangerous.

Bacteria are more exacting in their requirements than either the yeasts or the molds. This means that yeasts and molds can multiply under conditions unfavorable to the growth of bacteria. Bacteria require relatively large amounts of moisture, hydrogen-ion concentrations usually near the neutral point, and relatively low osmotic pressures, for growth and multiplication. Yeasts can tolerate less moisture, are less exacting in their pH requirements, and can multiply in solutions having higher osmotic pressures. Molds are the least exacting of the fungi. They can withstand relatively high acidities, require far less moisture, even grow on substances almost dry, and can tolerate extremely high osmotic pressures.

### METHODS EMPLOYED FOR THE PRESERVATION OF FOOD

The methods used at the present time were employed long before their modes of action were clearly understood. As their mechanisms became known, various improvements were made. The methods commonly employed for the preservation of food involve the use of (1) heat, (2) cold, (3) drying, (4) preservatives, and (5) high osmotic pressures.

**Heat.**—The use of heat is the method employed in home and commercial canning of meats, fruits, and vegetables. Heat is used to effect either a complete sterilization or a reduction in the number of organisms that may be present. In the latter case, the organisms that have not been killed are prevented from multiplying. Excessive heat is efficient in destroying all forms of microscopic life. The destructive action of heat is due probably to the coagulation of the protoplasm of living cells, rendering it incapable of carrying on its vital functions. It is not desirable greatly to exceed the minimum temperature required to effect sterilization, otherwise

alterations may occur in the appearance, flavor, and composition of foods. Since all bacteria are not necessarily killed, the term "processing" is generally employed in referring to heat-treated canned foods.

The diverse details of canning procedures necessarily vary with the nature of the product to be preserved. There are certain important operations common to all canning procedures: (1) cleansing operations, (2) blanching, (3) exhausting or preheating, (4) sealing the tin container, (5) heat-processing the sealed container, and (6) cooling the tin container after thermal processing (Fig. 194).

*Cleansing Operation.*—The first and one of the most important steps in commercial canning is the thorough cleansing of the food materials to be preserved. Cleansing serves two purposes. It makes a better looking product, and it serves to reduce substantially the load of spoilage bacteria that may place a heavy burden on the heat process.

Cleansing may be effected by various types of washers. The raw materials are subjected to high-pressure sprays or strong-flowing streams of water while passing along a moving belt or while being dropped in agitating or revolving screens. With certain food materials, dirt and other large, adhering particles are mechanically removed by means of revolving or agitating screens or by strong blasts of air.

*Blanching.*—The blanch involves the immersion of raw food materials (fruits and vegetables) into warm or hot water, or exposure to live steam. This is practiced for several reasons. Blanching may serve only as a hot-water wash where adhering materials cannot be removed with cold water. It may soften fibrous plant tissue so that it will either contract (lose water) or expand (take up water). This ensures proper filling of the container. During the blanching operation, respiratory gases are expelled. This prevents strain on the can during processing and favors the development of a higher vacuum in the finished product. Blanching inhibits the action of respiratory enzymes, especially those of oxidation, to give a product of superior quality and nutritive value. Lastly, blanching fixes the natural color of certain products and makes them more attractive in appearance.

*Exhausting or Preheating.*—All canning procedures provide for the exclusion of as much oxygen (air) as possible. The presence of oxygen is undesirable for two reasons: (1) It may react with the food material and the interior of the container and affect the quality and nutritive value of the food. (2) The presence of oxygen and other gases may cause undue strain on the container during the processing period.

The procedure followed in expelling gases consists of passing the open can, containing the raw food, through an exhaust box in which hot water or steam is used to expand the food and expel air and other gases from the contents and the head-space area of the can. After the gases are ex-

pelled, the can is immediately sealed, heat-processed, and cooled. During the cooling, the contents of the can contract, creating a vacuum. This is

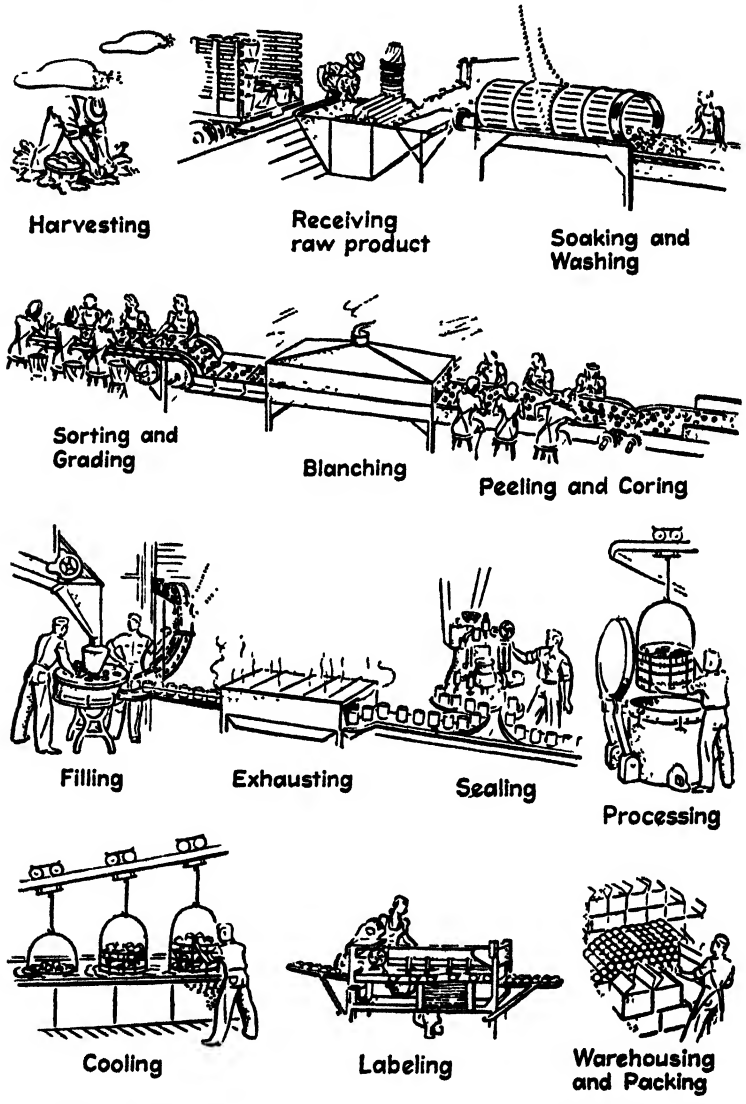


FIG. 194.—Basic operations in commercial canning. (*From "The Canned Food Reference Manual," American Can Company.*)

accepted as evidence of soundness of the canned product.

With some products, the same effect is produced by preheating the food in kettles, filling into cans while still hot, and immediately sealing

the containers. With other products, an exhausting effect is produced by adding boiling water, sirup, or brine to the food in the tin. With still other products, exhausting is accomplished by mechanical means rather than by the use of heat. Special machines are used for withdrawing the air from the cans and sealing at the same time. This latter process is known as vacuum packing.

*Sealing the Tin Container.*—Each can must be properly sealed before being subjected to the heat process. The heat destroys any organisms present in the raw food material, and the seal on the can prevents reinfection of the contents. The sealing operation is, therefore, one of the most important steps in the canning procedure.

*Heat-processing the Sealed Container.*—The processing operation usually involves the application of steam under pressure (autoclave). This destroys pathogenic and other organisms capable of causing spoilage of the contents. The seal on the can prevents the contents from becoming recontaminated by the same or other kinds of organisms.

The time required for processing canned foods depends upon various factors, such as character and composition of the food, types and numbers of organisms likely to be present, and hydrogen-ion concentration of the food. Heat penetrates to the center of cans by conduction and convection. Penetration of solid foods by heat takes place by conduction and is relatively slow. Penetration of liquid foods takes place by conduction and convection, with the result that the action is more rapid. The size of the food particles also influences the speed of penetration by heat. The larger the particles, the slower the penetration.

Bacteria are usually more easily killed in an acid or an alkaline environment than in a neutral one. Fruits and vegetables are, therefore, more easily processed than fish and meats. Also, fruits and vegetables are more easily penetrated by heat than are meats and fish. The temperature and time of processing must be determined for each kind of food. In general, nonspore-forming organisms in a liquid medium are destroyed at a temperature of 60°C. for 1 hr. or at 70 to 80°C. in a few minutes. Spores are not destroyed when subjected to the above temperatures. A temperature of 115°C. for 30 min., or 120°C. for 15 min., will usually destroy all forms of life.

Spiegelberg (1940*a*,*b*), in his studies on the spoilage of canned pineapple, concluded that at a pH of 4.5 or below, a temperature of 190°F. in the fruit following the cooker was adequate to ensure sterility. At a pH above 4.5, a temperature of 200°F. was required to eliminate nonspore-forming types of swells (*Lactobacillus plantarum*, *Leuconostoc mesenteroides*, and three unnamed types), whereas butyric swells (*Clostridium butyricum*) persisted with even much higher temperatures.

In the processing of foods, an excessive period of heating is avoided to

prevent injury to the product. A long exposure at a relatively low temperature is usually preferable to a short exposure at a higher temperature. This applies especially to canned fruits.

*Cooling the Tin Container after Thermal Processing.*—The last operation in the commercial process involves rapid cooling of the sealed cans. This is necessary in order to check the action of the heat and prevent undue softening or change in color of the contents. The cans may be cooled by means of air or of water.

Air cooling is accomplished in well-ventilated, specially designed, storage rooms where the cans are stacked in rows with ample space for efficient circulation of air. Water cooling is accomplished by allowing water to run into the autoclave in which the cans are processed, or the cans may be removed from the sterilizer and conveyed through tanks of cold water or through cold-water showers.

**Cold.**—Two methods are followed in the preservation of foods by cold temperatures: (1) cold storage and (2) chilling. Both methods have their advantages and disadvantages.

In the cold-storage method, the temperature is kept well below the freezing point. The food is frozen solid, producing some changes in its physical condition. Meats, fish, and poultry are usually preserved in this manner for long periods of time.

The food is frozen either in cold air or immersed in salt solution and then frozen. In the air method, the food is held at a temperature of about −15°C. A long time is required in freezing the food, and considerable moisture is lost by evaporation. The addition of salt to water lowers the freezing point. A salt concentration of 25 to 28 per cent lowers the freezing point to about −15°C. An important advantage of the brine process over the air method is that freezing takes place in a shorter time. An important disadvantage is that some substances are likely to diffuse out of the food and be replaced by salt.

After the food is frozen by either of the two methods, it is stored in a chamber where the air is kept below 0°C. Certain changes are known to take place during the storage period. Some water is lost by evaporation. Aromatic substances that impart flavor and odor to the food may disappear entirely. Fats and oils may be slowly hydrolyzed to glycerol and fatty acids. Microorganisms may slowly multiply on the surface of the food. The extent of these changes depends upon the temperature of the storage chamber, the relative humidity, and the air currents in the room.

In the chilling method, the temperature is kept just above the freezing point. This is the condition encountered in the usual ice or electric refrigerator in the home. The physical state of the food is unaltered. Eggs, vegetables, and fruits are better chilled rather than frozen solid.

Cold storage is preferable to chilling from a bacteriological standpoint, because there is less bacterial action when the food is frozen solid. Chilling

retards but does not prevent bacterial action. Chilled foods cannot be kept for many weeks.

Cold does not kill all bacteria, but it does slow down their generation times. This means that the organisms increase in numbers at a very slow rate. Few bacteria are capable of multiplying below 10°C. These low-temperature organisms are generally referred to as psychrophilic (cold-loving) forms.

Molds are able to multiply under conditions that prevent the growth of bacteria. Some species of molds are capable of growing on the surface of meat kept at temperatures below the freezing point, provided sufficient moisture is present in the air. In the absence of sufficient humidity, the molds lose moisture and die. For the prevention of mold growth, it is necessary to keep the temperature and humidity low. The temperature and humidity necessary to prevent the growth of molds vary with different types of foods.

Molds do little damage to foods. The chief objection to their presence is that they produce considerable discoloration. Under some conditions, their metabolic waste products may leave unpleasant tastes and flavors in the food but they are usually harmless.

**Drying.**—The preservation of foods by drying or dehydration is of ancient origin. Although the process was not greatly employed at first, it is now of great industrial importance. Practically every type of food is now prepared in dehydrated form, including nuts, vegetables, fruits, eggs, milk, fish, meat, and soups.

Dehydrated foods are probably never sterile. In this respect they differ from most canned foods. It is, therefore, of the utmost importance to prevent entrance of organisms capable of producing food poisoning, especially the toxigenic forms, such as *Clostridium botulinum* and certain strains of *Micrococcus pyogenes* var. *aureus*. Dehydrated foods should also be free from certain intestinal forms likely to be pathogenic to man by mouth, *e.g.*, members of the genera *Salmonella* and *Shigella*. The bacterial count of dried foods should be reasonably low so that no decomposition or development of undesirable flavors occurs during the period of reconstitution.

In order to prevent in food the formation of bacterial toxins and the development of organisms pathogenic to man, Haines and Elliot (1944) stated that the product should be dried at a temperature at which significant bacterial growth is unlikely to occur. They concluded that 50°C. is the minimum temperature below which dehydration should not be carried out. Where some heating below this temperature is unavoidable, owing to loss of quality in the product, the period of such heating should not exceed 4 hr. Haines and Elliot found that bacterial growth did not occur in foods when the water content was below 15 per cent.

Dehydration of foods is a valuable procedure for several important

reasons. Dried foods may be easily preserved for future use. This means that certain foods may be utilized over longer periods of time rather than for only a short season of the year. Dehydration greatly reduces the bulk of a product, conserves space, and facilitates handling. This is a decided advantage from the standpoint of transportation costs. Most of the dehydrated products, if properly prepared, are very good substitutes for fresh foods, being detected from the normal product with difficulty. Dried foods do not require sterilization or the maintenance of sterile conditions during preparation. They are more economical to use since no waste is involved. Only that amount necessary for use at one time need be prepared.

The use of dehydrated foods also presents several decided disadvantages. Dried products require a long soaking period to restore the water lost by evaporation. The period required for rehydration varies with different foods. If this is not carefully done, the results are likely to be unsatisfactory. Sometimes dehydrated foods become infested with insects owing to improper packaging or handling. Sometimes dried foods become moistened, with the result that conditions become favorable for the growth of bacteria, yeasts, and molds. This applies more especially to the hygroscopic foods or those which readily absorb moisture from the air.

**Preservatives.**—Sometimes chemicals are added to foods to preserve them. These act either by killing the organisms or by merely preventing them from multiplying.

An ideal antiseptic would be one that killed microorganisms or prevented them from multiplying without producing any harmful physiological effect. Apparently such a compound is not yet known. All the commonly used preservatives exert some physiological action on the human body and, unless employed in minute amounts, may produce harmful effects.

The inorganic chemicals commonly employed include boric acid and borates, nitric acid and nitrates, nitrous acid and nitrites, sulfurous acid and sulfites.

Boric acid is a weak antiseptic, saturated solutions being unable to destroy bacteria. However, it does prevent the growth of most bacteria and is used sometimes to preserve butter.

The color of fresh, unheated, muscle tissue is due to the presence of a red pigment known as myohemoglobin or myoglobin. This pigment is an integral part of the tissue that does not circulate in the blood stream.

Sodium nitrate and small amounts of sodium nitrite are usually added to salt solutions used for the pickling of meats. The nitrate was believed to react with the hemoglobin of the meat to produce nitric oxide hemoglobin. It is now known that the nitrite reacts with the myohemoglobin of muscle to produce nitric oxide myohemoglobin according to the reaction,

$$\text{NO} + \text{myohemoglobin} \rightarrow \text{nitric oxide myohemoglobin}$$

Some bacteria present on meat are able to reduce nitrate to nitrite. The same or other species produce small amounts of organic acids from the meat which convert the nitrate and nitrite to nitric and nitrous acids, respectively. The nitrous acid then reacts with the myohemoglobin to give nitric oxide myohemoglobin. An acid solution is necessary for the reaction to take place. This compound imparts a bright red color to meat, making it more attractive in appearance. Unlike fresh muscle tissue, cured meats retain their redness on cooking. The nitrate and nitrite also produce an inhibitory effect on the growth of bacteria likely to be present on meat. The nitrite has been shown to be more effective in this respect than the nitrate.

Hall (1935) recommended a new method for pickling meats. He used a pickling solution containing salt, nitrate, nitrite, and a small amount of citric acid or some other appropriate acid. The purpose of the acid is to convert the nitrate and nitrite to nitric and nitrous acids. The nitrous acid is then capable of reacting with myohemoglobin to produce the attractive red color.

Sulfurous acid and sulfites are added to alcoholic liquids, especially wines. The addition of sulfite to greenish, discolored meat restores the original red color to the product. It enjoyed great popularity as an addition to hamburger prepared from old, scrap meat. The use of sulfite for this purpose is now prohibited by law.

The organic chemicals added to foods include benzoic acid and benzoates, salicylic acid and salicylates, formaldehyde, and creosote.

Benzoic acid and benzoates are used for the preservation of vegetables. A small amount of sodium benzoate is sometimes added to tomato catchup. Salicylic acid and salicylates are used as preservatives of fruits and vegetables. Formaldehyde was formerly used as a preservative of milk and cream, but its use for this purpose is forbidden by law. The use of wood smoke in the curing of meats is due to the presence of small amounts of creosote furnished by the burning wood.

**High Osmotic Pressures.**—Some foods are protected from attack by microorganisms in the presence of appreciable amounts of moisture. This applies to those foods which contain high concentrations of sugar or salt. It is generally stated that these compounds act entirely by osmosis or the withdrawal of water from the protoplasm of the organism. This causes a shrinkage of the protoplasm, resulting in the death of the cell.

Rockwell and Ebertz (1924) found that the preserving effect of salt involved more than its dehydrating action. Magnesium sulfate has greater dehydrating effect on proteins than sodium chloride, yet is not as efficient in preventing growth of *Micrococcus pyogenes* var. *aureus*. They concluded that the preserving action of sodium chloride on proteins involved four additional factors: (1) the direct effect of the chloride ion,

(2) the removal of oxygen from the medium, (3) the sensitization of the test organism to $CO_2$, and (4) interference with the rapid action of proteolytic enzymes.

Practically no multiplication occurs in salt concentrations of 25 per cent or greater. Bacteria that are capable of resisting high osmotic pressures are called halophilic (salt-loving) organisms. A strength of 10 per cent markedly inhibits the growth of the great majority of bacterial species. The pathogenic or disease-producing organisms are less resistant to strong saline solutions than the saprophytic bacteria. Cane sugar in a concentration of 60 to 70 per cent usually prevents the growth of all types of microorganisms. Occasionally, molds may be seen growing on the surface of a closed jar of jam or jelly. This is due to the fact that water evaporates from the jam or jelly and, not being able to escape, condenses back to water producing a layer of less concentrated sugar solution on the surface. Some molds are capable of multiplying in this less concentrated solution.

Bacteria are not so sensitive to osmotic changes as are higher plant and animal cells. For this reason, solutions having extremely high osmotic pressures must be used either to kill bacteria or prevent them from multiplying.

## BACTERIOLOGY OF MEAT

There is still a controversial question concerning the presence of living bacteria in the tissues and blood of healthy individuals. Some investigators have reported the presence of organisms in living tissues; others have been unable to verify such findings. Burn (1934*a*,*b*) and Jensen and Hess (1941) brought forth evidence to show that the invasion of tissues by bacteria is agonal and postmortem rather than antemortem.

**Autolysis of Tissues.**—Frozen or cold-storage meats can be kept for long periods of time without showing any signs of spoilage. On the other hand, meats kept at higher temperatures (chilled) show spoilage in much shorter periods. The changes that take place result from the action of autolytic enzymes normally present in the meat and those elaborated by the contaminating organisms. Proteins are first hydrolyzed to amino acids and then putrefied with the liberation of bad odors. A short action of the proteolytic enzymes is beneficial in tenderizing meats, but a prolonged action will result in decomposition and putrefaction.

The bacteria found on meat surfaces are usually species of *Achromobacter* and *Pseudomonas*. The molds commonly present include species of *Thamnidium*, *Rhizopus*, and *Mucor*. These organisms, growing on meat, are probably not dangerous to health. The putrefactive changes produced by bacteria are objectionable from the standpoint of odor and taste, and the growth of molds lowers the appearance of meat.

Under ordinary conditions, beef is held at 2.2 to 3.3°C. for 5 days after slaughter before it is released. If the meat is to be ripened to increase its

tenderness and flavor, it is stored at low temperatures for longer periods. For example, beef held at the following temperatures and times shows the same degree of tenderness (Jensen, 1944):

21 days at 1.1°C.
8 days at 4.4°C.
5 days at 8.3°C.
3 days at 15.6°C.

Meats are also tenderized by the use of enzyme preparations. Many proteolytic enzymes are employed for this purpose. The enzyme is allowed to act until the meat shows the proper degree of tenderness. The meat is then heated to destroy the enzyme, otherwise it tends to become mushy and butyrous in texture during culinary heating. Meat in this condition is organoleptically undesirable.

**Bacterial Count of Meats.**—Weinzirl and Newton (1914*a*,*b*) examined samples of meats purchased in the open market and found that the numbers of aerobic organisms present varied from 270,000 to 88,000,000 per gram. This applied to ground meat as well as to tissue in larger pieces. They failed to judge the quality of meat by the number of organisms present. Others have reached a similar conclusion. There appears to be no correlation between the bacterial population and the sanitary quality of meat. This is owing to the fact that it is not so much the numbers as it is the kinds of organisms that determine the sanitary quality of meat. Some samples showing high counts of saprophytic organisms may produce no harmful effects when ingested; others showing low bacterial counts may produce harmful effects. Apparently, the best criteria for judging the quality of meat are appearance, feel, and smell. This is sometimes referred to as the organoleptic test.

**Bacterial Flora of Meats.**—Both aerobic and anaerobic organisms are concerned in the spoilage of meats, fish, and other high-protein-containing foods. The aerobic organisms act first and create an environment favorable to the growth of the anaerobes. Then the anaerobes attack the proteins, resulting in the liberation of foul-smelling compounds. The process of putrefaction in nature involves the action of both aerobes and anaerobes, but the changes that occur are chiefly anaerobic in character.

Many types of organisms concerned in putrefaction may be grouped as follows: (1) Gram-positive, aerobic, spore-bearing rods, (2) Gram-negative, aerobic, nonspore-forming rods, (3) cocci, (4) anaerobes, and (5) molds and yeasts. The Gram-positive, aerobic, spore-bearing group includes *Bacillus subtilis*, *B. albolactis*, and *B. mesentericus*. These organisms are saprophytes and are capable of liquefying gelatin very rapidly. The Gram-negative, aerobic, nonspore-forming group includes *Escherichia coli*, *E. coli communior*, *Proteus vulgaris*, *P. mirabilis*, *Aerobacter cloacae*, and *Pseudomonas fluorescens*. The members of the genus *Proteus* are

capable of initiating some of the changes produced by anaerobes. They are very proteolytic in their activities, being able to carry protein degradation further than any of the other aerobes in this group.

Some of the cocci that have been isolated include *Micrococcus pyogenes* var. *aureus*, *M. candidus*, *M. aurantiacus*, *M. candicans*, *M. saccatus*, *M. flavescens*, *M. roseus*, and *Sarcina aurantiaca*. All these organisms are Gram-positive. The most pronounced changes on meats are produced by the anaerobic, spore-bearing organisms. Some of the anaerobes that have been isolated from fresh and spoiled meat are *Clostridium perfringens*, *C. tertium*, *C. bifermentans*, and *C. sporogenes*. These organisms are responsible for putrefactive changes on proteins, resulting in the liberation of foul-smelling compounds. The molds that have been found growing on meat belong to the following genera: *Aspergillus*, *Penicillium*, *Mucor*, *Cladosporium*, *Sporotrichium*, *Alternaria*, and *Monilia*. Those belonging to the genera *Penicillium* and *Mucor* have been isolated with greater frequency than any of the others. Spores of such molds are commonly present in air. Several species of yeasts have been isolated from meat kept under refrigeration.

Molds are aerobic and grow on or near the surface of meat. Most molds produce pigments that impart discolorations to meat. Molds may be removed by wiping or trimming the surface layer with a knife. If molds are permitted to grow without being checked, they may impart unpleasant odors and flavors to meat.

**Dried Beef.**—Meat is dried in order that it may be preserved for long periods of time. Dried beef is prepared in the following manner: Beef sets are obtained from the carcasses of freshly slaughtered animals and kept under refrigeration until ready to be used. The meat is usually cured in barrels or in tierces. In barrel curing, about 4½ gal. of pickle are used for each 100 lb. of meat. In tierce curing, about 300 lb. of meat and 8 to 12 gal. of pickle are added to each container. The length of the pickling period varies, depending upon the temperature. Sets are usually cured in from 75 to 120 days.

Beef ham sets must be well cured, otherwise decomposition may occur during the drying and smoking operation. Beef hams increase in weight about 8 per cent in the curing process. The pickle used is often plain brine and saltpeter, but frequently sugar and a small amount of sodium nitrite are added. A typical pickling solution has the following composition:

| | Parts |
|---|---|
| Sodium chloride ($NaCl$) | 25 |
| Sodium nitrate ($NaNO_3$) | 0.05 |
| Sodium nitrite ($NaNO_2$) | 0.10 |
| Sugar (sucrose) | 4 |
| Water, to make | 100 |

After the meat is cured, it is soaked well to remove an excessive amount of salt. The soaking water is usually maintained at a temperature of 60 to 80°F. (16 to 27°C.). Two changes of water are usually employed. The length of the soaking period depends upon the kind of meat, nature of the pickle, length of time in the pickle, etc. Overcured, very salty, and hard-cured meats are soaked longer than mild-cured meats. The soaking period is usually from 12 to 24 hr. Meats are soaked for shorter periods in summer than in winter.

The meat is now ready to be dried, or dried and smoked. The meat is handled in a dry room heated by means of steam coils or by a hot-air furnace, and provided with good air circulation to remove moist air. By this method, the drying may be completed in from 5 to 9 days. The room is kept at a temperature of 135°F. for the first two days, then dropped to 115°F. for the remainder of the drying period. Sometimes both drying and smoking are practiced. Wood smoke is produced and distributed in smoke-houses in conjunction with heat and air circulation to preserve, color, and flavor cured meats. Smoke is produced by burning wood or sawdust. Hardwoods, such as hickory and maple, are used extensively and impart very desirable flavors to cured meats. Preservation is due not only to desiccation but to absorption of gases and fumes of creosote, pyroligneous acid (a mixture of acetic acid, acetone, methyl alcohol, etc.), and other antiseptic substances found in wood smoke. Thorough drying of the product is essential.

The final operation consists in chilling the smoked beef at a temperature of 34°F. (1°C.) so that it can be easily sliced. The outside slices show less moisture than those from the center. The sliced product is now ready to be packed for commerce.

For more information on the microbiology of meats, see Jensen (1942, 1944).

**Sweetened Condensed Milk.**—Sweetened condensed milk is prepared in the following manner: Clean, sweet milk is pasteurized at a temperature of 80 to 90°C. for about 1 min. to inactivate the enzymes and kill most of the bacteria in the milk, which may cause undesirable physical and chemical changes. Cane sugar is added as dry crystals, or in the form of a boiled, concentrated solution. The preparation is heated under reduced pressure so that it will boil at a temperature of 50 to 60°C. The milk is reduced to almost one-third of its original volume. The final product contains about 25 per cent water, 40 per cent cane sugar, at least 8 per cent milk fat, and 28 per cent total milk solids including fat. The evaporated and cooled milk is transferred to sterile containers and capped.

Sweetened condensed milk is not sterilized before being placed in cans. The increased osmotic pressure of the preparation, due to the added sugar, is sufficient to prevent multiplication of most organisms. For this reason,

only a small percentage of cans of sweetened milk are ever sterile. Occasionally some cans show evidence of attack by organisms. The ends of the cans become blown, owing to the fact that the organisms produce gas from the added sucrose and from the lactose normally present in the milk.

The organisms that have been isolated include yeasts, molds, micrococci, streptococci, diplococci, anaerobes, gas-producing rods, aerobic spore-forming bacilli, thermophilic and other aerobic bacteria. The coccus forms are encountered in practically every sample of sweetened condensed milk. These organisms are able to survive the temperatures employed in its preparation. Anaerobic organisms cause little trouble, being rarely found in condensed milk. Organisms of the *Escherichia-Aerobacter* group are occasionally encountered but offer little trouble. The medium is unfavorable, and the rods ultimately die. There is no evidence that the aerobic gas-producing organisms are responsible for the blown appearance of cans showing fermentation. Bacilli of the aerobic, spore-bearing types are almost always found in both normal and decomposed cans of milk. These organisms are actively proteolytic and may initiate changes on the milk proteins. It is questionable whether aerobic, sporulating rods ever produce swells in cans of sweetened condensed milk. The cans contain an insufficient amount of oxygen and too much sugar for active growth. Organisms of this group play an insignificant role in the peptonization of sweetened condensed milk. Thermophilic bacteria are often present. They are neither actively proteolytic nor gas producers and, therefore, play no part in the peptonization and fermentation of sweetened condensed milk.

Yeasts are believed to be the most common organisms causing spoilage of sweetened condensed milk. Many of these organisms are active fermenters, attacking the sucrose with the liberation of gas. This results in the cans having a blown appearance. Not all species found in milk are capable of attacking sucrose. Unless the sugar is fermented, the presence of yeasts does not mean that abnormal changes have taken place. Even the fermentative types grow with difficulty, owing to the fact that the environmental conditions are not favorable. The sources of yeasts in canned milk are (1) contamination of the original fresh milk, (2) contamination of the air of the cannery, and (3) contamination of the added sugar. It is not believed that any of the yeasts found in milk are harmful to man. Decomposed milk is objectionable but practically free from any toxic substances.

## BACTERIOLOGY OF EGGS

Fresh eggs are not always free from microorganisms. Results have shown that about 10 per cent of fresh eggs contain living viable bacteria. Organisms have been isolated from both the whites and the yolks of eggs.

The bacteriology and mycology of eggs have not been studied sufficiently and systematically to give an accurate picture of their flora.

An egg shell is about 1/60 in. in thickness and composed largely of calcium carbonate. It is a porous structure, the pores being sufficiently large to permit the passage of gases and microscopic solid particles. When fertile eggs are incubated, these pores are the means by which air passes through the shell to furnish oxygen to the developing chick embryo. Organisms are always found on the shells of eggs. The number varies from 400 to 1,600,000 per gram of shell. Damp and soiled eggs soon become contaminated, owing to the fact that moisture aids in carrying organisms through the pores. This applies to molds as well as to bacteria.

Stuart and McNally (1943) made a study of the initial bacterial flora of the normal egg shell just prior to and following the physiological act of laying. They found that the egg shell as laid down in the oviduct of the normal hen is sterile. Also, it seemed probable that the shell is not contaminated during the act of laying and that the egg is sterile when deposited in the nest. They concluded that all bacterial contamination on the shells of normally laid eggs could be attributed to contact with nesting material, the feet and body of the bird, and the subsequent handling procedures.

It has been shown repeatedly that bacteria can penetrate the shell of the egg and cause spoilage on subsequent storage. Eggs should never be washed (Sayers, 1943). Reports from egg processors throughout the country indicate that one out of every five eggs has been washed before reaching their plants. During washing, bacteria are carried into or through the shells where they eventually penetrate into the egg white and yolk. Thus, the problem of producing and marketing clean, uncontaminated eggs is purely one of sanitation.

In their studies on the rate of penetration of bacteria through the shell and shell membrane, Stuart and McNally (1943) swabbed 50 per cent of the shell area of normally clean eggs, immediately after they were laid, with a 48-hr. culture of *Pseudomonas aeruginosa*. Each egg was sterilized by soaking in 70 per cent alcohol and burning, before sampling and culturing the yolk and albumin. They found that the penetration of organisms through the outside shell onto the shell membrane may be very rapid. The test organism was recovered from 60 per cent of the membranes immediately after inoculation. After standing for 3 and 6 hr., respectively, the test organism was recovered from 80 per cent of the shell membranes; after 18 and 24 hr., it was recovered from 95 per cent of the membranes.

To quote from Stuart and McNally,

> It would appear that bacteria penetrate the shell to the membrane with little or no interference but in the majority of cases are held up at this point. In about

50 per cent of eggs that have been contaminated, as was the case in this experiment, the membrane cannot prevent rapid penetration of some organisms into the albumin and yolk. However, the egg seems to have the ability in an appreciable number of cases to completely destroy the invading organisms. In this study that was particularly true of the shell membrane and albumin.

The existence of the bactericidal agent "lysozyme" in the whites of eggs is well established and we can assume with safety that the decrease in the number of infected whites and the counts on storage with infected whites is due to the action of this substance.

The shell membrane was also found to contain a substance that had a definite bactericidal effect for *Pseudomonas aeruginosa*. It appeared that the shell membrane may be a much more important agent in preventing contamination of the interior of the egg than the so-called mucoid layer covering the shell.

**Preservation of Eggs.**—A number of methods are employed for the preservation of eggs: (1) cold storage, (2) freezing, (3) drying, (4) immersion in sodium silicate solution (water glass), (5) packing in brine or sawdust, (6) coating with vaseline, (7) wrapping in oiled paper, (8) coating with paraffin, and (9) immersion in lime water (solution of calcium hydroxide).

In the cold-storage process the eggs are kept at a temperature of about −6°C. If the temperature goes below this point, a nonreversible change takes place in the albumin, preventing the egg from being restored to its normal transparent condition.

Frozen eggs are kept at a temperature of about −18°C. or lower until needed. They are used in large quantities by candymakers, bakers, egg noodle and macaroni manufacturers, and in other industries. The eggs are removed from the shell, placed in large metal containers, and frozen. Often the whites and yolks are separated because they may be used for different purposes. They are usually cheaper than fresh eggs because they may be prepared during the months of high production.

Eggs may be dried unseparated or first separated into whites and yolks and then dried. Dried yolks, whites, and unseparated eggs are used to a considerable extent in prepared cake and doughnut flours, in ice creams, in macaroni and noodles, and in bakery products, such as meringue and marshmallows. Dried eggs have good keeping qualities if kept dry and cool. However, the presence of a small amount of moisture and a warm atmosphere may be sufficient to permit the growth of organisms and cause spoilage in a short time. It is desirable, therefore, to store dried eggs in a cool place, protected from an excessive amount of moisture.

The other methods used for preserving eggs are designed to prevent the passage of air (oxygen) through the pores of the shell and into the egg. Regardless of which one of these procedures is followed, the eggs must be

kept under cold conditions to prevent decomposition by the enzymes normally present in the egg.

For more information on frozen eggs, see Schneiter, Bartram, and Lepper (1943).

## FOOD POISONING

Food poisoning refers to the ingestion of food contaminated either with harmful bacteria or with certain soluble excretory products known as toxins. It does not include the toxic effect that follows the consumption of noxious plants, such as mushrooms, or poisonous mussels, or decomposed foods containing chemical poisons, or idiosyncrasies connected with certain plant and animal poisons.

At one time, food poisoning was believed to be caused by the consumption of food containing certain chemical compounds known as ptomaines. The term "ptomaine" is from the Greek and means a dead body. Ptomaines are produced in putrefying meat and other proteinaceous foods. They are basic substances and belong to the group of compounds known as amines. They result chiefly from the decarboxylation of amino acids. A typical reaction is the following:

$$\underset{\text{Alanine}}{CH_3{\cdot}CHNH_2{\cdot}COOH} + \underset{\text{Ethylamine}}{CH_3{\cdot}CH_2NH_2} + CO_2$$

The amino acid alanine loses carbon dioxide and is converted into the ptomaine ethylamine. Ptomaines appear only when putrefaction is in an advanced stage. Ptomaines are poisonous when injected into the tissues, but there appears to be very little evidence that they produce any toxic action when taken by mouth. The ptomaine theory of intoxication is a misconception.

The fact that bacteria are present in foods does not mean necessarily that they are harmful. Many saprophytic organisms can attack proteins and release ptomaines during the later stages of decomposition. However, most bacteria that are capable of putrefying proteins with the formation of ptomaines are harmless when taken by mouth. Putrefactive aerobic and anaerobic species normally present in the intestinal tract of man and capable of producing the above changes are harmless when ingested with food.

**Organisms Concerned.**—The organisms chiefly responsible for true food poisoning belong to three well-defined types: (1) *Micrococcus pyogenes* var. *aureus*, (2) several types of *Clostridium botulinum*, chiefly type A in this country and type C in Europe, and (3) members of the genus *Salmonella* (*S. enteritidis*, *S. schottmuelleri*, *S. aertrycke*, *S. typhimurium*, etc.). In addition, certain streptococci, *Escherichia coli*, and *Proteus vulgaris* have also been reported as being responsible for outbreaks of food poisoning.

*Clostridium botulinum* types produce their toxic effect by the secretion of soluble toxins. These organisms probably never multiply within the

body when ingested with foods. The disturbances they produce are essentially intoxications caused by the secretion of soluble exotoxins. The spores of *C. botulinum* are found in the soil and are likely to be present on many kinds of foods. If they are not destroyed in the heating process, conditions are created that usually permit germination of the spores. The vegetative cells then secrete a powerful toxin in the food. The mere tasting of such food may be sufficient to cause death. The toxin can pass through the walls of the stomach and intestines unchanged, differing in this respect from practically all bacterial toxins (Fig. 195).

Some strains of *Micrococcus pyogenes* var. *aureus* are also able to grow

FIG. 195.—Bacilli and spores of *Clostridium botulinum*

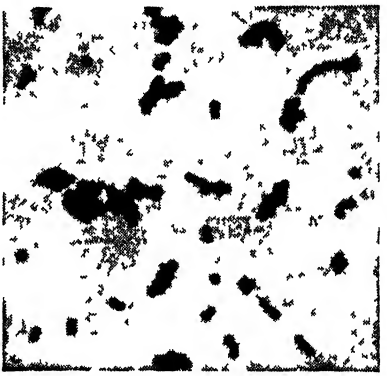

FIG. 196.—*Micrococcus pyogenes* var. *aureus*, an enterotoxin-producing strain.

in food and secrete a soluble toxin. The ingestion of such food produces an intoxication, but the symptoms are considerably less severe than those produced by *C. botulinum*. Death rarely occurs after ingestion of food contaminated by *M. pyogenes* var. *aureus* toxin (Fig. 196).

Some strains of *M. pyogenes* var. *aureus* are capable of liquefying gelatin; others are not. Stone (1935, 1939, 1943) believed that there was a correlation between gelatin liquefaction and ability of a strain to produce an extracellular toxin. He developed a simple method for separating the gelatin liquefying strains from those not capable of digesting gelatin. A plate containing beef extract gelatin agar was streaked with a loopful of the organism under examination and incubated for 24 hr. at 37°C. Then a strong solution of ammonium sulfate was poured over the surface of the plate. The reaction was usually complete in about 5 min. The ammonium sulfate is capable of precipitating protein (gelatin) to give an opaque appearance to the agar. The gelatin in the area immediately surrounding each colony is digested, with the result that the agar is not opaque (Fig. 197). A typical reaction shows clear zones around each colony, with the re-

mainder of the agar appearing opaque. The test does not always show perfect correlation between toxigenicity and ability of a strain to liquefy gelatin.

Members of the *Salmonella* produce chiefly endotoxins. The organisms

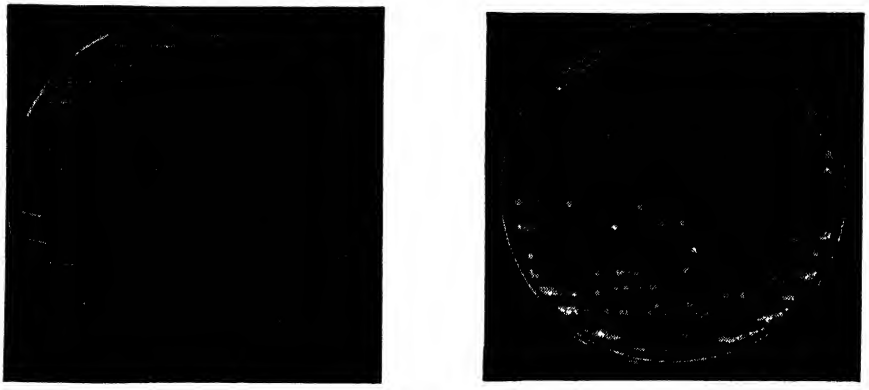

FIG. 197.—Left, strain of *Micrococcus pyogenes* var. *aureus* not capable of liquefying gelatin; right, strain capable of liquefying gelatin. The gelatin agar plates were flooded with a strong solution of ammonium sulfate to precipitate the gelatin Clear zones around the colonies indicate that the gelatin was digested by a gelatinase.

are ingested with the food and reach the intestinal tract without being destroyed. They multiply in the intestines and liberate their endotoxins after the death and digestion of the bacterial cells (Fig. 198).

Cherry, Scherago, and Weaver (1943) examined 250 samples of various types of meats obtained from retail markets and found 5.2 per cent of them to contain species of *Salmonella*. The evidence indicated that the most probable source of the organisms was the animals from which the meats were obtained.

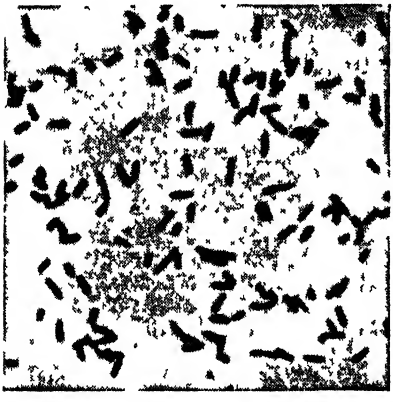

FIG 198.—*Salmonella enteritidis*, an organism that multiplies in the intestinal tract and produces food poisoning by the elaboration of an endotoxin.

Spoiled canned foods or those which are suspicious of being spoiled should never be tasted. If the container is contaminated with *C. botulinum*, sufficient toxin may be present in a minute portion of the food to cause death. The suspected food should not be fed to animals or chickens as they may be highly susceptible to the toxin. Spoiled canned foods are best disposed of by thorough cooking to destroy the toxin.

According to Meyer and Geiger (1942), the incubation period, treat-

TABLE 50

| General food poisoning | Botulism |
|---|---|
| **Incubation period** | |
| Usually 2 to 4 hr., staphylococcus toxin; if over 12 hr., salmonella bacilli | Usually 24 to 38 hr. |
| **Treatment** | |
| Supportive and eliminative | Botulinus antitoxin, specific type; give both *A* and *B* antitoxin; absolute quiet; eliminative |
| **Investigation procedure** | |
| 1. Use incubation period for basis of determining the causative meal | 1. Use incubation period for basis of determining the causative meal |
| 2. Always suspect freshly cooked or warmed-over foods, cakes, pastry, minced meats, etc. Preserved foods are rarely at fault. The foods are usually all right as to taste, appearance, odor, and texture | 2. Always suspect preserved foods; likewise, meat products such as sausages. Spoilage of food is noted in many instances |
| 3 Bacteriologic examination of excreta of patients and the suspected food for the salmonella group, staphylococcus group, and other organisms | 3. Test suspected food for toxin by animal inoculation; mice, guinea pigs, or rabbits. Test for type with specific antitoxin. Cultures of suspected food for the presence of spores, particularly if food has been previously boiled |
| 4. Bacteriologic and epidemiologic search for human carriers and possible contamination from animal sources | 4. Search for domestic animals, such as chickens with symptoms of limberneck, for corroborative field and laboratory evidence |
| 5. Complications: appendicitis, cholecystitis, persistent elevation of temperature (paratyphoid infection) | 5. Complications: bronchopneumonia |
| | 6. Human outbreaks are usually due to type *A* toxin, occasionally to type *B* |
| **Symptomatology** | |
| Sudden onset; nausea, vomiting, abdominal pain, prostration, diarrhea, and rise of temperature | Delayed onset; marked muscular weakness; gastrointestinal symptoms, rare; disturbances of vision with diplopia and blepharoptosis; loss of ability to swallow and talk; constipation; rapid pulse and subnormal temperature; rarely any pain; death from respiratory failure |
| Mortality, 0 to 1 per cent | Mortality over 60 per cent |
| Case-infectivity rate high | Case-infectivity rate usually 100 per cent |

ment, investigation procedure, and symptomatology of the three chief types of organisms responsible for food poisoning are given in Table 50.

For additional information, see American Can Company (1943), Cruess (1938), Dack (1943), Dewberry (1943), Dolman (1943), Hucker and Pederson (1942), Prescott and Proctor (1937), Public Health Bulletin No. 280 (1943), Sherman, Smiley, and Niven, Jr. (1943), Spiegelberg (1944), and Tanner (1944).

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# CHAPTER XXIII

## BACTERIOLOGY OF SOIL

Generally speaking, soils are excellent culture media for the growth of many kinds of organisms. This is especially true of the cultivated and improved soils. The microscopic life of the soil includes bacteria, yeasts, molds, algae, diatoms, and protozoa. The latter includes amoebas, flagellates, ciliates, and rotifers. In addition, there are present various nematodes, insects, etc.

Since most of the soil inhabitants are aerobic, the organisms are found in greater numbers in the surface layers. The numbers decrease as the depth of the soil increases. A well-aerated soil contains more organisms than one lacking in an abundance of oxygen. The numbers and kinds of organisms found in soil depend upon the nature of the soil, the depth, season of the year, state of cultivation, reaction, amount of organic matter, temperature, moisture, etc.

Methods are available for counting the organisms in soil as well as for isolating the various species in pure culture. Since the organisms may vary considerably in their growth requirements, many types of culture media must be employed. The organisms may be aerobic, anaerobic, or facultative types.

Many of the species of organisms present in soil grow in associations with others. The phenomena of symbiosis, synergism, commensalism, and antagonism are believed to be of common occurrence. Two species growing together frequently elaborate metabolic waste products not produced by either organism when grown in pure culture. A product of metabolism of one organism may serve as a nutrient for another species. Antagonistic organisms are usually present and serve to combat other species, especially certain important plant pathogens. This explains why it is exceedingly difficult to determine from laboratory studies on pure cultures what actually takes place in the soil (see Chap. XVIII for a discussion of the various types of associations).

In general, the same media and methods employed for the cultivation and isolation of heterotrophic bacteria are used for the propagation and separation of the majority of organisms found in the soil. However, the soil contains some species that are specific and do not grow on the usual culture media. Special media and methods must be employed for their cultivation. These include symbiotic nitrogen-fixing bacteria, nonsym-

biotic nitrogen-fixing bacteria, sulfur-oxidizing forms, sulfate-reducing species, urea-decomposing bacteria, cellulose-decomposing organisms, and ammonia-oxidizing species.

## FUNCTIONS OF SOIL ORGANISMS

One of the important functions of soil organisms is to decompose various kinds of organic matter of plant and animal origin. This includes stable manures, green manures, plant stubble, plant roots, organic fertilizers, and other products. The decomposition of such compounds is the result of the activities of bacteria, molds, protozoa, worms, and other organisms present in the soil. Each group selects certain constituents of the organic matter suitable for synthesizing its own characteristic protoplasm.

The organic compounds added to the soil as the result of biological action include various sugars, amino acids, pentosans (compounds that yield pentoses on hydrolysis), celluloses, lignins, proteins, fats, waxes, tannins, and pigments. These compounds are further decomposed by soil organisms, resulting in the liberation of soluble organic and inorganic constituents. Some of the inorganic compounds, notably ammonia, may be utilized by plant life as a source of nitrogen.

Waksman (1942) concluded that organic materials, especially stable manures and green manures, produce four distinct effects upon soil processes and upon plant growth:

1. They supply inorganic nutrients to plants, especially nitrogen and phosphorus.
2. They affect the physical conditions of the soil, especially the moisture-holding and buffering capacities.
3. They supply certain specific elements that may be limiting factors for the growth of some plants.
4. They favor the development of organisms that secrete substances antagonistic to the growth of certain specific forms responsible for plant diseases.

**Nitrogen Cycle.**—Nitrogen is the cornerstone of the structural requirements of all living organisms. It is, therefore, absolutely necessary for the growth of bacteria and other microorganisms. Without an available supply of this element, life cannot continue.

A continuous transformation of nitrogen takes place in the soil by various groups of organisms. In the breakdown of protein, the first step is a hydrolysis of the molecules to their constituent building stones or amino acids. The amino acids are then deaminized with the liberation of ammonia:

1. Proteins + $H_2O$ → amino acids
2. Amino acids + $H_2O$ → ammonia + carbon residue

After ammonia has been liberated from various nitrogenous compounds, it may be (1) assimilated by soil organisms and again synthesized into proteins, (2) used by higher plant life as a source of nitrogen, (3) absorbed by the colloidal substances in soil and bound as ammonia, (4) acted upon by other soil forms and oxidized first to nitrites and then to nitrates.

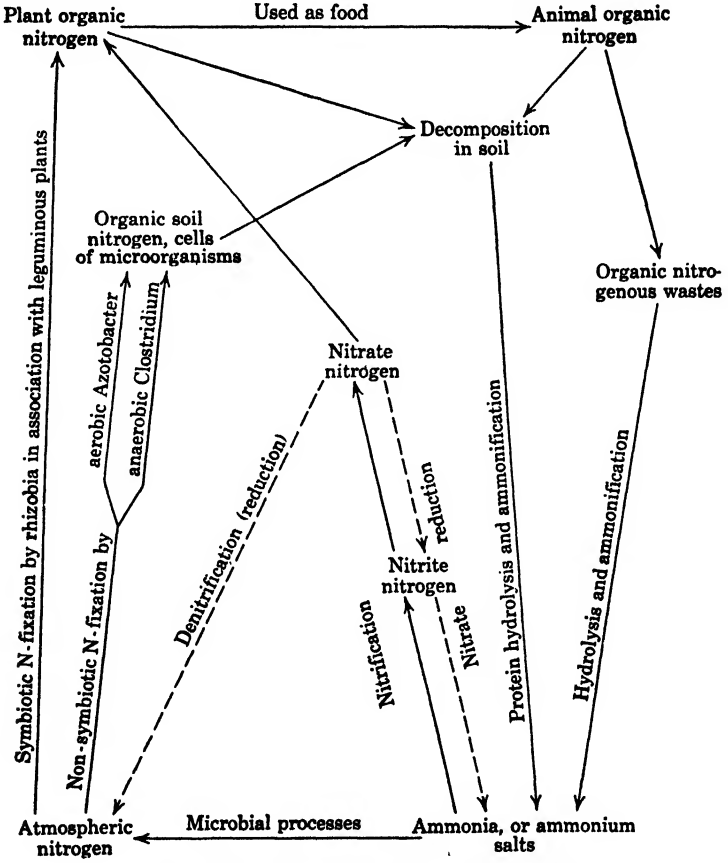



FIG. 199.—The nitrogen cycle. (*After Allen.*)

The organisms responsible for this last set of reactions belong to the autotrophic group of bacteria and are incapable of utilizing organic compounds for structural or energy purposes.

The nitrates may be utilized by various microorganisms and by higher plants and synthesized into proteins, or they may be reduced first to nitrites and finally to free nitrogen. The free nitrogen is lost as far as being available to plant and most microscopic soil life. However, certain bacteria found in the soil have the ability of utilizing free atmospheric nitrogen and making it available to plant life.

Two groups of organisms are responsible for nitrogen fixation. The organisms in one group are the nonsymbiotic nitrogen fixers; those in the other group fix nitrogen only when growing in symbiosis on the roots of certain plants. The free nitrogen is transformed into nitrogenous compounds and made available to plants and soil microorganisms. The nitrogen is again synthesized into proteins. The nitrogen cycle, according to Allen (1935), is diagramed in Fig. 199.

## QUANTITATIVE EXAMINATION OF SOIL

The soil is the natural habitat of a considerable number of bacterial species, varying from strict autotrophs to heterotrophic forms. Because

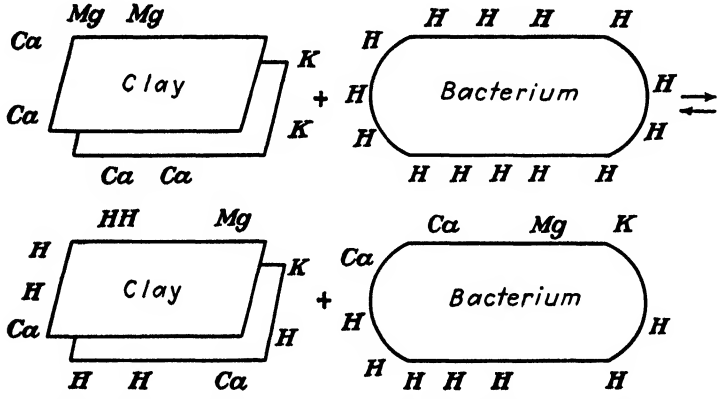



FIG. 200.—Mechanism of ion exchange between soil bacteria and colloidal clay. (*After McCalla.*)

of this fact, many types of culture media are required to obtain an accurate appraisal of the numbers of organisms present. Such a procedure would be out of the question where a rapid determination of the approximate numbers present in a sample of soil is desired.

**Soil Colloids.**—Bacterial organisms are found chiefly in the layer of colloidal material surrounding the inorganic particles of soil. Conn and Conn (1940), McCalla (1940), and others noted that bacteria grew better in the presence of colloidal clay than in its absence. McCalla attributed the stimulation of bacterial growth to the catalytic effect of the clay in speeding up biochemical reactions, either by providing a more efficient utilization of nutritive material or by decreasing the toxic effects of waste products by adsorbing them.

Bacteria in contact with soil colloids adsorb cations. Under normal conditions, the solid material in the soil constitutes about 80 per cent and the water content about 20 per cent. The water is present around and between the particles of soil. Normally, the bacteria probably live in the

water films that adhere to the surface of the colloid particles containing adsorbed ions. To quote from McCalla,

The bacterial cell is undoubtedly in close proximity to the soil particles and, assuming that the bacteria may adsorb ions and hold some of them in the outer surface of the cell, this would permit contact exchange of adsorbed ions. Ions with large oscillating volumes would overlap, and exchange between systems could readily take place. Other ions which are strongly adsorbed would not be expected to wander far from the surface of the colloid. On the basis of the displacement of adsorbed methylene blue, the ions would be expected to be adsorbed by the bacteria from the soil colloids in the following series: H > Al > Fe > Mn > Ba > Ca > Mg > K > $NH_4$ > Na.

From the foregoing facts and theoretical considerations it is suggested that in the adsorption of nutrients from the soil by the bacteria, and possibly by living cells in general, an exchange of adsorbed bases takes place between the bacteria and soil colloid as depicted in Fig. 200. In bacterial metabolism large amounts of carbon dioxide and water are formed. In the presence of $H_2O$, H-ions are produced from the carbon dioxide which may be adsorbed at the cell's surface. When a colloidal clay particle, saturated with adsorbed bases contacts a bacterium saturated with H-ions, an exchange of ions takes place until an equilibrium is reached. As this equilibrium is upset by the more complete utilization of the adsorbed basic ions in bacterial metabolism, a further exchange may take place, the colloidal clay functioning as a constant reservoir for basic ions utilized in the growth of such organisms.

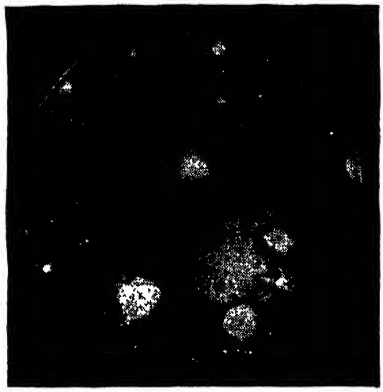


FIG. 201.—Quantitative examination of soil by the agar plate method.

It is difficult to remove or separate the organisms from this colloidal layer. This means that the number of colonies appearing on an agar plate is not an accurate index of the population of a soil sample. Also, no single culture medium is satisfactory for the growth of all species present in the soil.

Three methods may be employed for estimating the numbers of microorganisms in soil: (1) the plate method, (2) the dilution method, and (3) the direct microscopic method.

**Plate Method.**—The plate method is used more than the others. The procedure is as follows: A weighed sample of soil is mixed with a known volume of sterile water. The sample is well shaken to wash out as many organisms as possible from the colloidal material surrounding the soil particles. A series of dilutions is usually prepared from the soil suspension. Aliquot portions from each dilution are transferred to Petri dishes and

mixed with melted agar. The plates are incubated from 2 to 14 days. The colonies appearing on the plates are counted and computations made for the number of organisms per gram of soil (Fig. 201).

The enumeration of the soil population by this method presents several very serious errors. Conditions are not favorable for the growth of the anaerobic organisms. The autotrophic bacteria do not multiply on an organic medium. The nonsymbiotic, nitrogen-fixing organisms grow to a limited extent only. Many of the cellulose-decomposing organisms fail to grow on the commonly used media. Sulfate reducers grow only in an inorganic medium containing sulfate. The counts as determined by this method represent only a fraction of the total bacterial population of the soil. It does, however, give an estimate of the number of organisms present in soil capable of growing on a nutrient agar medium.

The temperature at which soils are stored greatly influences the number of organisms developing on agar plates. Greaves and Jones (1944) stored soils for 24 months at 10, 20, 30, and 40°C. and examined them at the end of 6, 12, 18, and 24 months for numbers of organisms. The greatest number developed when the soil was stored at 10°C. and the fewest at 40°C.

The numbers of organisms found in soil by the agar plate method vary from 200,000 to 100,000,000 per gram. The counts are considerably less than the actual numbers present, owing to the limitations already mentioned. According to Waksman (1932), the colonies appearing on agar plates consist of 10 to 40 per cent actinomycetes, 50 to 80 per cent nonspore-forming bacteria, and 3 to 10 per cent spore formers.

Molds are commonly present in soil although the numbers appearing on agar plates show only a small percentage of the total counts. Yeasts are even less prevalent than molds, their number increasing in acid soils and in vineyards and orchards.

**Dilution Method.**—In this method, a suspension of soil is prepared as already given. Aliquot portions of the suspension are transferred to different types of culture media favoring the growth of the specific soil forms. A urea medium is used for the growth of the urea-decomposing organisms; a cellulose medium is employed for the detection of the cellulose-hydrolyzing bacteria; a medium devoid of nitrogen is used for the isolation of the nitrogen-fixing organisms; etc. The accuracy of the method depends upon the preparation of a series of dilutions of the original soil suspension. Thus, if a dilution of 1:10,000 failed to show the presence of urea-decomposing bacteria whereas a dilution of 1:1000 did, then the number of organisms capable of liberating ammonia from urea would be between 1000 and 10,000 per gram of soil. More accurate counts could be obtained by employing a larger number of dilutions over a narrower range. This method is to be preferred for the isolation of the specific forms present in the soil.

**Direct Microscopic Method.**—The direct microscopic procedure is considered more accurate than either of the cultural methods already mentioned for the determination of the abundance of the soil population.

The method is as follows: One part of soil is suspended in ten times its weight of a 0.015 per cent solution of agar in water. The purpose of the agar is to fix the organisms to the slide. A known volume of the soil suspension is placed on a ruled glass slide and spread uniformly over the marked area. The slide is dried on a flat surface over a water bath and then covered with a staining solution consisting of 1 per cent rose bengal dissolved in a 5 per cent aqueous solution of phenol. The slide is finally washed in tap water, dried, and examined under the oil-immersion objective.

In a good preparation, the bacteria take a deep pink or red color whereas the mineral constituents do not stain. Some of the dead organic matter appears light pink, but most of it stains either yellow or not at all. If the bacteria appear faintly stained or if everything is colored pink, a new preparation should be made. The former condition generally means that the slide has been washed too long; the latter indicates that the staining solution is too old.

The direct counts are from five to twenty times greater than the agar plate counts. Much of the discrepancy is due to bacteria that fail to grow on the plates rather than to the presence of large clumps of organisms that do not break up in the plating process. Also, the direct method records dead organisms that are eliminated in the agar plate procedure.

The direct microscopic method for the enumeration of soil organisms is subject to several serious errors, especially in the hands of the inexperienced worker. The organisms must be evenly distributed over the slide. It is of great importance to prepare smears as uniformly as possible. It is usually considered advisable to prepare several slides and take an average of the counts. Another disturbing factor is the difficulty in recognizing the bacteria. Many of the soil forms are too small to be easily distinguished from the soil particles. Also, many particles of soil resemble bacteria. Considerable experience is, therefore, necessary in making accurate determinations of the soil population by the direct method.

For more information, consult the reports by Conn (1918, 1926).

**Variations in Soil Counts.**—The numbers of organisms in a soil are not uniform, even over a very small area. In order to increase the accuracy of the determination, several samples from the same plot of ground should be collected and an average taken of all the soil determinations for computing the final soil count. A single determination may be considered as valueless for computing the soil population.

Soil counts have been shown to vary from day to day. These daily changes appear to be related to fluctuations in the numbers of active

protozoa. Russell and Hutchinson (1909, 1913) found that, if soil were partly sterilized with steam or with a volatile antiseptic such as toluene, the bacterial counts showed first a decrease followed by a sharp increase in numbers and activity. If a little untreated soil were added, the bacterial counts again decreased. The partial sterilization of the soil destroyed the protozoa but not the bacteria, and was followed by a sharp increase in the latter. The addition of an unheated soil reintroduced protozoa and resulted again in a decrease in the numbers of bacteria. The important protozoa responsible for the daily variations in the bacterial counts include ciliates, flagellates, and rhizopods. These organisms have been found to be of world-wide distribution in the soil.

Cutler, Crump, and Sandon (1922) found that an inverse relationship existed from day to day between the numbers of bacteria and active amoebas in the soil. A decrease in the numbers of protozoa was followed by an increase in the bacterial population, and vice versa. This means that, if a soil analysis is to be of any value, it is of great importance to examine samples from different parts of the same plot as well as at frequent intervals over a long period of time.

For further reading, see James and Sutherland (1942, 1943*a*,*b*).

## QUALITATIVE EXAMINATION OF SOIL

Lochhead (1940) reported that the qualitative nature of the soil microflora was markedly influenced by the growing plant. In the rhizosphere (the zone influenced by root excretions), the Gram-negative rods were increased in numbers, and the Gram-positive rods, coccus forms, and spore bearers were less abundant.

Organisms in the rhizosphere showed greater physiological activity than those more distant from the plant. There were present (1) a greater proportion of motile forms, (2) a pronounced increase in the number of chromogenic bacteria, and (3) a higher incidence of organisms capable of liquefying gelatin and of attacking glucose.

West and Lochhead (1940*a*) stated that bacteria of the rhizosphere of flax and tobacco showed more complex nutritive requirements than those of the control soils. The roots were found to stimulate the growth of those types which required thiamin (vitamin $B_1$), biotin, and amino nitrogen for growth. This suggested that the plant roots excreted significant amounts of these essential substances. The difference in the microflora of the rhizosphere and control soil was more pronounced where the latter was poor than where it was supplied with an abundance of organic matter. This was due, no doubt, to the liberation of growth substances in the decomposition of the organic matter, which were essential for the growth of the typically rhizosphere forms.

West and Lochhead (1940*b*) employed three synthetic media and were

able to classify most soil bacteria into five different groups on the basis of their nutritional requirements. Later, Lochhead and Chase (1943) examined more extensively the nutritional requirements of soil bacteria and were able to cultivate a greater number of bacteria by employing seven different kinds of media.

For additional information, see Katznelson and Chase (1944).

## AMMONIA PRODUCTION

Most species of bacteria are capable of decomposing proteins and protein-split products with the liberation of ammonia as one of the compounds. The ammonia is released prior to the utilization of the organic acid residue chiefly for energy purposes. The production of ammonia is an essential stage in the formation of nitrate in the soil. Most plant crops are dependent largely upon soil nitrates for structure and growth.

It is important to note that the decomposition of nitrogenous compounds for energy takes place only in the absence of a rapidly utilizable carbohydrate. In the presence of a rapidly fermentable carbohydrate, bacteria derive their energy from this source, utilizing the nitrogenous compounds for structure and growth only. This results in a greatly lowered production of ammonia and nitrate. Therefore, maximum ammonia production takes place only in the absence of a rapidly utilizable carbohydrate.

**Urea-decomposing Organisms.**—Proteins and their degradation products are not the only compounds capable of yielding ammonia as the result of bacterial action. Some nitrogenous compounds of a nonprotein nature are also acted upon by bacteria, resulting in the release of ammonia.

Stable and barnyard manures are often used as fertilizers because of their nitrogen content. They help to replenish the nitrogen supply of the soil. A high content of urine is often present in such waste material. The most important nitrogen compound present in urine is urea. Many organisms have the power of converting the urea to ammonium carbonate and finally to free ammonia and carbon dioxide, according to the equation:

$$O{=}C(NH_2)_2 + 2H_2O \rightarrow (NH_4)_2CO_3$$

$$(NH_4)_2CO_3 \rightarrow 2NH_3 + CO_2 + H_2O$$

The presence of urea-decomposing organisms in the soil is determined by incorporating urea in the culture medium. The liberation of ammonia from the culture medium indicates the presence of the specific organism or organisms capable of utilizing this nitrogenous compound. Some of these organisms are *Micrococcus ureae*, a Gram-positive, aerobic coccus appearing singly, in pairs, and in clumps. Its optimum temperature is 25°C.; *Bacillus freudenreichii*, a Gram-positive, motile, facultative aerobic, spore-forming

rod, the cells appearing singly and in chains. The optimum temperature is 30°C.; *Sarcina ureae*, a Gram-positive, motile, aerobic coccus, occurring singly, in pairs, and in packets. The organism does not produce spores but resists heating to 80°C. for 10 min. The optimum temperature is 20°C.; *Pseudomonas ureae*, a Gram-positive, motile, facultative aerobic rod, appearing singly and in pairs. Its optimum temperature is 20°C.

The urea bacteria thrive best in media containing urea, especially when made alkaline with ammonium carbonate. The organisms are capable of rapidly converting the urea to ammonium carbonate, resulting, in many instances, in the death of the bacteria. The organisms are commonly found in air, water, soil, and manure. About 2 per cent of the organisms present in surface soil and about 10 per cent of those present in manure are capable of decomposing urea. The isolation of the urea-decomposing organisms is a simple matter, provided urea is added to the culture medium.

In addition to the above organisms, Stuart, Van Stratum, and Rustigian (1945) reported that, in strongly buffered medium, members of the genus *Proteus* gave evidence of urea utilization. In weakly buffered medium, however, urea utilization occurred by many coliform and paracolon cultures, especially of the *Aerobacter* type.

**Urease-like Activities in Soils.**—In a series of investigations, Conrad (1940*a*,*b*,*c*, 1942*a*,*b*, 1944) percolated urea solutions through cultivated and uncultivated soils and showed that the hydrolysis of the compound was due largely to a thermolabile catalyst or catalysts rather than to microbial action. Determinations were made both in the presence and in the absence of antiseptics. The antiseptics added were toluene, chloroform, and carbon disulfide, all capable of destroying bacterial action. These antiseptics had little, if any, inhibiting action on the hydrolysis of urea in the soils studied. The natural soil catalytic activity was inactivated or greatly reduced by preheating the moistened soil to 85°C. This activity was much more resistant to decomposition than was that of added laboratory urease. If the activity of soils was due to the presence of the enzyme urease, it must have been associated with some other material which gave it some protection against decomposition. Since enzymes are elaborated by living cells, soil organisms must be considered as potential producers of any enzymes present in soils.

## DIGESTION OF CELLULOSE

The greater part of the organic matter in the soil is decomposed by bacteria in the process of acquiring energy. The simpler carbohydrates, including the monosaccharides, the disaccharides, and some of the polysaccharides are easily attacked and decomposed by a large number of soil bacteria. The addition of such compounds to the soil causes a rapid increase in the bacterial population.

Cellulose is one of the most important constituents added to the soil. It finds its way into the soil as the chief component of crop residues and of natural vegetation. Under normal conditions of temperature and moisture, cellulose disappears almost completely and quite rapidly. Cellulose is a polysaccharide and yields glucose on hydrolysis. The cell walls of plants are composed of cellulose, whereas those of animals are made up of protein. This is one of the distinguishing characteristics between plant and animal cells.

The first stage in the utilization of cellulose is a hydrolysis of the molecule to cellobiose and finally to glucose. The two enzymes, cellulase and cellobiase, are concerned in the reaction. Cellulase hydrolyzes cellulose to cellobiose and cellobiase splits cellobiose into two molecules of glucose (see pages 276 and 279).

The two enzymes cellulase and cellobiase accompany each other. The enzymes are of tremendous importance in the dissolution of insoluble cellulose in the soil. Since animals do not elaborate a cellulase, the presence of cellulose-decomposing bacteria in the intestines of herbivorous animals is responsible for the hydrolysis of the insoluble compound, making it available for food.

**Organisms Concerned.**—Cellulose is attacked by a number of aerobic, anaerobic, and thermophilic species in the process of acquiring energy.

*Aerobic Bacteria.*—According to Fuller and Norman (1943*a*,*b*), almost all the aerobic mesophilic cellulose bacteria so far isolated have been placed in three genera: *Cellvibrio*, *Cellfalcicula*, and *Cytophaga*. In addition to these, there are a few aerobic species in the genera *Vibrio*, *Bacterium*, *Bacillus*, *Pseudomonas*, and *Achromobacter*. With the exception of *Pseudomonas erythra*, all are versatile organisms, being capable of growing well on many carbohydrates. The species *Bacillus aporrhoeus* produces colonies that move about on the surface of the agar medium. A portion of a colony may move in a thin line away from the main body at the rate of about 3 mm. in 2 hr. Not all colonies are motile.

The presence of aerobic organisms can be easily demonstrated by placing some soil in a culture medium composed of certain inorganic salts and cellulose (filter paper) and incubating at room temperature for several days. The filter paper slowly disintegrates and finally disappears (Fig. 202).

*Anaerobic Bacteria.*—Almost all the anaerobic cellulose-hydrolyzing species are found in the genus *Clostridium*.

Khouvine (1923) reported the presence in the human intestine, of an obligately anaerobic spore-forming bacillus which attacked cellulose with the formation of acetic acid, accompanied by smaller amounts of butyric acid, ethyl alcohol, carbon dioxide, and hydrogen. Cellulose served as the only source of carbon. The organism could be cultivated in an

inorganic salt medium containing 0.1 per cent peptone, about 1 per cent cellulose, and some fecal extract. The organism failed to develop on a medium in which cellobiose or glucose was substituted for the cellulose. The organism was named *Bacillus cellulosae dissolvens* (*Clostridium dissolvens*).

Hungate (1944) isolated a cellulose-digesting anaerobe, *C. cellobioparus*, from the rumen of cattle. The organism grew well in an inorganic medium containing biotin and either cellulose or any one of a variety of sugars. The fermentation products consisted of acetic, formic, and lactic acids, ethyl alcohol, carbon dioxide, and hydrogen. Cellobiose was the chief digestion product of cellulose; glucose was not formed.

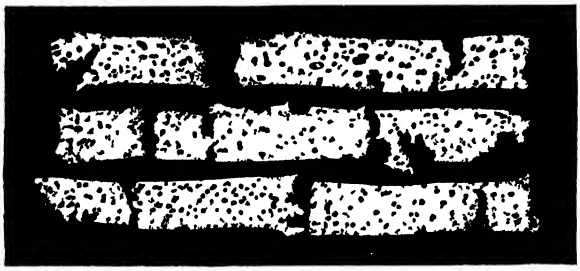


FIG. 202.—Strips of filter paper showing disintegrated areas due to bacterial attack. (*After Omelianski; from Lipman's "Bacteria in Relation to Country Life," The Macmillan Company, New York.*)

*Thermophilic Bacteria.*—Some organisms found in the soil are capable of dissolving cellulose at a temperature of 60 to 65°C. These are thermophilic organisms. Both aerobic and anaerobic species have been isolated. The anaerobic organisms may be isolated by inoculating an inorganic medium containing cellulose with an infusion of rapidly decomposing manure and incubating at 65°C. If the organisms are present, the cellulose is completely dissolved usually in from 6 to 8 days.

*Other Organisms.*—A considerable number of molds and mold-like organisms have been described as being capable of utilizing cellulose. The species that have been most extensively investigated are members of the genera *Aspergillus, Penicillium, Monilia, Alternaria, Trichoderma, Mucor, Rhizopus, Merulius, Paxillus, Fusarium, Hormodendrum, Chaetomium, Polyporus, Actinomyces,* and others. Molds are commonly found growing on old paper, especially if kept in a damp or humid atmosphere. They have been responsible for the destruction of many valuable manuscripts (Fig. 203).

For additional information on cellulose-digesting organisms, see Beckwith, Swanson, and Iiams (1940), Boswell (1941), Hungate (1946), and Stanier (1942*b*).

**Specificity of the Reaction.**—It has been said that the organisms hydrolyzing cellulose attack the compound as a source of energy and that

they are unable to utilize any other carbon source. Stanier (1942*a*) brought forth evidence to substantiate the statement that there are probably no obligate cellulose-hydrolyzing bacteria. He showed that glucose and other carbohydrates could replace cellulose in the cultivation of a number of species of the genus *Cytophaga* which have been generally regarded as obligate forms. It is highly probable that glucose can replace cellulose in

FIG. 203.—Photomicrograph of a cellulose-digesting mold growing on paper. (*Slide prepared by W. H. Swanson.*)

the metabolism of other cellulose-decomposing groups. These findings refute the statement of Winogradsky (1929) that there is a direct oxidation of the cellulose molecule. The similarity in the rates of cellulose, cellobiose, and glucose oxidation by *C. hutchinsoni* provides good evidence to believe that cellulose is first hydrolyzed to glucose and then the monosaccharide is oxidized as a source of energy.

## SYMBIOTIC NITROGEN FIXATION

It was shown several generations ago that the growth of certain plants in the soil resulted in a stimulation of the succeeding plant crop. The fertility of the soil was greatly increased. The plants responsible for this

stimulation were found to be members of the family *Leguminosae*. Late in the last century, it was clearly shown that the stimulation was due to an increase in the nitrogen supply of the soil, which was the result of the presence of small tumor-like growths or nodules on the roots of the leguminous plants. In the absence of nodules, no stimulation of growth of the succeeding plant crop occurred.

The formation of nodules on roots is caused by the associated growth of the leguminous plant and a bacterial organism. The bacteria are members of the genus *Rhizobium* and are commonly referred to as the root-nodule bacteria. The organisms live in the cells of the plant roots where their growth and metabolic activities cause a swelling or nodule to form on the root. The organisms utilize the nitrogen of the atmosphere and synthesize it into a nitrogen compound. The plant obtains its nitrogen from the synthetic activities of the organisms while the bacteria derive their food from the plant. The plant and bacteria live together for mutual benefit. Such an association is known as symbiosis.

The organisms responsible for symbiotic nitrogen fixation are members of the genus *Rhizobium*. This genus is characterized as follows:

Rods, 0.5 to 0.9 by 1.2 to 3.0 microns. Motile when young, commonly changing to bacteroidal forms (*a*) upon artificial culture media containing alkaloids or glucosides, or in which acidity is increased; or (*b*) during symbiosis within the nodule. Gram-negative. Aerobic, heterotrophic, growing best with extracts of yeast, malt, or other plant materials. Nitrates may be reduced to nitrites. Nitrites are not utilized. Gelatin is not liquefied or is very slightly liquefied after long incubation. Optimum temperature 25°C. This group is capable of producing nodules on the roots of *Leguminosae*, and of fixing free nitrogen during this symbiosis.

The genus includes six species: *Rhizobium leguminosarum*, *R. phaseoli*, *R. trifolii*, *R. lupini*, *R. meliloti*, and *R. japonicum*. A specific name has not been given to the organism that produces nodules on plants of the so-called "cowpea" group. Walker and Brown (1935) proposed the name *R. japonicum* to include both the cowpea and the soybean groups.

**Cross-inoculation Groups.**—The leguminous plants may be divided into seven groups on the basis of the species of *Rhizobium* present in the nodules. These seven groups are sometimes referred to as cross-inoculation groups, meaning that one organism will infect all the plants in a given group but will not attack those in another. Occasionally, an organism in one group will infect a plant in another group. No satisfactory explanation has been advanced to account for the above groupings. However, it has been shown that the seed proteins of those plants belonging to the same group are very closely related when tested serologically by the precipitin technique.

The organisms within a given cross-inoculation group do not appear to be all alike. For example, the bacteria from alfalfa and yellow clover are

interchangeable, but best results are usually produced by inoculating alfalfa plants with alfalfa-specific organisms.

For additional information, consult Appleman and Sears (1942), Burton and Erdman (1940), Kleczkowska, Nutman, and Bond (1944), and Kleczkowski and Thornton (1944).

**Formation of the Nodule.**—The nitrogen-fixing organisms live in the soil in the free state. Recent work points to the fact that they fix nitrogen only when growing in association with the plant. They gain entrance to the plant through the root hairs or other epidermal cells. The bacteria multiply

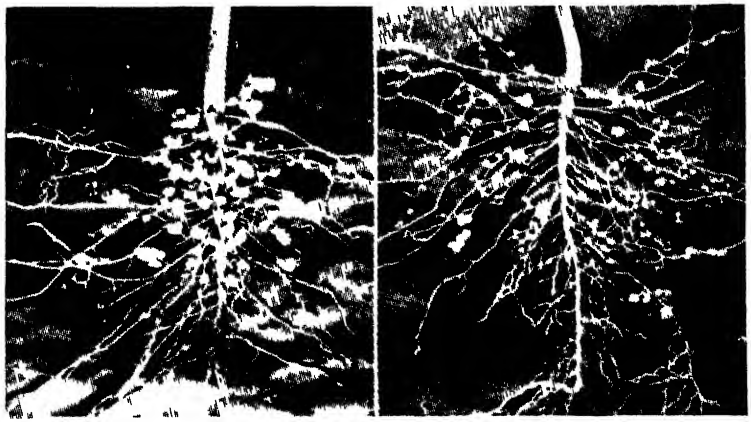


FIG. 204.—Formation of nodules on *Enterolobium cyclocarpum* inoculated with different strains of *Rhizobium*. (*After Allen and Allen.*)

very rapidly, forming long filaments in the root hairs and into the parenchyma of the root. The organisms cause a rapid proliferation of the surrounding tissue in the innermost cells of the root cortex, which results in the formation of a young nodule (Fig. 204). The young nodule pushes out the overlying parenchyma and epidermis and produces a swelling on the side of the root. A nodule consists of a mass of thin-walled parenchyma cells, which are usually almost filled with the specific organism. A corky layer and branches of a vascular system are also present. This system provides the bacteria with their nutrients, and the plant in turn takes away the nitrogen compounds synthesized by the bacteria.

**Nitrogen Fixation in Excised Nodules.**—Allison, Hoover, and Minor (1942) removed nodules from the roots of sweet clover, cowpea, crown vetch, soybean, and hairy vetch, and attempted to determine whether such nodules possessed the ability to fix atmospheric nitrogen when maintained in culture solutions in the laboratory. On the basis of their experimental data, they came to the following conclusions:

1. No nitrogen fixation was observed with whole or crushed nodules kept in a mineral culture solution containing sucrose, glucose, or mannitol.

2. A 1 to 3 per cent increase in nitrogen, which is scarcely significant, was obtained in a few cases, where nodules were kept in thin layers of a mannitol-glucose medium at 4 to 12°C. for 1 to 3 days.

3. Pure cultures of rhizobia did not fix nitrogen when supplied with oxalacetic acid (see page 564). A few similar experiments with excised nodules gave negative or inconclusive results.

4. Fifteen series of experiments, in which many variables were considered, gave no evidence that the juices extracted from nodules and sterilized by passing through filter candles can fix atmospheric nitrogen when incubated in mineral-sugar solutions.

**Plants Involved.**—Something like 10,000 species of leguminous plants have been described. All but a few of them show the presence of root

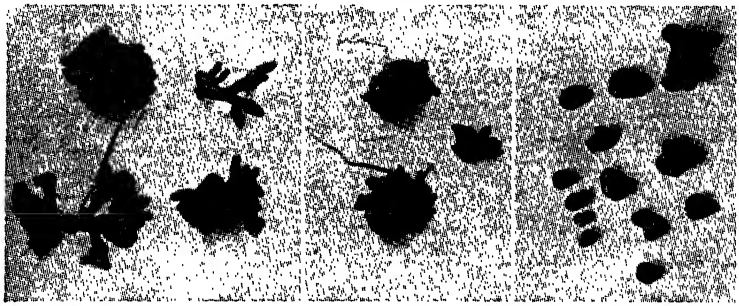


FIG. 205.—Left, nodules from *Acacia horrida;* center, nodules from *Inga edulis;* right, nodules from *Andira inermis.* (*After Allen and Allen.*)

nodules. The nodules vary in size, shape, and position on the different plants (Fig. 205). It is generally stated that nodule formation occurs only on the leguminous plants. It is true that nodule formation occurs most successfully on the *Leguminosae*, but a few plants in other families are also involved.

**Organisms.**—Great masses of organisms are present in the nodule. Their presence may be easily demonstrated by crushing a washed nodule between two glass slides, fixing the smear, and staining by the usual technique. It is a relatively simple matter to isolate a pure culture of the organism from a previously washed and sterilized nodule. The usual culture media are not satisfactory for the cultivation of the organisms. A medium that has yielded very good results is known as Ashby's mannitol phosphate agar, which is an inorganic medium to which is added mannitol as a source of energy. Colonies appear in from 5 to 10 days when the plates are incubated at 25°C.

The colonial characteristics of the organisms show some variation, depending upon the plants from which they are isolated. Some species show large, raised, opaque, and sticky colonies whereas others appear as small, slightly raised, transparent colonies.

The symbiotic, nitrogen-fixing organisms are characterized as minute

rods, which are motile when young. Branching forms are abundant and characteristic in nodules. The organisms are obligate aerobes. Cells from nodules are commonly irregular with X-, Y-, star-, pear-, and club-shaped

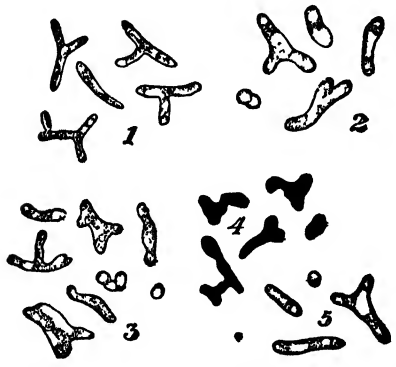



FIG. 206.—Bacteroids from root nodules. 1, *Melilotus alba;* 2, 3, and 5, *Medicago sativa;* 4, *Vicia villosa.* (*After Harrison and Barlow; from Lipman's "Bacteria in Relation to Country Life," The Macmillan Company, New York.*)

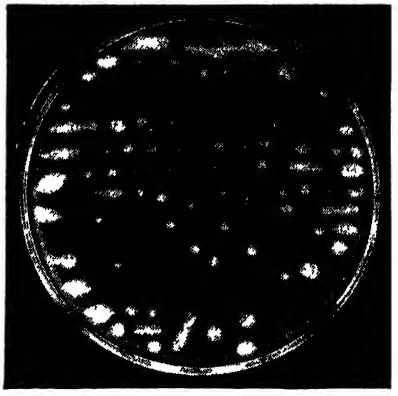


FIG. 207.—Agar plate culture of *Rhizobium leguminosarum* incubated 6 days at 100m temperature. Note the mucoid appearance of the colonies, showing considerable tendency to run together.

forms. Swollen or vacuolated forms appear to predominate. These forms are also known as bacteroids (Fig. 206). Round or coccus cells may also be present. The cocci arise from the swollen or vacuolated cells, which later return to the rod form. Some have described the various forms as orderly stages in the life cycle of the organism.

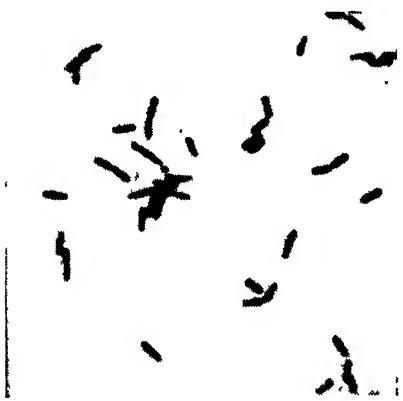


FIG. 208.—*Rhizobium leguminosarum* from culture on mannitol agar.

Although the bacteroid form predominates in the nodule, it is usually absent when nodular material is streaked on agar plates (Figs. 207 and 208). After death and dissolution of the nodule, the organisms round up and pass back into the soil in the form of small cocci.

The organisms in the various cross-inoculation groups show some differences in their fermentation reactions. Fermentation usually results in a weak production of acid. This is due to the slow rate of growth of the organisms on laboratory media.

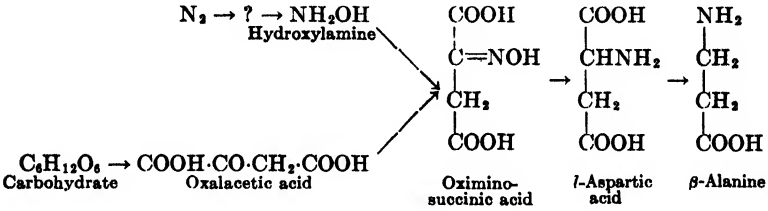
Some of the root-nodule bacteria show a strong tendency to produce large quantities of gum when grown on carbohydrate media. Gum forma-

tion may be so pronounced as to render the medium strongly viscous. Organisms in different cross-inoculation groups show considerable variation in the amount of gum produced. The gum is nitrogen-free, soluble in water, and precipitated from solution on the addition of alcohol or acetone. On heating with a mineral acid, the gum is hydrolyzed to a reducing sugar.

**Mechanism of Nitrogen Fixation.**—Several theories have been advanced to explain the mechanism of biological nitrogen fixation but the one proposed by Virtanen and Laine (1939) appears to be the most plausible.

Legumes were inoculated with a root-nodule organism and grown in a sterile, nitrogen-free culture medium. Nitrogen compounds appeared immediately after nodule formation. The nitrogenous compounds were found to be excreted into the soil from the root nodules rather than from the roots. The excreted nitrogen was chiefly amino-N characterized as *l*-aspartic acid. Some β-alanine was also present, which was formed from the *l*-aspartic acid by the legume bacteria. In addition small amounts of oxime-N and nitrite-N were found in the excretion products.

On the basis of the experimental facts, they proposed the following scheme for the biological fixation of nitrogen:




From their experimental data, Virtanen and Laine came to the following conclusions:

1. Over 90 per cent of the nitrogen excreted was amino-N. In addition, 1 to 2 per cent oxime-N and some nitrite-N were present.
2. The major portion of the amino-N was present as *l*-aspartic acid, if the legumes were harvested at a young stage, long before flowering. The amount of aspartic acid excreted decreased with the age of the plant.
3. β-Alanine was also found among the excretion products, which was slowly formed from *l*-aspartic acid by the root-nodule bacteria. Its formation explained the decrease of *l*-aspartic acid with age.
4. The oxime was present as oximinosuccinic acid; the nitrite-N was formed from this oxime.
5. A small amount of fumaric acid was also detected among the excretion products.

**Artificial Inoculation of Plants.**—The efficiency of symbiotic nitrogen fixation has been greatly increased by (1) choice of the proper legumes, (2) development of new varieties of leguminous plants, (3) artificial inocula-

tion of seeds with pure cultures of root-nodule bacteria, and (4) adjustment of the environment to optimum conditions.

Cultures of the organisms have been added to the soil to increase the nitrogen content. This may be practiced either by adding the culture to the soil or by inoculating the seeds with the bacteria. The latter procedure is much easier to carry out and appears to yield superior results. Soaking the seeds in a culture of the specific root-nodule bacteria has become a well-established practice especially when a legume plant is seeded for the first time.

For successful results in the use of cultures of *Rhizobium* for the inoculation of seeds, it is extremely important that the strain of organism selected is one known to produce nodules on the legume. Greenhouse tests should be supplemented with actual field tests to make sure of a good selection of strains for the mass production of commercial cultures.

The addition of an abundant supply of nitrates to the soil results in a luxuriant plant growth. Under such conditions, nodules probably do not appear on the plant roots, even though the seeds and soil have been inoculated with a pure culture of the specific organisms. The suppression of nodule formation is not due to a direct effect of the nitrate, but rather to a change in the metabolism of the plants. On the other hand, the addition of calcium and phosphorus to the soil results in a stimulation of nodule production. Other factors influencing nodule formation include reaction of the soil, temperature, moisture, and oxygen content.

For additional information, see Albrecht (1944), Appleman, Barnes, and Sears (1942), Burris, Eppling, Wahlin, and Wilson (1942, 1943), Collins (1944), Davis (1944), Erdman (1944), Fred, Baldwin, and McCoy (1932), Kleczkowska (1945), Wilson (1939, 1940), Wilson, Burris, and Coffee (1943), Wilson and Westgate (1942), and Wyss and Wilson (1941).

## NONSYMBIOTIC NITROGEN FIXATION

In addition to the organisms discussed in the preceding section, the soil harbors certain nonsymbiotic bacteria that are also capable of fixing atmospheric nitrogen. The presence of a readily available carbohydrate for energy appears to be necessary for the reaction to take place. Many organisms possess the power to fix nitrogen in small quantities, but only a few species are capable of utilizing the gas in relatively large amounts.

**Organisms Concerned.**—The first nonsymbiotic, nitrogen-fixing organism isolated was *Clostridium pastorianum* (*butyricum*), which is a motile, Gram-positive, anaerobic, spore-forming rod (Fig. 209). *C. butyricum* is usually found in all soils and fixes small amounts of atmospheric nitrogen.

However, the most important organisms concerned in the fixation of nitrogen nonsymbiotically belong to the genus *Azotobacter*, which is characterized as follows:

Cells without endospores. Relatively large rods or even cocci, sometimes almost yeast-like in appearance. The type of flagellation in this genus has been definitely established as peritrichous. Gram-negative. Obligate aerobes, usually growing in a film on the surface of the culture medium. Capable of fixing atmospheric nitrogen when provided with carbohydrate or other energy source. Grow best on media deficient in nitrogen. Soil and water bacteria.

Three species are recognized: *Azotobacter chroococcum*, *A. agile*, and *A. indicum*.

Horner and Allison (1944) reported that in the absence of atmospheric nitrogen a number of organic nitrogenous compounds including urea,

FIG. 209.—*Clostridium butyricum*, showing rods, clostridia, and spores. Nonsymbiotic nitrogen-fixing bacteria. (*After Omelianski, from Lipman's "Bacteria in Relation to Country Life," The Macmillan Company, New York.*)

aspartic acid, asparagine, adenine, and glutamic acid could be assimilated. With the exception of urea, they were not so readily utilized as inorganic ammonium salts, nitrites, and nitrates. The decrease in nitrogen fixation by cultures, owing to addition of the nitrogen compounds, corresponded very closely to the amount of fixed nitrogen utilized. They concluded that the relative availability and probable occurrence of fixed nitrogen compounds in soils are such that they would be unlikely markedly to retard nitrogen fixation by *Azotobacter* under most conditions.

Investigations with the electron microscope and with the Hofer and Wilson (1938) modification of Gray's flagella stain showed that cells of *Azotobacter* possessed an unusually large number of flagella and that they were peritrichous (Hofer, 1944). Drawings of a number of species are shown in Fig. 210 (see also page 70).

The presence of nitrogen-fixing organisms may be easily demonstrated by inoculating a small amount of fertile soil into an inorganic medium containing glucose. *Clostridium butyricum*, together with other bacteria, will develop in such a solution. If the glucose is replaced by mannitol, organisms belonging to the genus *Azotobacter* will be found to predominate in the culture.

The inoculated medium is best incubated at a temperature of about 25°C. After several days at this temperature, a pellicle begins to form on

the surface of the medium. The pellicle is at first gray in color, later becoming brownish in appearance A microscopic examination reveals the presence of many typical cells of *Azotobacter* surrounded by slimy capsules. New cultures are prepared by transferring some of the pellicle to a flask of fresh medium. After several transfers, the culture becomes heavily enriched in *Azotobacter*. A loopful of the pellicle is streaked over the surface of a solid medium and incubated. A pure culture is obtained by transferring a typical colony developing on the plate to a tube of fresh medium.

The members of the genus *Azotobacter* are widely distributed in soil. The absence of the organisms from certain soils is due probably to the pH of the soil. *Azotobacter* cells cannot develop if the pH is lower than 6. If the reaction is adjusted to the proper pH, the organisms appear in the soil.

The size and shape of the cells depend upon certain environmental factors, such as the composition of the medium, the conditions of cultivation, and the amount of oxygen. The presence of an ample supply of oxygen causes a lengthening of the cells, accompanied by an increase of motility. The addition of colloidal organic substances or aluminum salts to the medium results in the cells remaining young for a long period of time. On the other hand, alkali salts added to the medium stimulate the cells to maturity.


FIG. 210.—Drawings of flagella of *Azotobacter*. *A*, *Azotobacter chroococcum*; *B*, *Azotobacter beijerinckii*; *C*, *Azotobacter agile*; *D*, *Azotobacter agile* (another strain); *E*, *Azotobacter indicum*. (*After Hofer.*)

**Stimulation of Nitrogen Fixation in Mixed Culture.**—Lind and Wilson (1942) isolated an aerobic spore-forming organism as a contaminant from a culture of *Azotobacter vinelandii* which markedly stimulated nitrogen fixation by this organism. Attempts to replace this contaminant with other aerobic spore-bearing species failed.

Conditions in the soil are probably seldom optimum for nitrogen fixation by *Azotobacter*. The unusually high rates of fixation obtained in the laboratory are rarely, if ever, duplicated in nature. Associated microorganisms may stimulate the normal slow rate of fixation by liberating essential elements from soil minerals, by providing superior sources of carbon, or by assimilating free ammonium ions. Since *Azotobacter* cells

are capable of synthesizing biotin, inositol, nicotinic acid, pantothenic acid, pyridoxin, riboflavin, and thiamin (Jones and Greaves, 1943) these factors, as well as a supply of nitrogen, are made available to the associated organisms.

**Mechanism of Nitrogen Fixation.**—Virtanen and Laine (1939) believed that the reaction mechanism of nitrogen fixation occurring in *Azotobacter* and *Rhizobium* was probably the same. *Azotobacter* and *Rhizobium* showed marked differences in the amounts of their excretion products. The former excreted very small amounts of nitrogen compounds, whereas the latter excreted 60 to 80 per cent of the total fixed nitrogen. The difference in the amounts of excretion products of the two groups of organisms is probably due to the fact that *Azotobacter* uses the nitrogen compounds largely for its own cell protein whereas *Rhizobium* excretes most of it into the soil.

Wilson, Hull, and Burris (1943) cultivated *Azotobacter vinelandii* in the presence of normal nitrogen compounds in an atmosphere containing the stable isotope of nitrogen ($N^{15}$). Isotopic analysis furnished a means for determining the ability of various compounds to compete with the nitrogen-fixation reaction. To quote,

> Ammonia and compounds readily converted to ammonia are used to the virtual exclusion of molecular nitrogen. With ammonium compounds, and probably urea, the change from a metabolism involving only $N_2$ to one based on combined nitrogen is rapid and complete. With other compounds, notably nitrate, a period of "adaptation" is essential, otherwise fixation is not entirely suppressed. With asparagine, an increased but not complete inhibition of nitrogen fixation was observed after adaptation. Nitrogen compounds which the organism assimilates only with difficulty (aspartic and glutamic acids) or not at all (arginine) do not inhibit the fixation of $N_2$ to a marked extent. It is concluded that present evidence based on research with isotopic nitrogen favors the view that $NH_4^+$ is a key intermediate.

Wilson (1939, 1940) demonstrated that molecular hydrogen acted as a specific inhibitor of nitrogen fixation by inoculated red clover plants. Later, Wyss and Wilson (1941) made estimations of nitrogen fixation by three species of *Azotobacter* and obtained essentially the same result. To quote,

> *Azotobacter* cultures grown in an atmosphere in which the $pN_2$ is reduced to 0.3 atm., the $pO_2$ kept at 0.2 atm. and the abstracted $N_2$ either unreplaced or replaced with helium or argon, fix atmospheric $N_2$ at the same rate as that observed with cultures grown in air. If, however, $H_2$ is used to replace the $N_2$, a significant decrease is observed in both rate and extent of fixation. Since the symbiotic nitrogen fixation system of red clover responds to $H_2$ in the atmosphere in essentially the same manner, it is concluded that the mechanism of nitrogen fixation by the symbiotic system is similar, if not identical, with that of the fixation system in the free-living *Azotobacter*.

**Inoculation of the Soil.**—Many attempts have been made to increase the nitrogen content of the soil by inoculation with pure cultures of the specific organisms. In most cases, the results have been negative. In a few experiments a detrimental influence was noted. The failures have been attributed to the following factors: (1) absence of suitable environment, such as proper temperature, moisture, amount of oxygen, food, and reaction of the soil, (2) absence of a source of carbon, and (3) injurious effects due to the end products liberated in the decomposition of added carbohydrate.

The specific organisms are cultivated in the laboratory under optimum conditions for growth. Also, they are grown in pure culture, not in association with other species. When such cultures are introduced into the soil, conditions are encountered that are generally not ideal. This means that most of the organisms soon die. Also, there are so many more bacteria of other genera already in the soil that it is extremely difficult for a laboratory culture to gain a foothold. In order that soil inoculations be successful, the chemical, physical, and probably biological conditions of the soil must be made suitable for the growth of *Azotobacter*.

For additional information on nonsymbiotic nitrogen fixation, consult Fife (1943*a*,*b*), Lee and Burris (1943), Lee, Wilson, and Wilson (1942), Lee and Wilson (1943), Wilson, Lee, and Wilson (1942), Wilson and Burris (1947), Wilson and Lind (1943), and Wilson and Wilson (1943).

## AUTOTROPHIC BACTERIA

Most bacterial species and other lower forms of life utilize inorganic and complex organic compounds for structure and energy. They are unable to synthesize carbohydrates, fats, and proteins or their hydrolytic products from water, carbon dioxide, and nitrogen of the atmosphere but must have their food preformed as organic and inorganic compounds. Such organisms are classified under the heterotrophic group.

A few bacterial species are able to obtain their carbon from carbon dioxide and their energy from the oxidation of nitrogen, sulfur, iron, hydrogen or carbon, either free or in the form of inorganic compounds. These organisms are classified under the autotrophic bacteria.

Higher plants are also able to effect a synthesis of their own organic compounds. They take carbon dioxide from the air and combine it with water to form carbohydrates. Nitrogen is absorbed from the soil in the form of nitrate or other nitrogen-containing compound and synthesized into proteins.

The true autotrophic bacteria show several distinctive physiological characteristics:

1. They grow and multiply in strongly elective mineral media, containing the specific inorganic oxidizable substances.

2. Their existence is dependent upon the presence of these minerals which undergo oxidation as a result of the life processes of the organisms.

3. These oxidations are the only source of energy for the bacteria.

4. The organisms do not require any organic nutrients for either structure or energy.

5. They assimilate carbon dioxide chemosynthetically as the only source of carbon.

6. The presence of small amounts of certain organic compounds may be stimulating at least to some of the autotrophs. Since these organisms are soil inhabitants, they are in practically all cases in contact with soluble organic matter.

The number of obligate autotrophic species is very small. A greater number of species are facultative autotrophs, being capable of existing both autotrophically and heterotrophically. The obligate forms include the nitrifying organisms and some of the sulfur and iron bacteria. The facultative forms can obtain their energy (1) from the oxidation of inorganic substances and the reduction of carbon dioxide for the synthesis of their own protoplasm, or (2) from purely organic compounds. Some of the sulfur, iron, and hydrogen bacteria are facultative autotrophs.

The autotrophic group includes forms varying considerably in their morphological appearance. Some of the forms exist as minute cells, greatly resembling in appearance the members of the *Eubacteriales*. Others exist in the form of long filaments. Still others appear to be closely related to the algae in size, shape, and mode of division. Some of the forms contain a pigment known as bacteriopurpurin. This is a red-colored pigment that appears to function in a manner similar to chlorophyll when the bacteria are exposed to the light. The autotrophic bacteria may be classified as follows:

Order I. *Eubacteriales.*[1]
Suborder I. *Eubacteriineae.*
Family I. *Nitrobacteriaceae.*
Tribe I. *Nitrobactereae.*
A. Bacteria oxidizing ammonia to nitrite.
Genus 1. *Nitrosomonas.*
Genus 2. *Nitrosococcus.*
Genus 3. *Nitrosospira.*
Genus 4. *Nitrosocystis.*
Genus 5. *Nitrosogloea.*
B. Bacteria oxidizing nitrite to nitrate.
Genus 6. *Nitrobacter.*
Genus 7. *Nitrocystis.*
Tribe II. *Hydrogenobactereae.* Bacteria oxidizing hydrogen to water.
Genus. *Hydrogenomonas.*

[1] See Chap. XVI for descriptions of the various orders, suborders, families, tribes, and genera.

Tribe III. *Thiobacilleae.* Bacteria oxidizing sulfur or sulfur compounds.
Genus. *Thiobacillus.*
Family II. *Pseudomonadaceae.*
Tribe. *Pseudomonadeae.* Bacteria oxidizing methane to carbon dioxide and water.
Genus. *Methanomonas.*
Suborder II. *Caulobacteriineae.*
Family. *Gallionellaceae.* Bacteria oxidizing iron compounds.
Genus. *Gallionella.*
Suborder III. *Rhodobacteriineae.*
Family I. *Chromaticidaceae.* Cells not filamentous and contain both sulfur granules and bacteriopurpurin. Cells divide in three directions of space. They are sometimes called the *Thiorhodaceae.*
Genus 1. *Thiosarcina.*
Genus 2. *Thiopedia.*
Genus 3. *Thiocapsa.*
Genus 4. *Thiodictyon.*
Genus 5. *Thiothece.*
Genus 6. *Thiocystis.*
Genus 7. *Lamprocystis.*
Genus 8. *Amoebobacter.*
Genus 9. *Thiopolycoccus.*
Genus 10. *Thiospirillum* (Fig. 211).
Genus 11. *Rhabdomonas.*
Genus 12. *Rhodothece.*
Genus 13. *Chromatium* (Figs. 212 and 213).
Appendix: Doubtful genera.
Genus 1. *Thiosphaerion.*
Genus 2. *Pelochromatium.*
Genus 3. *Thioporphyra.*
Family II. *Chlorobacterioidaceae.*
Genus 1. *Chlorobium.*
Genus 2. *Pelodictyon.*
Genus 3. *Clathrochloria.*
Genus 4. *Chlorobacterium.*
Genus 5. *Chlorochromatium.*
Genus 6. *Cylindrogloea.*
Family III. *Athiorhodaceae.* Cells not filamentous, contain bacteriopurpurin but no sulfur granules.
Genus 1. *Rhodopseudomonas.*
Genus 2. *Rhodospirillum.*
Order II. *Chlamydobacteriales.*
Family I. *Chlamydobacteriaceae.* Bacteria oxidizing iron compounds.
Genus 1. *Sphaerotilus.*
Genus 2. *Clonothrix.*
Genus 3. *Leptothrix.*
Family II. *Crenothricaceae.*
Genus. *Crenothrix.*
Family III. *Beggiatoaceae.* Cells filamentous, nonmotile, differentiated into base and tip.
Genus 1. *Thiothrix* (Fig. 214).

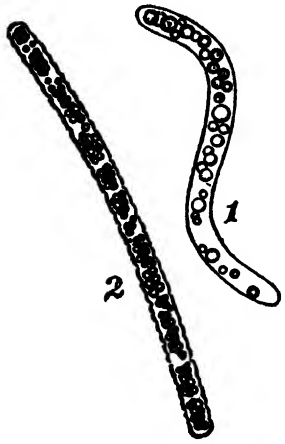



FIG 211 —1, *Thiospirillum winogradskii* (*After Omelianski.*) 2, *Beggiatoa alba* (*After Corsini, from Lipman's 'Bacteria in Relation to Country Life,' The Macmillan Company, New York.*)

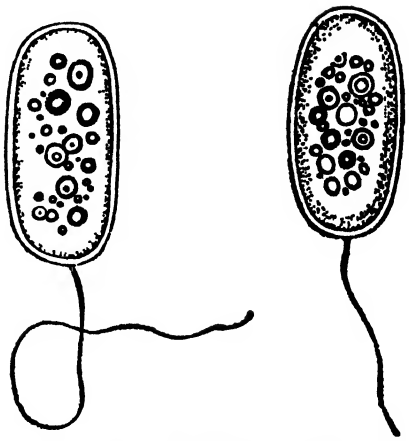


FIG 212.—*Chromatium okenii.* The round bodies inside of the cells are sulfur granules. (*After F. Forster.*)

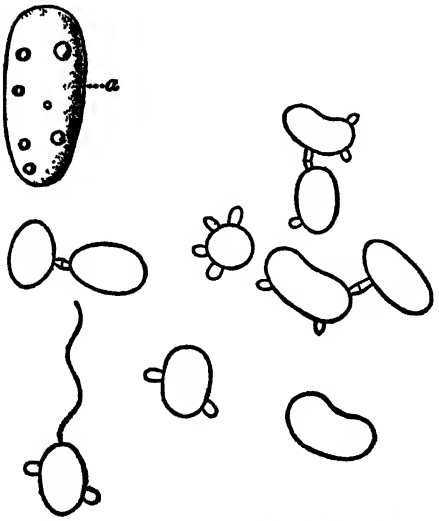



FIG. 213.—*Chromatium minus.* Artificial cultures show the development of buds similar to those observed in yeasts, Motile by means of one large polar flagellum. (*After Bavendamm.*)

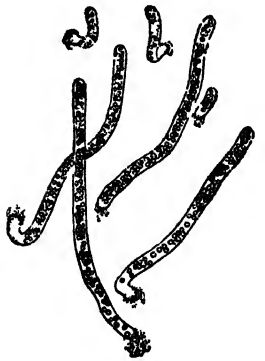


FIG. 214.—*Thiothrix nivea.* Young, unicellular, sulfur-filled filaments with a slime cushion at the base of each. (*After Winogradsky.*)

Genus 2. *Beggiatoa* (Figs. 215 and 216).
Genus 3. *Thioploca.*

Family IV. *Achromatiaceae.* Cells not filamentous, containing sulfur granules but no bacteriopurpurin.
Genus 1. *Achromatium* (Fig. 217).
Genus 2. *Thiophysa.*
Genus 3. *Hillhousia.*

## NITROSIFICATION

The organisms responsible for the oxidation of ammonia to nitrite in the soil are members of the genera *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosocystis* and *Nitrosogloea.* The cells are rod-shaped, spherical, or spiral, aerobic, nonsporulating, and grow best at a temperature of about

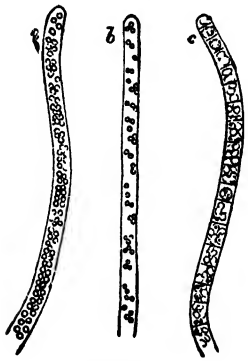



FIG. 215.

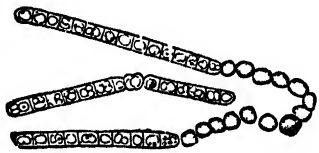


FIG. 216.

FIG. 215.—*Beggiatoa alba.* *a*, filament growing in a medium rich in $H_2S$; *b*, same filament after 24 hr. in a medium free of $H_2S$, only a few sulfur granules are present. *c*, same filament after 72 hr. Note the disappearance of the sulfur granules and the appearance of cross walls. (*After Winogradsky.*)

FIG. 216.—*Beggiatoa alba.* Degenerate thread due to lack of $H_2S$. (*After Winogradsky.*)

25°C. The organisms secure their growth energy from the oxidation of ammonia to nitrite. They grow very poorly or not at all on a medium containing organic matter.

In order easily to isolate the organisms from soils, conditions must be made unfavorable for the growth of other species except those which are able to oxidize ammonia to nitrite. A simple inorganic medium is employed containing an ammonium compound, magnesium carbonate, and other salts. Since the organisms are strongly aerobic, the cultures should be exposed to the air in shallow layers. Erlenmeyer flasks are suitable for the cultivation of the nitrifiers.

Nitrite formation takes place best in neutral or slightly alkaline soils. The ammonia of the medium is oxidized to nitrous acid. As the nitrous acid content increases, the percentage of ammonia decreases. This continues until all of the ammonia has been oxidized to nitrite. The addition

of more ammonium salt results in a continuation of the process as long as there is an excess of magnesium carbonate to neutralize the nitrous acid. When all of the carbonate has been decomposed, the organisms should be transferred to a flask of fresh medium. Colonies may be obtained by streaking the organisms over the surface of an inorganic medium solidified by silicic acid. The inorganic silicic acid is superior to agar for the cultivation of nitrifying organisms.

According to Anderson and Macsween (1942), silica gel may be prepared as follows:

Commercial water glass (sodium silicate) is diluted with water to a specific gravity of 1.3. One volume of the solution is mixed with 9 volumes of culture

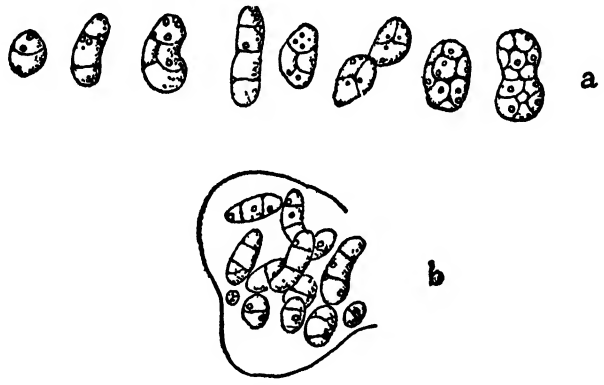



FIG. 217.—*Achromatium oxaliferum*. *a*, zoospores, *b*, zoospores being liberated from mother cell. (*After Virieux.*)

medium and 25 cc. of 0.04 per cent bromothymol blue indicator per liter. The solution is sterilized through a Seitz filter as soon as possible after mixing. A delay of even an hour or two considerably slows down filtration, whereas standing for two or three days results in gel formation. The filtered broth-silicate mixture is transferred aseptically to a sterile container. The pH is then adjusted by the addition of 8*N* or 4*N* phosphoric acid under sterile conditions. Gel formation occurs between pH5 to 9, but considerably more rapidly on the alkaline side. The optimum appears to be about pH7.2, but it can be varied to suit the requirements of the organism under investigation. To bring the broth-silicate mixture to pH7.2 requires a volume of 8*N* phosphoric acid approximately one-tenth that of the broth. Gel formation at this pH takes about 2 min., allowing ample time for pouring plates. It is advisable to incubate plates for 24 hr. in an inverted position before use, in order to allow complete extrusion of fluid from the gel by syneresis. If this is not done, the inoculum may be washed off or a spreading growth result. The extruded fluid can be removed in a sterile manner if desired, although it is not really necessary. This preliminary incubation also serves as a check on sterility.

The nitrifiers are widely distributed in nature, being present in practically all neutral and alkaline soils. They are the agents primarily re-

sponsible for the appearance of nitrate in the soil, which is in turn utilized by higher plants in the synthesis of proteins and other nitrogenous compounds.

## NITRIFICATION

Autotrophic organisms that are unable to oxidize ammonia to nitrite but can oxidize nitrite to nitrate are included in the genera *Nitrobacter* and *Nitrocystis* under the family *Nitrobacteriaceae*. The oxidation of nitrite to nitrate is known as nitrification, as distinguished from nitrosification.

The medium used for the cultivation of the organisms is similar to that employed in the preceding section except that sodium nitrite is substituted for the ammonium sulfate. The inoculation of such a medium with a suitable soil sample results in a gradual decrease in the nitrite content accompanied by a corresponding increase in the amount of nitrate. Several transfers to fresh medium are necessary to obtain a culture rich in nitrifying organisms. The medium is not specific for the growth of the nitrate-producing organisms alone but may show the presence of other species.

The nitrifiers produce neither pellicle nor uniform turbidity but a slimy layer on the bottom and sides of the flask. The organisms grow on the surface of an inorganic medium solidified with agar. The colonies are very minute, usually brownish, and vary considerably in shape. A pure characteristic colony is transferred to nitrite broth and incubated at a temperature of 28°C. for several days. The organism is identified by the appearance of nitrate in the medium.

All neutral or slightly alkaline soils show the presence of organisms capable of oxidizing ammonia to nitrite and finally to nitrate. Martin, Buehrer, and Caster (1942) presented experimental evidence for the existence of a threshold pH value of 7.7 ± 0.1 for the nitrification of ammonia in alkaline desert soils. The nitrifying bacteria are peculiarly sensitive to alkalinity, oxidizing little, if any, nitrite to nitrate at values just above the threshold value. Just below this value, the oxidation of accumulated nitrites to nitrates proceeds so rapidly that the purely microbiological nature of the transformation is open to question. It is significant that the bacteria exhibit such a marked change in activity within this narrow pH range.

## SULFUR CYCLE

Sulfur occurs in the soil in both the free and the combined form. In the combined state, it exists in the form of both organic and inorganic compounds. It is present in such organic compounds as taurine and its derivatives, in mucin, mucoid, proteins, cystine, cysteine, methionine, methyl mercaptan, etc. The inorganic compounds consist chiefly of sulfates, sulfites, and sulfides.

Sulfur finds its way into the soil from the decomposition of native rock, from organic manures, and from rain water. The gases emitted from vol-

canoes contain sulfur dioxide and hydrogen sulfide. It is present in large quantities in sulfur springs. Free sulfur is found in the neighborhood of volcanoes, being emitted through these from the interior of the earth. The commercial source of sulfur is chiefly as crude brimstone, obtained from the sides of volcanoes or mined in certain parts of the world. Extensive deposits are found on the island of Sicily and in Louisiana. Commercially, it occurs as brimstone or as flowers of sulfur, which is prepared from the crude brimstone.

Sulfur is one of the elements absolutely essential to living organisms. It enters into the composition of all plant and animal cells. The addition of sulfur to a soil low in this element results in a marked stimulation of growth. As the plants and animals die and decompose, the sulfur finds its way into the atmosphere. Volcanoes emit large quantities of sulfur dioxide and hydrogen sulfide. The burning of coal also releases gaseous compounds of sulfur. The gases are dissolved by rain water and again returned to the soil. Some soil organisms are able to convert the sulfur-containing gases to sulfates. Some of the sulfates are utilized by growing plants and some are leached out by waters and carried off to the ocean. The sulfates in the ocean may be reduced to sulfides and then precipitated by iron as iron sulfide, or they may be converted into insoluble calcium sulfate. Deposits of gypsum (calcium sulfate) are believed to have been formed in this manner. Deposits of sulfur probably resulted from the reduction of sulfate to sulfite and then to free sulfur, or from the oxidation of hydrogen sulfide. The sulfur cycle may be diagramed as shown in Fig. 218.

**Sulfur Bacteria.**—Sulfur organisms are commonly present in water containing dissolved hydrogen sulfide. The gas results from the decomposition of organic matter by saprophytic organisms. The bacteria may be present in such organic materials as decomposing seaweed, rock pools containing dead algae and other lower forms of plant life, stagnant woodland pools, and sewage. Sulfur bacteria have been found in hot sulfur springs and in sulfur mines. Some species are able to grow in water pipes and cause serious obstructions. Foul odors and tastes are produced after death and decomposition of the organisms. A large number of the purple-colored bacteria growing together cause a body of water to appear purple.

**Thiobacillus.**—The members of the genus *Thiobacillus* are small, rod-shaped organisms capable of deriving their energy from the oxidation of sulfides, thiosulfates, or free sulfur, forming sulfur, persulfates, and sulfates under acid or alkaline conditions. They obtain their carbon from carbon dioxide or from bicarbonates and carbonates in solution. With one exception, all species are aerobic. Some of the aerobic species are obligate and some are facultative autotrophic. Two important members are *T. thioparus* and *T. thiooxidans.*

*Thiobacillus thioparus.*—In an inorganic medium containing sodium thiosulfate, the organisms are capable of oxidizing the compound with the formation of sulfates and free sulfur. The free sulfur is further oxidized to sulfuric acid:

$$5Na_2S_2O_3 + H_2O + 4O_2 \rightarrow 5Na_2SO_4 + H_2SO_4 + 4S$$
$$4S + 6O_2 + 4H_2O \rightarrow 4H_2SO_4$$

A pellicle, consisting of a mixture of bacteria and sulfur granules, is produced on the surface of the medium in from 24 to 48 hr. The addition of agar to the same medium, followed by inoculation, results in the appearance of colonies from which a pure culture may be obtained.

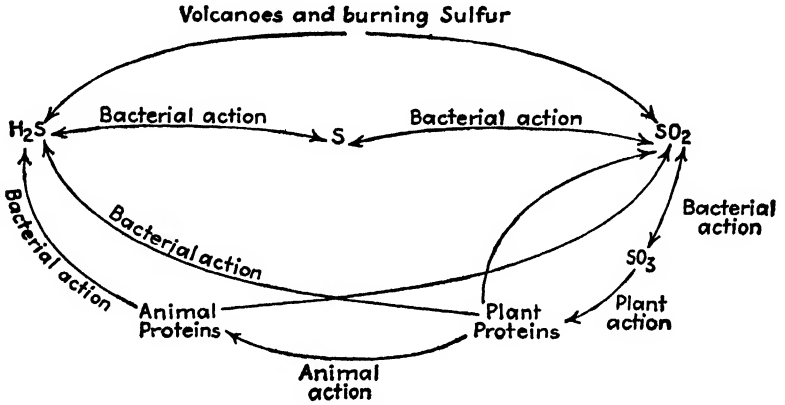



FIG. 218.—The sulfur cycle.

In the presence of air containing carbon dioxide but no dissolved carbonate or bicarbonate, growth proceeds at a much slower rate. In the absence of both carbon dioxide and dissolved carbonate or bicarbonate, no growth takes place even in the presence of various organic compounds. The organisms are unable to accumulate sulfur within their cells, although an abundant production takes place outside of the cells. The production of sulfur and sulfate from thiosulfate is an exothermic reaction resulting in the release of considerable energy. The energy is utilized for the reduction of sodium bicarbonate and for the synthesis of compounds for structure. The sodium thiosulfate may be replaced by a metallic sulfide, tetrathionate, or hydrogen sulfide. Carbon dioxide cannot be replaced by organic carbon compounds as a source of carbon. Agar colonies of the organisms are very sensitive to artificial conditions of cultivation and die after a period of about a week.

*Thiobacillus thiooxidans.*—The cells are characterized as short rods with rounded ends, occurring singly and in short chains. They are nonmotile. The organisms grow best in an acid environment. The cells use

carbon dioxide as a source of carbon. Bicarbonates are utilized only in small amounts and carbonates not at all.

The addition of sulfur to soil results in an accumulation of sulfuric acid:

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

If powdered insoluble calcium phosphate is added to a mixture of sulfur and soil, it is transformed into soluble phosphate by the sulfuric acid formed from the oxidation of the sulfur:

$$Ca_3(PO_4)_2 + 3H_2SO_4 + 6H_2O \rightarrow 3CaSO_4 \cdot 2H_2O + 2H_3PO_4$$

The tricalcium phosphate is converted to phosphoric acid. The amount of phosphate dissolved is directly proportional to the sulfuric acid content of the soil.

The rods are easily cultivated in an inorganic salt medium without carbonate, and having free sulfur and an acid reaction. Since the organism is obligately aerobic, the exposure of the medium in flat, shallow layers hastens the growth of the rods. A microscopic examination of the sulfur granules shows them to be surrounded by the specific organisms. At the same time there is a great increase in the acidity of the medium. The organism grows in the pH range of 1.0 to 6.0 with an optimum at 2.0 to 4.0. The presence of a calcium salt in the medium results in the precipitation of insoluble calcium sulfate. *T. thiooxidans* produces more acid than any other organism yet reported. The reaction of the medium drops to a pH of 0.6 or less.

In a series of investigations on *T. thiooxidans*, Vogler (1942*a*,*b*), Vogler, LePage, and Umbreit (1942), Vogler and Umbreit (1942), and LePage (1942) showed that there existed a measurable oxygen uptake in the absence of sulfur. It was possible to differentiate the endogenous respiration from that on sulfur by means of certain inhibitors of which sodium azide is a striking example. The respiration was shown to be due to the utilization of organic materials which must have been previously synthesized by the chemosynthetic process, providing evidence that the autotrophic bacteria contain a dissimilatory process which involves the breakdown of organic materials and furnishes energy for cell maintenance during periods in which the specific nutrient is absent. The organic storage material was found to consist of a polysaccharide formed during growth on sulfur. Phosphorylated intermediates appeared to be involved in the breakdown of the storage material.

For additional reading on *T. thiooxidans*, see Knaysi (1943) and Umbreit and Anderson (1942).

**Bacterial Photosynthesis.**—The pigmented, higher sulfur bacteria are generally divided into two groups on the basis of their nutritional requirements: (1) the *Thiorhodaceae*, comprising the green and purple sulfur bacteria that develop in a mineral medium in the presence of hydrogen

sulfide, and (2) the *Athiorhodaceae*, embracing the purple bacteria that require organic substances for growth.

The most important contributions on the photosynthetic activities of the higher sulfur bacteria have been reported by van Niel (1935, 1936*a*,*b*, 1941), and Gaffron (1933, 1935, 1940, 1944, 1946).

van Niel came to the following conclusions:

*Thiorhodaceae.*—1. Bacteria exist that can develop in inorganic media containing hydrogen sulfide, in the presence of sunlight, and under completely anaerobic conditions.

2. These organisms fail to develop in the absence of hydrogen sulfide.

3. In a mineral medium containing sodium bicarbonate, ammonia nitrogen, potassium, phosphorus, and magnesium, the amount of growth is proportional to the concentration of hydrogen sulfide present.

4. Growth of the organisms fails to occur in the absence of carbon dioxide (carbonate, bicarbonate).

5. Oxygen is not produced.

6. During growth of the green sulfur bacteria, hydrogen sulfide is oxidized to free sulfur which is deposited outside of the cells:

$$CO_2 + 2H_2S \xrightarrow{+\text{light}} CH_2O + H_2O + 2S$$

In the case of the purple sulfur bacteria, the $H_2S$ becomes oxidized to sulfuric acid:

$$2CO_2 + H_2S + 2H_2O \xrightarrow{+\text{light}} 2CH_2O + H_2SO_4$$

7. The reaction of the medium becomes more and more alkaline, owing to utilization of the carbon dioxide.

8. A stoichiometrical relationship exists between the amount of hydrogen sulfide oxidized and the quantity of carbon dioxide that disappears. For the green sulfur bacteria, 0.5 mole of carbon dioxide disappeared to each mole of hydrogen sulfide oxidized to sulfur; for the purple sulfur bacteria, almost 2 moles of carbon dioxide disappeared to each mole of hydrogen sulfide oxidized to sulfuric acid.

9. The carbon of the carbon dioxide that disappears can be recovered from the bacterial bodies as organic carbon.

10. Growth does not occur in the dark even though the environment is anaerobic. The hydrogen sulfide is not oxidized to sulfur or to sulfuric acid and carbon dioxide does not disappear.

The biological conversion of carbon dioxide into organic matter in the presence of light is known as photosynthesis. Since the green and purple sulfur bacteria (*Thiorhodaceae*) are capable of producing this reaction, they are considered to be photosynthetic organisms.

The photosynthetic reaction is believed to be as follows:

$$CO_2 + H_2S \xrightarrow{+\text{light}} CH_2O + H_2O + 2S$$

in which the hydrogen sulfide acts as a hydrogen donor.

The photosynthetic bacteria have been cultivated in media containing simple organic compounds instead of hydrogen sulfide or other sulfur compounds. If the organic compound employed is represented by the formula $H_2A$, the reaction now becomes

$$CO_2 + 2H_2A \xrightarrow{+\text{light}} CH_2O + H_2O + 2A$$

in which the organic compound serves as a hydrogen donor to reduce the carbon dioxide.

*Athiorhodaceae.*—The members of the *Athiorhodaceae* also contain bacteriopurpurin and are light-sensitive under anaerobic conditions, but they show the following dissimilarities to the *Thiorhodaceae:*

1. They do not contain sulfur granules within their cells.
2. It is generally stated that they develop only in the presence of organic matter, but this is not strictly correct.

Carbon dioxide can be reduced by organic compounds instead of by hydrogen sulfide (Gaffron 1933, 1935). In the presence of butyric acid, the reaction becomes

$$CO_2 + C_3H_7{\cdot}COOH + H_2O \xrightarrow{+\text{light}} 5CH_2O$$

The *Athiorhodaceae* are also capable of assimilating carbon dioxide in the presence of hydrogen, according to the reaction:

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

Approximately two molecules of hydrogen react with one molecule of carbon dioxide. In addition, the organisms utilize hydrogen sulfide photosynthetically in the presence of organic matter and carbon dioxide.

From the foregoing, it may be concluded that the two groups of organisms are photosynthetic only in the absence of free oxygen and require unusual hydrogen donors for the photochemical reduction of carbon dioxide. The general reaction

$$CO_2 + 2H_2A \xrightarrow{+\text{light}} CH_2O + H_2O + 2A$$

may be used to express the metabolism of both groups of organisms.

For excellent discussions on the true autotrophic bacteria, and on the culture, general physiology, morphology, and classification of the nonsulfur purple and brown bacteria, see van Niel (1943, 1944).

## IRON BACTERIA

Iron is, next to oxygen, the most abundant element necessary for living cells. In some species only minute amounts have been detected but, even if the quantities required are small, the element is absolutely necessary for the growth and well-being of all plants and animals.

Magnesium, rather than iron, is present in chlorophyll, the green color-

ing matter of plants. However, in the complete absence of iron, leaves do not become green. Also, if iron is withheld from a plant in which the chlorophyll is well developed, the color gradually fades to yellow.

Certain organisms found in water and soil are capable of taking up iron and accumulating it on the surfaces of their cells where it quickly oxidizes to ferric hydroxide. These organisms are known as the iron bacteria. The cells are classified under the orders Eubacteriales and Chlamydobacteriales.

The autotrophic iron bacteria are capable of oxidizing ferrous iron to ferric iron, and the energy so obtained is utilized for the chemosynthetic assimilation of carbon. These organisms should be distinguished from those forms which are capable of precipitating iron as a result of the decomposition of organic iron compounds. With the organisms in this last group, the iron plays no role of particular importance in their development. The bacteria obtain their energy from the oxidation of the organic portion of the compounds.

The reaction for the oxidation of iron by the autotrophic iron bacteria is as follows:

$$4FeCO_3 + O_2 + 6H_2O \rightarrow 4Fe(OH)_3 + 4CO_2 + 40 \text{ Cal.}$$

It may be seen that this reaction yields very little energy. To satisfy their energy requirements the organisms must precipitate large quantities of iron as ferric hydroxide. This explains why voluminous accumulations of ferric hydroxide are found in waters containing autotrophic iron bacteria.

Ferruginous waters usually show the presence of a yellowish- or reddish-colored slime on the stream bottom. The color is due to the deposition of iron in the outer sheaths of the filaments. The accumulation of iron and its oxidation to ferric hydroxide results in the formation of a hard and inelastic membrane, which eventually leads to the death of the organisms. Old filaments show a higher iron content than do young filaments. In some cases, young cells are completely lacking in a deposition of iron in their sheaths. The iron hydroxide may be removed by the application of dilute hydrochloric acid, after which the outer membrane becomes visible.

Five genera of iron bacteria are recognized: (1) *Sphaerotilus*, (2) *Clonothrix*, (3) *Leptothrix*, (4) *Crenothrix*, and (5) *Gallionella*. The first four are members of the order Chlamydobacteriales; the last one is placed under the Eubacteriales.

**Sphaerotilus.**—The organisms are attached, colorless threads, which show false branching. The filaments consist of rod- or oval-shaped cells surrounded by a thin sheath. The sheath is composed entirely of a deposit of colloidal ferric hydroxide. Multiplication occurs through the formation of conidia within the sheath of the vegetative cells. The conidia swarm at one end, float about for a time, and finally attach themselves to

solid objects where they develop into delicate filaments. The motile cells have a tuft of flagella near one end.

**Clonothrix.**—The filaments are attached and show false branching as in the genus *Sphaerotilus*. The base of a filament is broader and tapers toward the tip. A sheath is always present and later becomes encrusted with a deposit of iron. The cells are colorless and cylindrical. Multiplication takes place by means of small, nonmotile, spherical conidia. They result from the disk-shaped cells near the tip by longitudinal division and rounding up of the contents.

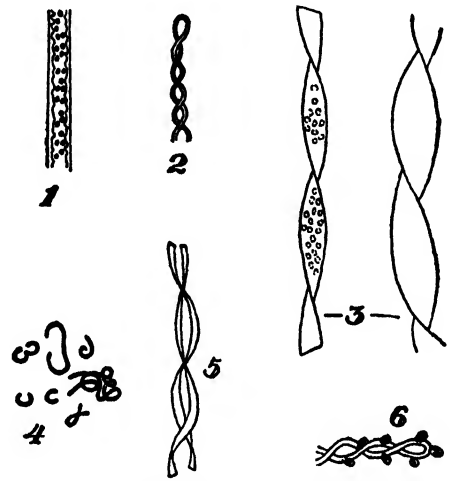



FIG. 219.—Iron bacteria. 1, *Leptothrix ochracea;* 2, 3, 4, 5, 6, *Gallionella ferruginea.* (*After Ellis, from Lipman's "Bacteria in Relation to Country Life," The Macmillan Company.*)

**Leptothrix.**—The filaments are composed of cylindrical, colorless cells, surrounded by a sheath which is at first thin and colorless, later becoming thicker and yellow or brown in color. The filaments are surrounded with iron oxide. The iron is easily dissolved by dilute hydrochloric acid, exposing the inner cells. Multiplication results by the division and abstraction of cells and by motile, cylindrical, swarm cells. Swarm cells sometimes germinate in the sheath giving the filaments the appearance of true branching. The organisms are found in rivers, lakes, ponds, and swamp waters containing iron in combination with organic matter. The best known species, *L. ochracea*, is world-wide in distribution (Fig. 219).

**Crenothrix.**—Filaments are unbranched, attached to a firm substrate, and show no differentiation of base and tip. Sheaths are easily seen, being thin and colorless at the tip, and thicker and encrusted with a deposit of iron at the base. The cells are cylindrical to spherical in shape, dividing in three planes to produce spherical nonmotile conidia. The conidia may

escape and germinate into new cells or germination may occur inside of the sheath. *C. polyspora* is the only species recognized (Fig. 220).

The organisms are found in stagnant and running water containing

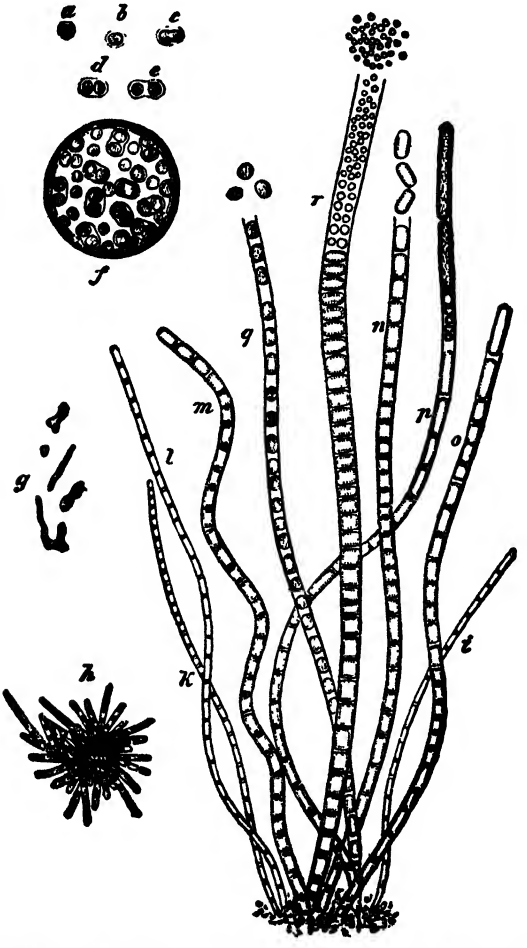



FIG. 220.—*Crenothrix polyspora.* *a–e*, reproduction of the cocci; *f*, zoogleal mass of cocci; *g*, same as *f*, natural size; *h*, same as *f*, beginning to germinate; *i–r*, threads of different diameter. (*After Zopf.*)

organic matter and iron salts. They grow in thick masses imparting a brownish or greenish color to the water, but do not grow on artificial media. When the filaments die, they are rapidly decomposed by saprophytic organisms present in water, resulting in the liberation of bad odors.

**Gallionella.**—This genus includes some of the stalked bacteria. The long axis of the rod-shaped cells is set at right angles to the axis of the stalk.

The cells occur in the form of filaments, twisted singly or two together (Fig. 219). Young cells are colorless, later becoming brown to rust red through the accumulation of iron. The twisted filaments are easily identified since no other organism of a similar character has ever been observed to take such a form. When the loops of the coils become encrusted with a deposition of iron, the filament resembles a row of beads. The presence of a sheath has not been demonstrated. The organisms attach themselves to pipes and cause extensive deposits of iron, which seriously interfere with the flow of water. For this reason, they are sometimes known as the "water-pest" bacteria. The organisms are widely distributed in nature.

For more information, see Cholodny (1926) and Starkey (1945).

## PHOSPHORUS CYCLE

Phosphorus is never found in nature in the free state. It is a constituent of many inorganic and organic compounds, being present in phosphates, nucleic acids, lecithins, phytin, coenzymes, etc. Phosphorus is found in the soil and in natural deposits. In both instances, it is present chiefly as phosphates. It is present in the protoplasm of nearly all cells as a constituent of nucleic acids. The framework of man and animals consists largely of calcium phosphate.

**Lecithin.**—Many soil organisms are capable of attacking organic phosphorus compounds. Lecithin is hydrolyzed with the liberation of glycerol, fatty acids, phosphoric acid, and choline. Many lecithins are possible, depending upon the nature of the fatty acid radicals present (see page 273).

**Phytin.**—Phytin is the phosphoric acid ester of inositol. It occurs abundantly in vegetable tissues, especially in seeds and grains. The enzyme phytase, which is elaborated by some organisms, is capable of hydrolyzing phytin to inositol and phosphoric acid (see page 275). The inositol is attacked and fermented like other carbohydrates or carbohydrate-like compounds.

**Calcium Phosphates.**—Insoluble calcium phosphates are converted into soluble phosphates by soil organisms. The solvent action is believed to be due to the presence of both organic acids and carbon dioxide formed by soil bacteria. Carbon dioxide appears to be more efficient than the organic acids for solubilizing the insoluble phosphates.

$$Ca_3(PO_4)_2 + 4H_2O + 4CO_2 \rightarrow Ca(H_2PO_4)_2 + 2Ca(HCO_3)_2$$
$$2CaHPO_4 \cdot 2H_2O + 2CO_2 \rightarrow Ca(H_2PO_4)_2 + Ca(HCO_3)_2 + 2H_2O$$
$$Ca_4P_2O_9 + 5H_2O + 6CO_2 \rightarrow Ca(H_2PO_4)_2 + 3Ca(HCO_3)_2$$

The various organic acids include butyric, lactic, acetic, citric, oxalic, and fumaric. These acids react with the phosphates to give butyrates, lactates, acetates, citrates, oxalates, fumarates, and phosphoric acid. The salts of the organic acids are usually further oxidized to carbon dioxide and carbonates.

The presence of ammonium salts in the soil results in their oxidation to nitrous and nitric acids by the autotrophic bacteria. The acid corresponding to the negative radical will also be released in the soil. If the salt is ammonium sulfate, some sulfuric acid will be liberated. The acids are capable of reacting with insoluble calcium phosphate converting it into soluble compounds. The reactions may be represented as follows:

$$Ca_3(PO_4)_2 + 2HNO_2 \rightarrow 2CaHPO_4 + Ca(NO_2)_2$$
$$Ca_3(PO_4)_2 + 4HNO_2 \rightarrow Ca(H_2PO_4)_2 + 2Ca(NO_2)_2$$
$$Ca_3(PO_4)_2 + H_2SO_4 \rightarrow 2CaHPO_4 + CaSO_4$$

A relatively high concentration of hydrogen ions is required to convert insoluble tricalcium phosphate into the soluble form. Most of the liberated

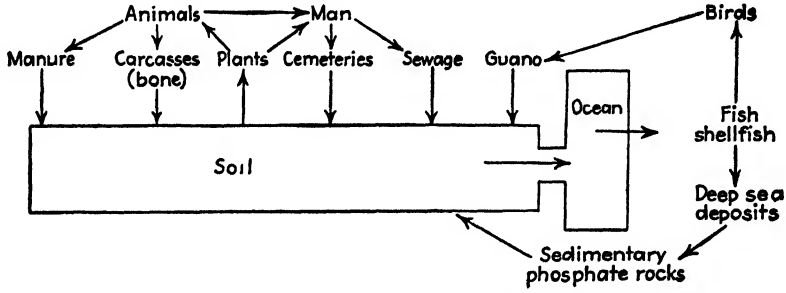



FIG. 221.—The phosphorus cycle. (*After Lotka.*)

nitrous acid reacts with calcium and magnesium carbonates and salts of organic acids. This means that the nitrous acid is replaced by weak, organic acids. In the presence of considerable acid, some insoluble phosphate is dissolved. However, the degree of acidity required for this purpose is so high that it would probably produce a destructive effect on plant growth. Therefore, the nitrifying bacteria are responsible for only a small amount of the soluble phosphate in the soil.

Sulfur is oxidized to sulfuric acid by members of the genus *Thiobacillus*. The dissolution of rock phosphate by sulfuric acid is very similar to its transformation by nitrous acid. The sulfuric acid reacts with calcium and magnesium carbonates and salts of organic acids in preference to insoluble phosphates. The high acidity (about pH3.0) required to dissolve phosphates is distinctly injurious to growing plants.

The phosphorus cycle may be diagramed as shown in Fig. 221.

**Nucleoproteins.**—Nucleoproteins are found widely distributed in plants and animals, being present in nearly all cells. They occur chiefly in the nucleuses of cells. Nucleoproteins are compounds of protein combined with nucleic acid. Different proteins and nucleic acids have been isolated, indicating that many kinds of nucleoproteins occur in nature. The pro-

teins present are basic in character, being members of the groups known as the protamines and the histones.

Nucleoproteins give an acid reaction and are insoluble in water. They are soluble in weak alkali but are precipitated from solution on the addition of acid. Nucleoproteins are very complex in composition and unstable chemically. In many cases, they appear to be mixtures of protein and nucleic acid rather than definite chemical compounds. The action of enzymes or weak acid on nucleoproteins results in a splitting off of some of the protein, transforming the compound into a mixture of protein and nuclein. The nuclein still contains some protein. More prolonged enzymatic action or treatment with acid removes the remainder of the protein, setting free nucleic acid. This may be represented as shown in Fig. 222.

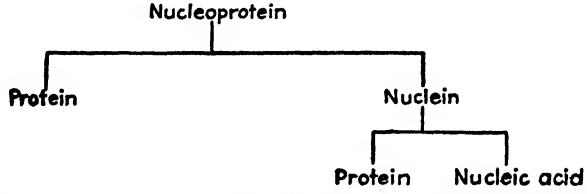
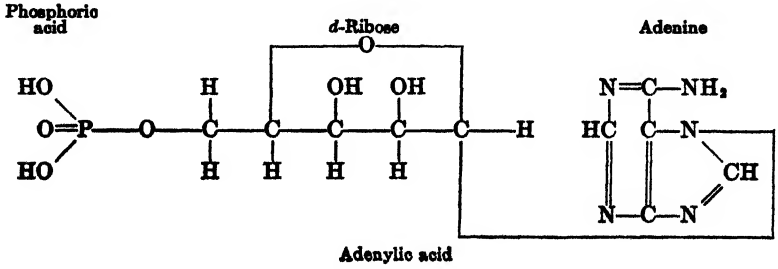



FIG. 222.—The hydrolysis of a nucleoprotein to nucleic acid and protein.

**Nucleic acids.**—Nucleic acids contain considerable amounts of phosphorus and are acid in reaction. There are two types: (1) ribonucleic acid and (2) desoxyribonucleic acid. The former type occurs in the cytoplasm of plant and animal cells in the form of granules; the latter is a constituent of the nucleus. On hydrolysis both types yield 4 molecules of phosphoric acid, 4 molecules of carbohydrate, 2 molecules of purines, and 2 of pyrimidines. Ribonucleic acid contains the pentose sugar *d*-ribose whereas desoxyribonucleic acid contains the desoxypentose *d*-2-ribodesose. The purines adenine and guanine are present in both types of nucleic acids. They differ in the kinds of pyrimidines present. Ribonucleic acid contains cytosine and uracil; desoxyribonucleic acid contains thymine and cytosine.

A nucleic acid molecule is known as a tetranucleotide being composed of 4 mononucleotides. The structure of a mononucleotide is as follows:



The above mononucleotide yields phosphoric acid, *d*-ribose, and adenine on hydrolysis. The nucleotide takes its name from the purine or pyrimidine base present. For example, a nucleotide containing guanine is known as guanylic acid; one containing cytosine is called cytidylic acid; etc. A nucleic acid molecule is composed of 4 mononucleotides joined to-

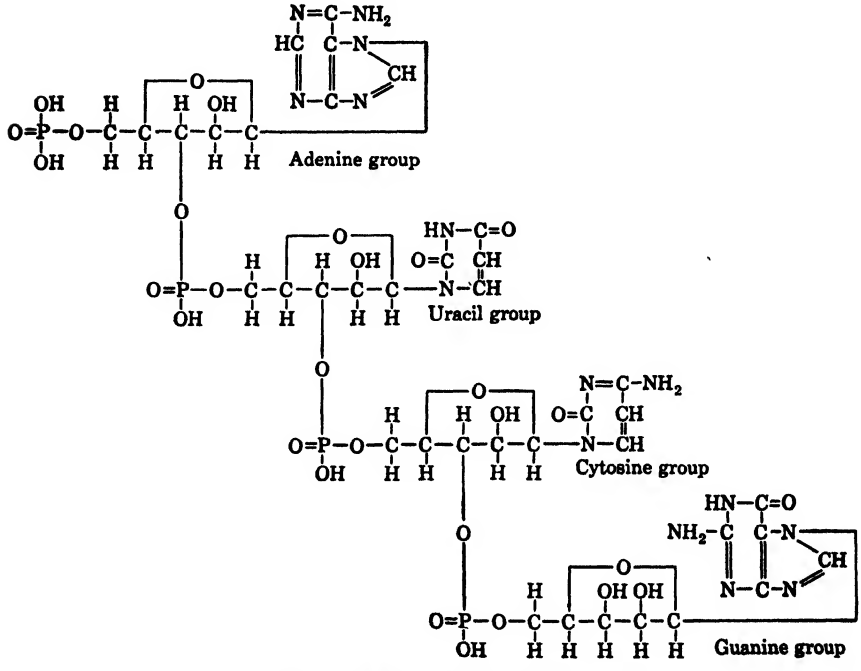



FIG. 223.—The structure of ribonucleic acid.

gether by ester linkages of the phosphoric acid with the center or third carbon atom of the sugar.

The structural formulas of ribonucleic acid and desoxyribonucleic acid are given in Figs. 223 and 224.

The hydrolysis of the nucleic acid molecules to their constituent units by enzymatic action is shown in Fig. 225.

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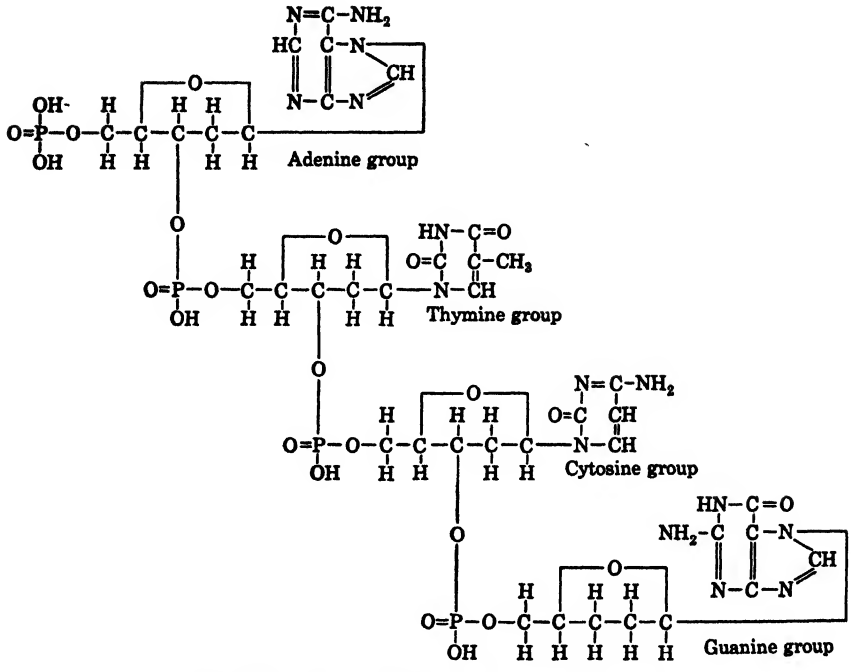


FIG. 224.—The structure of desoxyribonucleic acid.

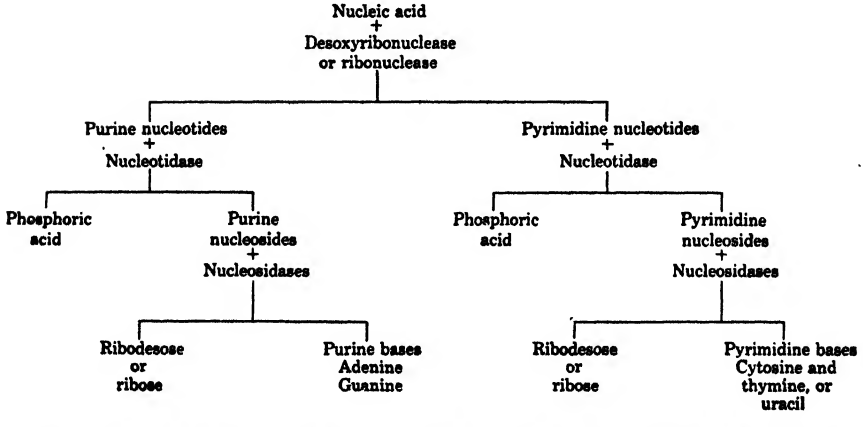


FIG. 225.—The enzymatic hydrolysis of both types of nucleic acids to their constituent units.

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## CHAPTER XXIV

## INFECTION AND IMMUNITY

**Infection.**—The term "infection" may be defined as the entrance, growth, and multiplication of organisms in the body, resulting in the development of a disease process.

**Contamination.**—The terms "infection" and "contamination" are not synonymous and should be distinguished from each other. A contaminated object is one that contains bacteria, especially those capable of producing disease. A drinking cup may be contaminated with the organism of tuberculosis, but it is not infected. Likewise, the hands may be contaminated with disease bacteria without being infected.

Bacteria are found in various parts of the body. Their presence does not mean necessarily that a disease process has been established. They are normally present on the skin, in the mouth, in the nasal passages, in the upper respiratory tract, on the conjunctiva, in the intestinal tract, etc. Some species are constantly present in each locality. Micrococci and streptococci are present on the skin, the colon organism (*E. coli*) is found in the intestinal tract, *Corynebacterium xerose* is present on the conjunctiva, streptococci and pneumococci are found in the normal mouth, etc.

**Incubation Period.**—The incubation period of a disease may be defined as the interval of time between the entrance of a disease organism into a host and the first appearance of symptoms. This period may be a few hours, several days, often weeks, and even months. For typhoid fever, it is usually about 10 days. Even though the length of the incubation period may vary, depending upon the virulence of the organisms, it can never be entirely eliminated. Just what happens during the incubation period is not clearly understood in every case. In rabies, the length of the incubation period is a measure of the time required for the virus to reach the central nervous system. The farther away from the central nervous system the bite from a rabid animal is located, the longer will be the incubation period.

**Communicable Disease.**—A communicable disease may be defined as one that is transferred naturally from one individual to another. All communicable diseases are infections produced by microscopic organisms, the causative agents of which are transferred from one person to another by contact, by coughing and sneezing, and in other ways. An infectious disease is not necessarily a communicable disease. For example, lockjaw, or tetanus, is an infectious disease but not communicable in the true sense

of the term. The organism produces an infection of wounds and has no natural means of reaching a similar wound in another individual. However, the great majority of infectious diseases are communicable. This includes such diseases as whooping cough (pertussis), mumps (parotitis), measles (rubeola), smallpox, influenza, etc. The most infectious communicable diseases are those which attack the upper respiratory tract, being usually transferred during coughing, sneezing, and talking.

**Endemic, Epidemic, Pandemic Disease.**—An endemic disease may be defined as one that occurs constantly among the population of a community. An epidemic disease is one in which a large number of cases develop in a community within a short time. A pandemic disease is an epidemic disease of wide distribution. The widespread occurrence of influenza during 1917–1919 was at first epidemic and then became pandemic, spreading over the entire world.

**Pathogenicity.**—A pathogenic organism is one capable of producing a disease. Thousands of bacterial species have been isolated but only a few of these are capable of producing disease in man. Some are pathogenic for man but not for animals. Conversely, some species produce disease in animals but fail to do so in man. The ability of an organism to invade and produce a disease process depends upon the species concerned. Therefore, it is necessary to name the host in order that the term "pathogenic" may be used correctly.

**Saprophyte and Parasite.**—Bacteria may be classed as saprophytes or parasites. The saprophytes are those organisms which live best on dead organic matter. They are the forms that are of great economic importance in reducing complex dead organic matter into simple soluble compounds, which may again be available to plants and bacteria. On the other hand, parasitic organisms are those which find conditions favorable to their growth on living cells. This latter group includes the disease-producing organisms.

Some exceptions have been noted. Occasionally a saprophytic organism becomes pathogenic, resulting in the establishment of a disease process. This is especially true when the defense mechanism of the host is lowered, following a severe illness. Also, not all parasites are pathogenic. The colon bacillus (*E. coli*) lives normally as a harmless parasite in the intestinal tract but only rarely invades the tissues to produce an infection.

**Virulence.**—Virulence may be defined as the degree of invasiveness of a pathogenic organism. Different strains of the same species may show great variability in their invasive powers. Also, the same strain kept under different conditions may show great differences in disease-producing ability.

As a rule, a pathogenic organism decreases in virulence when transferred from its natural environment to artificial culture media. Other

unfavorable environmental conditions also reduce the virulence of a pathogenic species. A strain that has been greatly reduced in virulence is said to be attenuated. Some organisms, like the pneumococcus, may lose their virulence entirely when transferred to culture media. Such a culture is said to be nonvirulent or avirulent. Other organisms retain their virulence even though cultivated on culture media for many generations.

Various methods are employed for decreasing the virulence of an organism. In addition to the use of culture media for this purpose, animal passage may be employed. For example, cowpox virus is smallpox virus that has been reduced in virulence for human beings by cultivation in the tissues of the cow.

An organism that is attenuated by passage through one animal species may be increased in virulence by passage through another species. For example, the virulence of the pneumococcus may be greatly increased by passage through white mice. In this way, a strain that was originally avirulent for mice may become so stepped up in virulence that 0.000001 cc. of a broth culture of the organisms will kill a mouse in 48 hr.

**Number of Organisms.**—The number of organisms plays a very important part in determining whether or not an infection will occur. A small number of virulent pathogenic organisms may be easily attacked and destroyed, whereas a larger number may not be completely eliminated by the defense mechanisms of the host. This explains why some individuals are attacked by an organism even though they have been previously immunized against the same species. The immunization will take care of a few invaders but breaks down when a mass attack occurs. In general, the number of organisms required to produce disease is inversely proportional to their virulence.

**Path of Infection.**—Bacteria gain entrance to the body in various ways. Some enter through the broken skin (occasionally through the unbroken skin), some by way of the respiratory passages, others by way of the alimentary tract. The portal of entry determines whether or not pathogenic bacteria are capable of producing an infection. The organism of typhoid fever, if rubbed into the broken skin, would probably not produce an infection but, if swallowed, may reach the intestinal tract and produce the disease. The organism of gas gangrene will have no effect if swallowed, but if rubbed into the broken skin may produce a fatal infection. Therefore, bacteria must enter the body by the route to which they are adapted. However, this is not the only factor that determines that an infection will result. Man and animals possess several defense mechanisms for destroying invading bacteria. If these mechanisms are vigorous and very active, they will usually defend the host against the disease organisms. On the other hand, if they are below normal and the invaders are very virulent, an infection may occur.

After bacteria invade the tissues, they may attack the host in a variety of ways. The organisms may produce a local inflammation or may localize in the liver, bone marrow, spleen, lymph glands, or in other places, giving rise to secondary abscesses or secondary foci of infection, also known as metastatic infections. Sometimes, organisms invade the blood stream producing a bacteremia or septicemia (blood poisoning).

**Bacterial Waste Products.**—Bacteria produce a large number of waste products in the culture medium in which they are growing. The formation of some of these compounds is dependent upon the presence of certain specific precursors in the culture medium. The formation of others is not dependent upon the composition of the medium but is a characteristic of the organisms themselves. The composition of the medium merely determines whether the compounds shall be produced in larger or smaller amounts.

To the former group belong such compounds as the ptomaines (amines), indole, skatole, phenol, and hydrogen sulfide. Specific amino acids must be present in the peptone of the medium, otherwise these compounds will not be formed. The latter group includes the true bacterial toxins. These are of two kinds: the exotoxins and the endotoxins. The exotoxins are elaborated by the bacterial cells and excreted into the surrounding culture medium. These may be recovered by passing the culture through an appropriate filter, which removes the bacterial bodies from the medium. Only a few pathogenic bacteria are capable of excreting true soluble toxins of great potency. The symptoms produced are due largely to the toxins excreted by these organisms. In other words, the injection of the cell-free filtrate produces symptoms characteristic of the disease. The best known members of this group are *Corynebacterium diphtheriae*, *Clostridium tetani*, *Cl. botulinum*, types *A*, *B*, and *C*, some of the sporulating anaerobes isolated from cases of gas gangrene, *Streptococcus pyogenes*, and *Micrococcus pyogenes* var. *aureus*. The endotoxins, on the other hand, are not excreted into the surrounding culture medium but remain confined within the bacterial cells. They are released only after the death and dissolution of the organisms. Most bacterial organisms fall in this group. An example is *Salmonella typhosa*, the organism of typhoid fever. If a young culture of this organism is filtered, the filtrate will produce only a slight toxicity whereas the organisms themselves may produce a very poisonous action. Filtrates of old cultures may be very toxic, owing to death and autolysis of many of the organisms resulting in the liberation of the endotoxins.

Some organisms have been shown to elaborate both exotoxins and endotoxins. The bacteria producing cholera and dysentery appear to belong to this group, although they elaborate considerably more endotoxin than exotoxin.

The exotoxins appear to be quite thermolabile, being destroyed by

moderate heating. They are easily decomposed on standing in the presence of oxygen (air). They are believed to be protein in nature, although their chemical composition has not been determined. The endotoxins show greater resistance to adverse conditions than the exotoxins.

**Mode of Action of Bacterial Toxins.**—Bacterial toxins exhibit their characteristic action by producing a specific effect on some organ or tissue. The toxins of *Clostridium botulinum* and *C. tetani* have been shown to exhibit a selective affinity for the nervous system. Some organisms, notably the hemolytic streptococci, attack and dissolve red blood corpuscles. Other organisms exhibit a selective action on the white blood cells or leucocytes. It is believed that the toxin produces a physical or chemical union, or both, with the specific tissue or organ involved. It has been shown that bacterial toxins, when injected into animals, rapidly disappear from the blood stream, indicating that a union with the specific tissue has taken place.

**Resistance.**—The fact that bacteria enter the body through the mouth, the nasal passages, or a break in the skin does not mean that an infection will take place. If this were true, man would have disappeared from the earth long ago. The power of the animal body to prevent growth and development of organisms after they have gained entrance is spoken of as resistance. The various defense mechanisms come into play and in most cases quickly remove the invading bacteria. Sometimes the resistance to a disease is characteristic of a species. It is then spoken of as immunity.

Various degrees of immunity have been shown to exist. One race may be immune to a certain disease; another may be susceptible. This does not mean that the former race cannot be given the disease. Small doses of the organisms may be easily disposed of, but massive doses are usually able to overcome the natural defenses of the host with the result that disease develops. Chickens are immune to anthrax because their body temperature is too high for the growth of the organism. If the body temperature is lowered to 37°C., chickens become susceptible to the disease.

## NATURAL IMMUNITY

A race or species may inherit a resistance to a certain infectious disease. This resistance is spoken of as natural immunity.

**Species Immunity.**—Many of the organisms that attack humans do not attack animals. Typhoid fever infections do not occur in animals except after massive experimental inoculations with the specific organism. Human leprosy has never been transmitted to animals successfully. Meningitis does not occur spontaneously in animals but may be produced experimentally. Many of the animal diseases do not occur spontaneously in man.

It is not known why differences in species susceptibility exist. It may

be due to differences in temperature, metabolism, diet, etc. Diseases of warm-blooded animals cannot ordinarily be transmitted to cold-blooded animals, and vice versa.

**Racial Immunity.**—The various races probably exhibit differences in their resistance to disease, although in many cases this may be due to differences in living conditions, to immunity acquired from mild infections in childhood, or to other causes. Negroes and American Indians are said to be more susceptible to tuberculosis than the white race. On the other hand, Negroes exhibit more immunity to yellow fever and malaria than the white race.

**Individual Immunity.**—Laboratory animals of the same species, kept under identical environmental conditions, exhibit only slight differences in their resistance or susceptibility to experimental disease. On the other hand, humans show wide differences in susceptibility to disease. For example, during an epidemic of influenza there are always some individuals who do not contract the disease even though in close contact with the virus. These individuals exhibit a higher degree of resistance than do the majority of people.

## ACQUIRED IMMUNITY

An individual of a susceptible species may acquire a resistance to an infectious disease either accidentally or artificially. This resistance is spoken of as acquired immunity.

**Accidental.**—Many of the infectious diseases, such as typhoid fever, scarlet fever, and measles, usually occur only once in the same individual. The resistance of the host to the disease is increased so that another exposure to the same specific organism usually produces no effect. This resistance or immunity may last for a limited time or for life.

**Artificial.**—Immunity may be acquired artificially by means of vaccines or by the use of immune serums. If the immunity is acquired by means of vaccines, it is spoken of as active immunity; if it is acquired by the use of immune serums, it is spoken of as passive immunity.

*Active Immunity.*—Active artificial immunity may be produced in a variety of ways: (1) by a sublethal dose of a virulent organism, (2) by a sublethal dose of dead bacteria, (3) by an injection of an attenuated culture, and (4) by immunization with bacterial products.

1. Sublethal Dose of Virulent Organisms.—This method has been employed experimentally with the organism of cholera. It is not applicable to very virulent organisms, such as *Bacillus anthracis*, the causative agent of anthrax.

2. Sublethal Dose of Dead Bacteria.—Active immunization by this method is practiced to a considerable extent against those organisms which produce only small amounts of soluble toxins (exotoxins). The method has

been used successfully against typhoid fever, the paratyphoid fevers, cholera, and to a lesser degree against *Micrococcus* and *Streptococcus* infections.

The organisms to be used for immunization are grown on an appropriate solid medium or in broth. If the organisms are grown on a solid medium, they are removed and suspended in salt solution. The broth culture of the organisms or the suspension in salt solution is sterilized by the application of heat or by the addition of an appropriate germicide. Portions of the suspension are then transferred to media to test the vaccine for sterility. Both aerobic and anaerobic cultures are prepared. If the vaccine is sterile, it is standardized to contain a definite number of organisms per cubic centimeter. This will vary depending upon the organism. *Micrococcus pyogenes* var. *aureus* vaccine is usually standardized to contain 500 million to 1 billion organisms per cubic centimeter. Typhoid vaccine usually contains 1 billion organisms per cubic centimeter. A germicide, such as tricresol or phenol, is added to preserve the product and measured amounts are distributed in sterile vials or bottles.

A vaccine, prepared from organisms grown on a solid medium, contains only the bacterial antigen in suspension. A vaccine prepared from a broth culture contains not only bacterial bodies but also various excretory products of the organisms. If the organism elaborates an extracellular toxin, the broth culture vaccine will be more valuable as an immunizing agent than one prepared from the growth on a solid medium. Immune bodies will be developed against both extracellular toxin and bacterial protein.

Vaccines prepared from laboratory stock cultures are known as stock vaccines. Various kinds of stock vaccines are prepared and may be purchased.

Vaccines prepared from two or more species are referred to as mixed vaccines. Probably the most commonly employed vaccine of this type is the common cold vaccine. A typical vaccine for colds and respiratory diseases in general has the following formula:

| | Million |
|---|---|
| *Hemophilus influenzae* | 300 |
| *Streptococcus* (several kinds) | 300 |
| *Diplococcus pneumoniae* (all types) | 300 |
| *Klebsiella pneumoniae* | 300 |
| *Micrococcus catarrhalis* | 200 |
| *Micrococcus pyogenes* var. *aureus* | 500 |
| *Micrococcus pyogenes* var. *albus* | 500 |
| Total per cubic centimeter | 2400 |

Vaccines prepared from a number of types or strains of the same species are referred to as polyvalent vaccines. An example of a commercial preparation of this type is *Streptococcus* vaccine.

Vaccines prepared from organisms freshly isolated from the patient to be treated are called "autogenous" vaccines. Such vaccines have been shown to be superior to those prepared from stock cultures because various strains of the same species may show some variation in antigenicity. Also, freshly isolated organisms are considerably more virulent than those carried on stock cultures.

The vaccines already discussed consist of dead bacteria in suspension. They are sometimes, and more correctly, referred to as bacterins. In a more restricted sense, the term "vaccine" is applied only to those preparations containing living organisms; but it has now taken on a broader meaning to include biologicals containing both dead and living organisms. The two most important vaccines containing living organisms (viruses) are smallpox and rabies vaccines.

3. Injection of an Attenuated Culture.—Pathogenic organisms rapidly lose their virulence by transfer on artificial culture media. Repeated passage through some animals increases the virulence, whereas the reverse effect may occur by passage through other animal species.

Pasteur, Chamberland, and Roux (1881) reduced the virulence of the anthrax organisms by cultivation at a temperature of 42°C. instead of at 37°C. Although the attenuated organisms were not capable of producing anthrax, they were satisfactory for immunization against the disease. Chamberland and Roux (1883) showed that the same result was achieved by growing the organisms in the presence of a dilute germicide (phenol 1:500). The bacteria lost their ability to produce spores (asporogenous) and became avirulent for sheep. However, the organisms were very satisfactory for purposes of immunization.

4. Immunization with Bacterial Products.—Some organisms excrete soluble compounds into the surrounding culture medium known as extracellular toxins or exotoxins. They may be recovered in an impure state by centrifugation of the culture followed by filtration through an appropriate filter to remove the living bacterial cells.

The symptoms produced by such organisms are largely the result of the action of the soluble products elaborated by the bacterial cells. Immunization against organisms of this group occurs following injection of gradually increasing doses of the filtrate or toxin. This method is followed in producing antitoxins against the toxins of *Corynebacterium diphtheriae*, *Clostridium tetani*, *C. botulinum*, *Streptococcus pyogenes*, *Micrococcus pyogenes* var. *aureus*, and the organisms responsible for gas gangrene. Unfortunately only a few organisms are capable of excreting potent extracellular toxins.

*Passive Immunity.*—It is evident that in active immunization a certain period of time is necessary before the cells of the host elaborate sufficient antisubstances to be of definite value in the prevention of disease. The

method is of value before symptoms of the disease appear. It is essentially a prophylactic treatment. Under some conditions, it may be used to incite antibody formation in certain chronic diseases.

In passive immunity, on the other hand, a temporary immunity may be acquired by injecting into the body an immune serum obtained from an immune animal or man. The protection enjoyed is due to the substances transferred to the patient, which are present in the immune serum. This type of immunization is practically limited to those diseases caused by organisms which elaborate powerful exotoxins. The active immunization of an animal following the injection of several doses of an exotoxin gives rise to an immune substance known as an antitoxin. The best representatives of this group are diphtheria and tetanus antitoxins, which have been of tremendous importance therapeutically. However, immune serums prepared against organisms that do not produce exotoxins are of value in some diseases. The antisubstances present in the serum of an animal injected with a suspension of bacteria (vaccine) are directed against the proteins of the bacterial bodies. The immune substances possess the power to attack the specific bacteria used in their production. These are known as antibacterial serums as distinguished from antitoxic serums.

Antitoxins are of great value prophylactically, especially after symptoms of disease have appeared. However, passive immunity lasts for only a relatively short period of time. It has been shown that antitoxin injected into humans becomes less and less from day to day and may be expected to disappear from the blood within a period of about 2 weeks. Antibacterial serums are employed before, at the same time that, or soon after infection takes place. Most of them are ineffectual and of minor importance therapeutically.

## ANTIGENS

An antigen may be defined as any substance which, when introduced parenterally into an animal, will cause the formation of antibodies. The antibodies are usually formed in the circulating blood and their presence may be recognized by appropriate tests.

As a rule, a substance is not antigenic unless it is foreign to the species receiving the injection. This statement applies only to antigens in circulation. For example, the injection of guinea pig serum into a guinea pig will not produce antisubstances but the injection of it into a rabbit will result in a vigorous antibody response. Certain proteins of the body that normally do not enter the circulation may also act as antigens in the same species. Protein of the lens of the eye from a guinea pig was found to be antigenic when injected into another guinea pig. Casein from the milk of lactating goats was reported to be antigenic when injected into the same goats.

**Nature of Antigens.**—Antigens are generally protein in character. It was formerly believed that all antigens were protein, but it has since been shown that there are some exceptions. A number of complex carbohydrates of bacterial origin and some lipid-carbohydrate compounds free from protein have been shown to be antigenic. A complex compound, consisting of carbohydrate, acetic acid, fatty acids, and phosphoric acid has been prepared by extracting *Salmonella typhimurium* with a weak solution of trichloracetic acid. This substance was undialyzable, opalescent in solution, toxic, and antigenic. Similar carbohydrate complexes have been isolated from a number of other organisms.

On the other hand, not all proteins are antigenic. In order that a protein be capable of inciting the production of antibodies, it must be soluble in blood plasma. Unless this occurs, it cannot reach the site of antibody formation. Proteins that have been irreversibly coagulated by heat usually fail to exhibit antigenic activity. There does not appear to be any relationship between protein toxicity and antigenic activity. Many proteins are nontoxic and yet elicit strong antibody responses.

It is not known why some proteins are antigenic and others are not. Gelatin is an example of a protein that is not antigenic. It is known that gelatin is lacking in aromatic amino acids, *i.e.*, acids having benzene rings. It contains no tryptophane nor tyrosine and only traces of phenylalanine. Some have concluded from this observation that the presence of aromatic acids is necessary for a protein to be antigenic, although there seems to be some evidence that this statement is not strictly correct.

**Molecular Size of Antigens.**—Antigenic substances are usually colloidal in solution. This means that they are composed of large molecules. They are held back by collodion membranes or ultrafilters which permit passage of smaller noncolloidal molecules. Diffusible compounds having relatively small molecular weights do not incite antibody formation because they readily pass into cells and are easily attacked and destroyed. Evidence seems to point to the fact that the larger the molecule, the better the antigenic response. An antigenic protein, when hydrolyzed to smaller units, such as peptones, peptides, and amino acids, becomes nonantigenic.

**Haptens.**—Carbohydrates and other nonprotein compounds are generally not antigenic but, when combined with protein, may determine the specific character of the antibody that the whole compound produces, and is capable of reacting only with that antibody. A substance of this type is known as a partial antigen or hapten. Haptens have been recognized in a number of organisms including *Diplococcus pneumoniae, Shigella dysenteriae, Klebsiella pneumoniae, Bacillus anthracis,* and *Mycobacterium tuberculosis.*

The pneumococcus (*D. pneumoniae*) has been classified into over 40 main serological types on the basis of the agglutination reaction. So far as

is known, all these types contain the same proteins but differ solely in the composition of the capsular polysaccharides. The polysaccharides are not antigenic but, when combined with protein, govern the specific character of the antibodies produced against each type.

**Flagellar and Somatic Antigens.**—Members of the genus *Salmonella* are mostly flagellated. Antigens of the flagella (flagellar or *H* antigens) are different from those of the bodies (somatic or *O* antigens). The flagellar antigens are of two types: those which are common to a group of species or types (nonspecific), and those which are peculiar to the species or type or possibly to a few species or types (specific). At one stage of the culture, specific flagellar antigens may be present; at another stage, the group antigens may be present. Therefore, a culture of an organism may consist of one phase or the other, or of a mixture of both. The somatic or *O* antigens are represented by Roman numerals; the specific flagellar antigens are represented by small letters; and the nonspecific or group flagellar antigens by Arabic numerals. The flagellar and somatic antigens of a few species of *Salmonella* are given in Table 51.

TABLE 51.—FLAGELLAR (*H*) AND SOMATIC (*O*) ANTIGENS OF SOME SPECIES OF *Salmonella*

| Group | Species | *O* antigen | *H* antigen | |
|---|---|---|---|---|
| | | | Specific | Nonspecific |
| A | *Salmonella paratyphi* | (I), II, XII | a | |
| B | *Salmonella abortus ovis* | IV, XII | c | 1, 4, 6 |
| B | *Salmonella schottmuelleri* | (I), IV, (V), XII | b | (1, 2) |
| B | *Salmonella typhimurium* | (I), IV, (V), XII | i | 1, 2, 3 |
| C-1 | *Salmonella choleraesuis* | VI, VII | (c) | 1, 5 |
| C-2 | *Salmonella morbificans bovis* | VI, VIII | r | 1, 5 |
| D | *Salmonella enteritidis* | (I), IX, XII | gomz, $z_2$ | |

For more information on the salmonellas, see Felsenfeld (1945).

**Vi Antigen.**—It was noted by Felix and Pitt (1934) that smooth strains of *S. typhosa* differed in their agglutinability by *O* antiserum. They found that the more virulent strains were generally the less agglutinable. This was later shown to be due to the presence in the virulent strains of a very labile antigenic substance known as the *Vi* or virulence antigen. Strains possessing maximal amounts of *O* and *Vi* antigens showed the highest degree of pathogenicity. The *O* or somatic antigen and the *Vi* antigen are believed to be composed of carbohydrate-lipid complexes but different from each other.

The *Vi* antigen is a surface antigen. It may be visualized as a discontinuous eruption from the deeper parts of the organism which extends out through the *O* fraction. This covering of the organism by the *Vi*

antigen may be so complete that the *O* fraction is obscured. Under these conditions, the organism is unable to exhibit the *O* antigen fraction and thus fails to agglutinate in the presence of the *O* antiserum or antibody.

The *Vi* antigen gradually disappears as the organism is carried on laboratory media, until it is completely lost. The strain passes through a number of phases before this occurs. On fresh isolation, the strain is inagglutinable by *O* antiserum. After a few transfers, the strain acquires *O* agglutinability. Then the strain is unable to stimulate the formation of *Vi* antibody, and finally ability to absorb *Vi* agglutinins from the immune serum is lost.

## ANTIBODIES AND THE ANTIGEN — ANTIBODY REACTION

Antibodies are specific substances produced by an animal in response to the introduction of an antigen. The antibodies are produced by certain cells of the animal, then thrown off into the blood stream. The serum containing the antibodies is known as an immune serum or an antiserum. An immune serum is capable of reacting either in vivo or in vitro with the homologous antigen.

The presence of antibodies in an immune serum may be demonstrated in a number of ways, depending upon the nature of the antigen. By such tests, five apparently different kinds of antibodies may be recognized: antitoxins, agglutinins, precipitins, lysins, and opsonins.

**Antitoxins.**—Antitoxins may be defined as immune bodies elaborated by living cells following the injection of bacterial filtrates containing soluble toxins. The bacterial bodies play no part in the reaction except to elaborate the soluble antigenic substances.

An antitoxin is generally prepared by injecting an animal with gradually increasing doses of toxin until a high concentration of antibodies is present in the serum. The animal is then either bled to death or a safe amount of blood removed. If the latter method is followed, the animal may be reimmunized a number of times. After the blood has clotted, it is allowed to stand until a clear straw-colored fluid (serum) separates from the clot. The serum contains the antibodies and is called an "antitoxin."

The injection of specific antitoxin into a patient suffering from a disease caused by a toxin-producing organism results in the neutralization of the toxin. The toxin is neutralized by the specific antitoxin without the destruction of either. The two components may be dissociated by acids, by high dilution, and in other ways. Several theories have been advanced to explain the mechanics of the reaction.

Arrhenius (1907) attempted to explain the neutralization of toxin by antitoxin on purely chemical grounds analogous to a reaction between a weak acid and base to give a salt. He believed that an equilibrium was reached in which there were present free toxin and antitoxin as well as some

combined toxin-antitoxin, the quantities depending upon the relative proportions of each in the mixture, according to the mass-action law:

$$\text{Toxin } (T) + \text{antitoxin } (AT) \rightleftharpoons \text{toxin-antitoxin } (TAT)$$

$$\frac{(T)(AT)}{TAT} = K$$

Bordet (1909), on the other hand, believed that the reaction could be explained as an adsorption phenomenon. He believed that the toxin and antitoxin did not unite in definite proportions similar to a chemical reaction but that the neutralization was purely physical. One serious objection to the adsorption theory is that it fails to explain the specificity of the reaction. Some believe that the neutralization is neither entirely physical nor entirely chemical but a combination of both.

**Exotoxins and Endotoxins.**—Exotoxins may be defined as high-molecular-weight compounds, generally protein in character, capable of damaging animal cells, and possessing antigenicity, *i.e.*, capable when injected into animals of causing the elaboration of substances known as antitoxins. The antitoxins are able to neutralize the damaging effects of the toxins. They are thermolabile, generally destroyed by proteolytic enzymes, detoxified by formaldehyde, and poisonous in extremely high dilution.

Some species produce only one exotoxin; others produce more than one. Oakley (1943) reported that strains of *Clostridium perfringens* produced seven different exotoxins, designated as $\alpha$-, $\beta$-, $\gamma$-, $\delta$-, $\epsilon$-, $\eta$-, and $\theta$-toxins. The $\alpha$-, $\delta$-, and $\theta$-toxins were hemolytic, *i.e.*, dissolved red blood cells, whereas the $\beta$-, $\gamma$-, $\epsilon$-, and $\eta$-toxins were nonhemolytic. Turner and Eales (1944) also found the $\delta$-toxin to be hemolytic.

With one exception, all exotoxins are digested by proteolytic enzymes. Therefore, they are not active when taken by mouth. On the other hand, the toxin of *C. botulinum* is not affected by proteolytic enzymes and is capable of producing its damaging effects when taken orally. This is rather surprising, since Lamanna, Eklund, and McElroy (1946) succeeded in crystallizing botulinum toxin and showed that it was a protein possessing the solubility properties of a globulin.

The toxic qualities of exotoxins may be destroyed by treatment with formaldehyde without damaging their antigenic or antitoxin-binding properties. Toxin so treated is known as toxoid or anatoxin. Mueller and Miller (1943) and Schoenbach, Jezukawicz, and Mueller (1943) cultivated *C. tetani* on a medium initially free from antigenic compounds and found that a potent exotoxin was produced. The exotoxin was easily detoxified by the addition of 0.2 per cent formalin and incubated at 37°C. for about 3 weeks.

A number of agents have been used for the concentration and purification of bacterial toxins and toxoids. Aluminum salts, especially alum

(aluminum potassium sulfate) appear to be superior for this purpose. The addition of about 2 per cent alum to a culture filtrate produces a flocculent precipitate of the toxin or toxoid which settles out in a few hours. The precipitate contains the active antigenic component of the culture. Elias (1944) found that the production of diphtherial toxin on gelatin-hydrolysate medium and its conversion to crude toxoid were easily accomplished. A satisfactory concentrated antigenic toxoid was obtained by adsorption of a slightly acidified medium on a suspension of aluminum hydroxide.

It is generally believed that the site of diphtherial toxin formation is within the cell although, until recently, experimental evidence for this statement was lacking. Morton and Gonzalez (1942) subjected thoroughly washed cells of *Corynebacterium diphtheriae* to sonic vibration whereby the cell walls were ruptured and the cellular contents liberated into the suspending fluid. Relatively large amounts of toxin were identified, which indicated that at least one of the sites of exotoxin formation—quite likely the only one—was within the bacterial cell.

Unlike the exotoxins, the endotoxins are poor antigenic substances which are not excreted but remain within the intact cell. Some are composed of protein and some of carbohydrate-lipid complexes. They are thermostable, generally resistant to the action of proteolytic enzymes, and not detoxified by formaldehyde. Burrows (1944) and Burrows, Mather Wagner, and McGann (1944) reported that the endotoxin of *Vibrio comma* (cholera) wa- resistant to peptic and tryptic digestion, stable to acid, unstable to $N/10$ sodium hydroxide at room temperature, readily soluble in methyl and ethyl alcohols, chloroform, and ether, but not in glycols, readily dialyzable, closely associated and possibly identical with a phospholipid, and poorly antigenic.

**Agglutinins and Precipitins.**—Gruber and Durham (1896) noticed that when bacteria (antigen) were mixed with specific antiserum, a gathering together or clumping of the organisms occurred. The clumping was due to the presence in the immune serum of antibodies known as agglutinins. The antigens (bacterial suspensions) are spoken of as agglutinogens (Fig. 226).

Later, Kraus (1897) observed that bacterial filtrates produced precipitates when mixed with specific immune serum. The precipitating antibodies in immune serum are spoken of as precipitins and the soluble antigens as precipitinogens (Fig. 227).

It is believed that agglutination and precipitation are produced by the same antibodies. In one case (agglutination), the antigen consists of particulate matter (bacteria or other cells); in the other (precipitation), the antigen is in solution. The two immune substances may be demonstrated in the same antiserum. For example, a typhoid antiserum will not only agglutinate typhoid organisms but will also produce precipitates with

culture filtrates. This holds true regardless of whether the serum was removed from an animal previously immunized against typhoid organisms or a culture filtrate.

Agglutination and precipitation are similar to the neutralization of toxin by antitoxin in that only two components are concerned in the re-

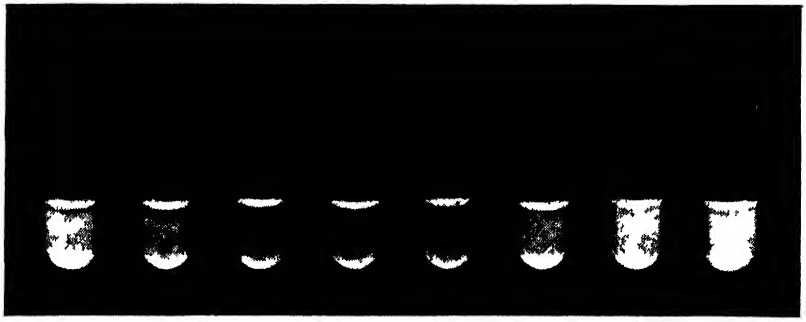

FIG. 226.—Agglutination. Various dilutions of typhoid antiserum were mixed with a suspension of *Salmonella typhosa* The immune serum dilutions increase from left to right. Tube No. 1 does not show agglutination (prozone phenomenon); tubes 2 to 6 inclusive show agglutination; tube 7 does not contain a sufficient number of antibodies to cause agglutination of the antigen. The last tube is the control.

FIG. 227.—Precipitation. Various dilutions of horse antiserum were mixed with horse serum. The tube to the left contained the most concentrated antiserum. Note the precipitation at the point of contact of the two solutions. The last tube to the right is the control.

action. These are the antigen (agglutinogen or precipitinogen) and the immune substances (agglutinins or precipitins).

According to Pauling (1940) and others, it seems likely that one antibody molecule is able to combine with more than one antigen particle, and vice versa, in such a manner as to form a framework or lattice of antigen particles. This gives rise to an antigen-antibody complex.

*Zone Phenomenon.*—If an immune serum is diluted sufficiently, it no longer agglutinates the cell suspension. This may also occur even though the dilution of antiserum contains a sufficient number of immune bodies to agglutinate the antigen.

In the series of test tubes shown in Fig. 227, the immune serum dilutions increase from left to right. Agglutination does not occur in the first tube to the left where the serum concentration is strongest. This is called the "prozone." It has been shown that an inhibitory substance, probably a globulin, is present in serum which interferes with agglutination. This inhibitory substance fails to function on further dilution of the antiserum. Agglutination is strongest in tube 3 and is absent in tube 7 where the antiserum concentration is too low to cause the cells to agglutinate.

**Normal Agglutinins and Precipitins.**—Normal serums of man and animals often possess the power to precipitate or agglutinate the specific antigen, provided the serums are not too highly diluted. It is not known if these normal agglutinins and precipitins are the same as the corresponding immune bodies, *i.e.*, those obtained following immunization.

Formalin (solution of formaldehyde), heat, ultraviolet rays, chemicals, etc., in concentrations just strong enough to kill bacteria do not destroy the agglutinating antibodies. However, most immune serums lose their agglutunating ability at a temperature of 60 to 65°C.

**Presence of Electrolytes.**—Bordet (1899) first noted that agglutination did not occur in the complete absence of an electrolyte, such as sodium chloride. However, the addition of only a minute amount of electrolyte to a nonagglutinating mixture of antigen and agglutinin caused agglutination to take place at once. This was shown to apply not only to bacteria, but to other cells. Agglutination is believed to proceed in two steps: (1) The antibody becomes fixed to the antigen and (2) the cells clump together in the presence of an electrolyte. The reaction is analogous to the precipitation of colloids by electrolytes. The union of antigen and antibody leads to the formation of an amphoteric colloidal suspension that is easily precipitable by electrolytes. Only minute amounts of electrolytes are required.

Hardy (1900) was the first to point out that the precipitation of colloidal particles is determined by the ion of the added electrolyte having an electrical charge opposite in sign to that of the colloidal particles. Since bacteria carry a negative electrical charge, the positively charged ions of the electrolyte will condition the agglutination of the organisms. The greater the positive charge, the smaller will be the quantity of electrolyte required to cause agglutination.

For an excellent discussion on the nature of the forces between antigen and antibody and of the precipitation reaction, see Pauling, Campbell, and Pressman (1943).

**Lysins.**—Nuttall (1888) found that, when normal blood was mixed with tissue containing anthrax organisms and incubated for a definite period of time, a reduction in the number of bacteria occurred. In other words, fresh normal blood exerted a bactericidal action. Buchner (1889*a*,*b*) observed that the bactericidal action of normal blood serum was destroyed

by heating to a temperature of 56°C. for 30 min. He named this thermolabile substance "alexin" (a defense substance that destroys bacteria) because of its resemblance to enzymes. Later, Pfeiffer and Issaeff (1894*a*,*b*) observed that, if cholera organisms were injected into the peritoneal cavity of a guinea pig (previously immunized against the same organism), they lost their motility, broke up into granules, and finally dissolved completely. They also showed that the injection of a mixture of cholera organisms and immune serum into a normal guinea pig resulted in a dissolution of the bacteria (bacteriolysis). Bordet observed that the reaction took place not only in the presence of unheated immune serum, but also in the presence of immune serum previously heated to 56°C. for 30 min. and mixed with a small amount of normal serum. These observations showed that two factors were concerned in the phenomenon of bacteriolysis: (1) a thermolabile substance present in normal and immune blood serum and (2) the immune substances that were produced during the process of immunization. The immune bodies are specific, whereas the thermolabile substance is not specific.

Specific, lytic immune serums may be prepared not only against bacteria (bacteriolysins) but also against other cellular bodies such as red blood corpuscles (hemolysins) and tissue cells (cytolysins).

If a bacterial serum is heated to 56°C. for 30 min., it will lose its ability to dissolve the specific antigen. The heating results in the destruction of the thermolabile substance known as alexin or complement. However, the addition of a small amount of normal serum restores the bacteriolytic power of the immune serum. Complement is present in all animal serums and is not increased in amount during immunization. The other component (known as immune body, immune substance, amboceptor, and sensitizer) is not destroyed when serum is heated to 56°C. for 30 min. and is increased in amount during immunization.

This group of antibodies requires three components for action: antigen, antibody, and complement. The antibody first reacts with the antigen. In the absence of complement, no dissolution of the antigen takes place; in the presence of this component, the cells are dissolved. On the other hand, agglutination, precipitation, and antitoxic immune reactions require only two components: antigen and antibody.

*Chemical Nature and Function of Complement.*—As has already been stated, antibodies are specific for the antigen used in immunization. On the other hand, complement is nonspecific and is not increased on immunization. Antigens (red blood cells, bacteria, etc.) undergo dissolution when acted upon by two factors in serum: (1) the relatively thermostable specific antibody and (2) the thermolabile nonspecific complement. The antibody first unites with the homologous antigen in the presence or absence of complement. Complement does not combine with the antigen or antibody

until antigen and antibody unite. When this occurs, the complement becomes bound and, under appropriate conditions, lysis of the antigen takes place.

Guinea-pig complement has been shown to consist of four functionally distinct components, designated by the symbols $C'1$ (mid-piece), $C'2$ (end piece), $C'3$ (third component), and $C'4$ (fourth component). The $C'1$ component is thermolabile, precipitated from serum by carbon dioxide or dilute acid, and is characterized as a euglobulin. The $C'2$ fraction remains in solution after the $C'1$ component is precipitated, is also thermolabile, and is characterized as a mucoeuglobulin. The $C'3$ component is inactivated by yeast or zymin, and is thermostable. The $C'4$ fraction is inactivated by dilute ammonia or hydrazine, is thermostable, and also a mucoeuglobulin.

The $C'1$, $C'2$, and $C'4$ components of complement first react with the antigen-antibody combination, after which the antigen is rendered susceptible to the action of $C'3$. This latter component behaves as if it were a catalyst causing a dissolution of the antigen.

Ecker and Seifter (1945) showed that, under the proper conditions of concentration, all the corresponding complement components of man and guinea pig were mutually substitutive. The same was found to hold true for human and rabbit complements.

For a review on the chemistry of complement, see Pillemer (1943).

**Complement Fixation.**—This is the name given to an immunological reaction first demonstrated by Bordet and Gengou (1901) and is of considerable importance in bacteriology. They noted that, when the serum of a patient who had recovered from bubonic plague was mixed with the specific antigen (plague bacilli), a binding or fixing of the complement of the serum occurred, resulting in a dissolution of the organisms. Since no free complement remained in the serum, they called the reaction "complement fixation." It is sometimes referred to as the Bordet-Gengou reaction.

Briefly, the test is as follows:

1. Antigen and antibody must first unite before dissolution of the cells by complement can occur.

2. The antigen is bound only by the specific antibody.

3. If the antigen and antibody are bound (sensitized antigen), the complement of the serum becomes fixed.

4. The absence of free complement in the serum indicates that it has been fixed by the antigen-antibody union.

5. The presence or absence of free complement in the serum may be detected by adding a mixture of sheep red corpuscles and sheep immune serum (previously heated to 56°C. for 30 min. to destroy complement) to the antigen-antibody mixture. In the presence of free complement, the sheep cells will undergo dissolution; in its absence no hemolysis will occur,

indicating that the complement has been bound by the antigen-antibody complex.

The test is of value in diagnosing the presence of certain disease organisms in patients suffering from disease or for the identification of certain antigens by means of specific serums. The test, as performed by Bordet and Gengou, may be outlined as follows:

| *A* | *B* |
|---|---|
| Antigen<br>(suspension of plague organisms)<br>+<br>Antibody<br>(plague-immune serum heated to 56°C. for 30 min.)<br>+<br>Complement<br>(fresh normal serum) | Antigen<br>(suspension of plague organisms)<br>+<br>Normal serum<br>(heated to 56°C. for 30 min.)<br>+<br>Complement<br>(fresh normal serum) |

To both *A* and *B* after 5 hr. was added:
Sheep red-cell-immune serum
(heated to 56°C. for 30 min.)
+
Sheep red blood cells

**Results:**

*A.* Hemolysis did not occur.
*B.* Hemolysis occurred.

**Opsonins.**—The immunological phenomena already discussed dealt with reactions between bacteria, bacterial excretory products (toxins), and other antigens with body fluids. These were the first immunological reactions recognized. It was subsequently shown by Metchnikoff (1901) and others that certain body cells also played a part in the defense mechanisms of the host.

The cells that are chiefly concerned in the reaction are the white blood corpuscles known as the polymorphonuclear leucocytes or phagocytes. These cells are capable of wandering to the site of infection, of engulfing bacteria, tissue fragments, etc., and of removing them from the infected area (Fig. 228). Since the cells chiefly concerned in the reaction are known as phagocytes, the process is generally referred to as phagocytosis. The leucocytes are sometimes called "scavenger cells" because they clear away bacteria and debris. Many of the leucocytes are destroyed in their attempt to remove the invading bacteria.

The power of phagocytosis is not limited to the polymorphonuclear leucocytes but is possessed by a number of other cells, some of which are fixed tissue cells, and some are wandering cells. The former includes the

endothelial cells that line the capillaries and sinuses in the liver, spleen, and lymph nodes, and other cells of the reticuloendothelial system; the latter includes the large mononuclear elements known as the macrophages.

It has been shown that, when leucocytes are washed free of serum and then mixed with bacteria, no phagocytosis occurs. If, however, a small amount of normal serum is added to a mixture of leucocytes and bacteria, active phagocytosis is restored. Antibodies known as bacteriotropins or opsonins are present in serums that prepare the bacteria for phagocytosis. Opsonins are present in normal serum and are increased during the process of immunization.

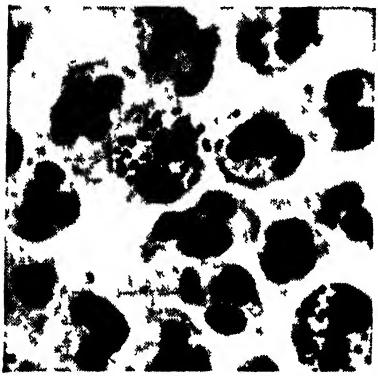

FIG. 228.—The phagocytosis of *Neisseria gonorrhoeae* by the polymorphonuclear leucocytes.

## SEROLOGY OF SYPHILIS

A number of tests are employed for the serodiagnosis of syphilis. Some of these are based on the complement fixation technique; others are flocculation tests. The most important ones are known as (1) the Wassermann test and its many modifications, (2) the Kahn test, and (3) the Kline test.

**Wassermann Test.**—A complement fixation test was proposed by Wassermann in which the antigen consisted of an aqueous extract of syphilitic fetus. It was later found that the aqueous extract of syphilitic fetus could be replaced by an antigen prepared by extracting beef heart muscle with alcohol and adding cholesterol or a mixture of cholesterol and lecithin to the alcoholic extract.

The lipoidal suspension is not an antigen in the true sense. It is not capable of inciting the production of immune bodies when injected into an animal. Since it is capable of binding syphilitic antibodies in the serum of the patient, it is generally spoken of as an antigen.

A type of antibody activity is developed as a result of infection with *Treponema pallidum*, the causative agent of syphilis. Since it is not known if this is a true antibody, it is generally referred to as a reagin. It has the power to react with the lipoidal antigen prepared from beef heart and reinforced by the addition of cholesterol and lecithin.

**Kahn Test.**—Precipitation tests for the diagnosis of syphilis have gained in popularity during the past several years. The first practical one of this type was the Kahn test. It is now the most commonly employed precipitation test for the diagnosis of syphilis.

The Kahn antigen is prepared by extracting dried beef heart with ether and the extract discarded. The residue is dried and extracted with

alcohol. The alcoholic extract is sensitized by the addition of a definite amount of cholesterol It is a specially prepared, concentrated antigen containing the colloidal material in large, unstable particles

**Kline Test.**—The Kline test is demonstrated as a microscopic slide reaction One-half cubic centimeter of serum is placed on a slide and mixed with one drop of the antigen The slide is rotated for 4 min at a speed of about 100 r p.m. and then examined under the low-power objective of the microscope. A positive test shows the presence of antigen in clumps,

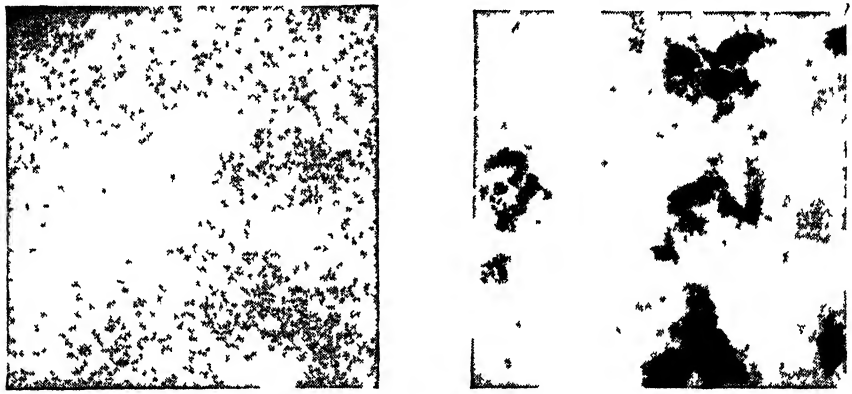

FIG 229.—Kline test for syphilis Left, negative test The antigen is not clumped but appears as a homogeneous suspension Right, positive test The antigen is clumped and the particles are floating in a clear liquid

a negative test a homogeneous emulsion without the presence of clumps (Fig. 229).

The antigen employed in the Kline test is made from the alcohol-soluble, acetone-insoluble portion of beef heart extract, concentrated by evaporation at 50°C.

The Kahn and Kline tests are simpler to perform than the older Wassermann test and its many modifications. These two tests, especially the former, are being slowly adopted in place of the Wassermann reaction, and it appears to be only a matter of time when they will replace the older method entirely.

The above methods do not give 100 per cent positive results. Usually only 80 to 90 per cent positive reactions are obtained with known sera. False, positive reactions may result from syphilitic individuals suffering from other infections, such as yaws, malaria, tuberculosis, and leprosy. In general, the tests give reliable results and, when used in connection with clinical findings and history, are of great importance in diagnosis and treatment.

For additional information, consult Boyd (1947), Kahn (1943), and Kahn, Marcus, McDermott, and Adler (1942).

## BACTERIOPHAGE (TWORT-D'HERELLE PHENOMENON)

Twort (1915) noticed certain transparent areas in a culture of a *Micrococcus* that were free from bacterial growth. He found that, if he touched one of these areas with an inoculating loop and then streaked it over the surface of an agar culture of the same species of *Micrococcus*, clear transparent areas developed along the line of streaking. Twort also found that, if the material from transparent areas was filtered through a Berkefeld filter, the filtrate contained a substance that was capable of dissolving a broth culture of the organisms. This lytic action was shown to be trans-

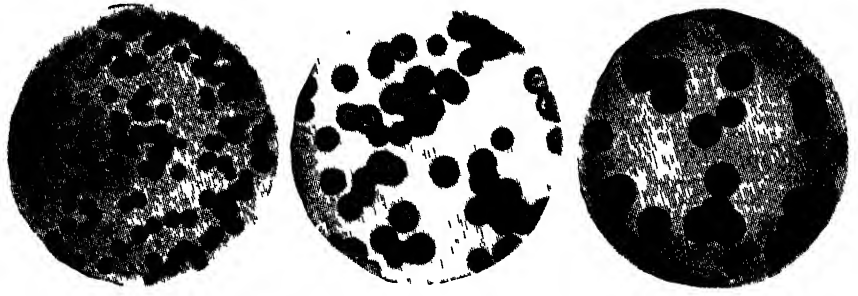

FIG. 230.—*Escherichia coli* bacteriophage. Appropriate dilutions of three different phage strains were mixed with a bacterial suspension, and 0.05 cc. of each mixture added to melted agar and poured into Petri dishes. Photographs were taken after an incubation period of 18 hrs. at 37°C. The circular areas are composed of plaques surrounded by halos. The plaques represent areas in which the bacteria have been lysed by phage. (*After Demerec and Fano.*)

missible in series. d'Herelle (1916) independently of Twort observed the same phenomenon and named the lytic principle bacteriophage (Fig. 230). The term "bacteriophage," sometimes referred to simply as phage, means literally "bacteria-eating agent."

**Demonstration of Bacteriophage.**—A number of bacteriophages, especially those active against intestinal bacteria, have been obtained from human feces, sewage, ground-up houseflies, chicken feces, and horse manure. Phages have also been isolated from old laboratory cultures, and many stock cultures contain a phage of some kind. Some strains of a bacterial species may carry a phage that will not cause lysis of the organisms. Separation of the lytic principle by filtration gives a solution that is capable of dissolving susceptible strains of the same species.

Many different phages have been isolated and studied. Bacteriophages are more or less specific in that they affect a single species or closely related species or types. Organisms having similar immunological characteristics are usually lysed by the same phage.

**Characteristics of Bacteriophage.**—Bacteriophages reproduce only in

the presence of living cells. They do not increase in the absence of susceptible bacteria nor in the presence of dead bacteria. It is generally believed that multiplication of phage requires the presence of young, actively growing cells. In most cases, the growth of phage leads finally to lysis of the bacterial cells, a phenomenon that manifests itself as a clearing of the culture medium. Multiplication does not occur to any extent in old cultures or in suspensions of resting bacteria.

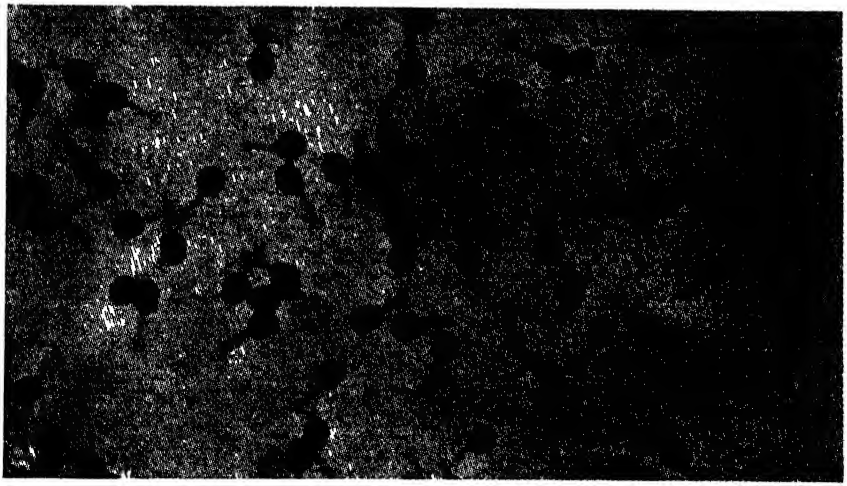

FIG. 231.—Electron micrograph of *Escherichia coli* ($T_2$) bacteriophage ×42,700. The particles are tadpole-shaped with a headpiece of well-rounded contours and a stubby tail-piece. (*After Sharp, Taylor, Hook, and Beard.*)

Electron micrographs have shown phages to be composed of particulate matter. Pure phages are quite uniform in size. They range from 10 to 100 *mu* in diameter, depending upon the strain. Some are cubical in shape; others are spherical. Phage particles usually consist of a head and tail (Fig. 231). The head is not homogeneous but displays an internal structure consisting of a pattern of granules.

Convincing evidence exists that one phage particle is sufficient to originate lysis of a cell. The phage particle is adsorbed on a living susceptible cell and, after a latent period of several minutes, causes lysis of the cell with the liberation of an average of about 100 infective units of phage (Figs. 232 and 233).

**Nature of Bacteriophage.**—d'Herelle believed that bacteriophage was an autonomous ultramicroscopic parasite, which possessed the power to break through the outer membrane and invade the bacterial cell. The bacteriophage multiplied within the cell and its accumulation resulted in cellular destruction or lysis.

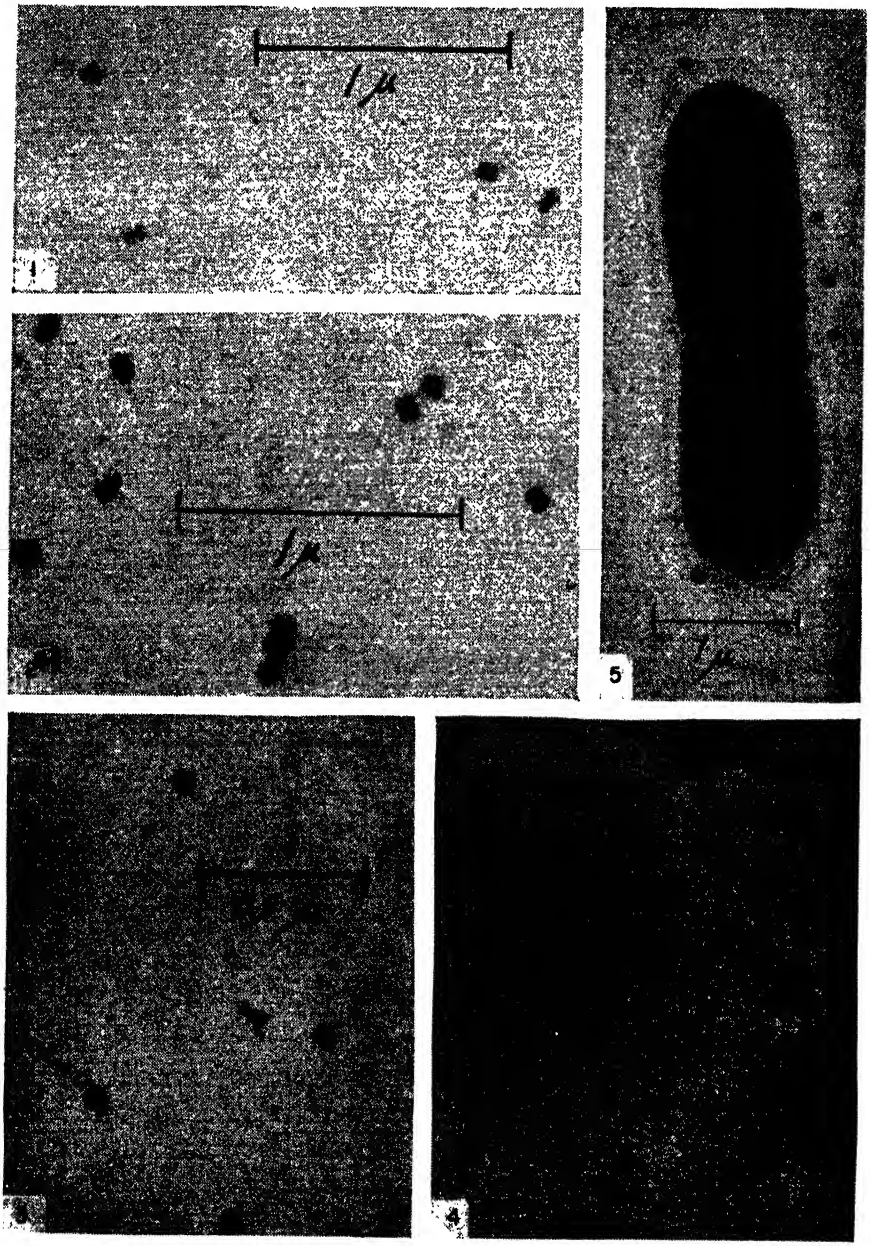


FIG. 232.—*Escherichia coli* bacteriophages. 1, Particles of *E. coli* ($\gamma$) phage ×36,000; 2, particles of *E. coli* ($\gamma$) phage ×40,000; 3, particles of *E. coli* ($\alpha$) phage ×47,000; 4, particles of *Staphylococcus* phage ×20,000; 5, *E. coli* + phage ($\gamma$), 15 minutes contact. A bacterium with adsorbed particles of phage ×20,000. (*After Luria, Delbrück, and Anderson.*)

Ellis and Delbrück (1939) found that the liberation of phage particles from *Escherichia coli* occurred in sudden bursts and showed that all the evidence was compatible with the assumption that in sensitive strains bursts of phage liberation occurred only when the cell was lysed (Fig. 234).

Northrop (1938) advanced the theory that phage production was a

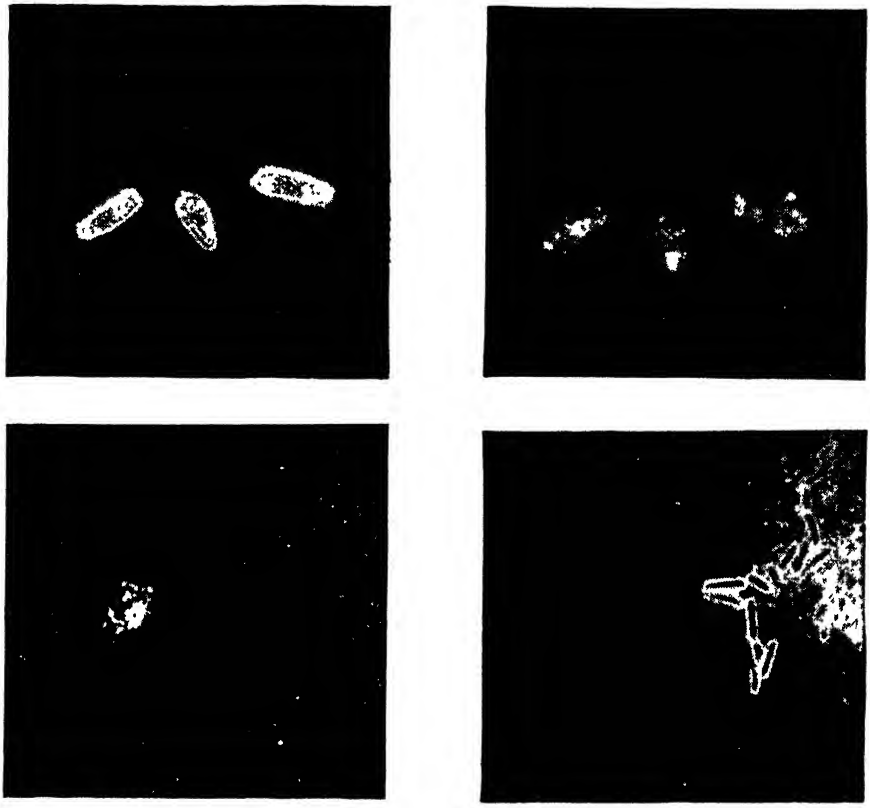

FIG. 233.—Lysis of *Escherichia coli* by bacteriophage. Upper left, three organisms showing phage particles inside, ×5000; upper right, same field 5 minutes later. The phage particles have been released and the bacilli have collapsed; lower left, phage particles free in the medium; lower right, resistant strain growing in spite of presence of abundant phage particles. (*After Merling-Eisenberg.*)

normal physiological function of the bacteria. He did not believe that phage was an autonomous living agent but a protein possessing many of the properties of an enzyme.

Delbrück (1940) reported that phage could lyse *E. coli* in two distinct ways, which he designated lysis from within and lysis from without. Lysis from within was caused by infection of a cell by a single phage particle and multiplication of this particle up to a threshold value. The cell con-

tents were then liberated without deformation of the cell wall. Lysis from without was caused by adsorption of phage above a threshold value. The cell contents were liberated by a distension and destruction of the cell wall. The adsorbed phage was not recovered upon lysis of the cell. No new phage was formed. Liberation of phage from a culture in which the bacteria were singly infected proceeded at a constant rate, after the lapse of a minimum latent period, until all the infected bacteria were lysed. If the bacteria were originally not highly in excess, this liberation was soon counterbalanced by multiple adsorption of the liberated phage to bacteria that were already infected. This led to a reduction of the final yield.

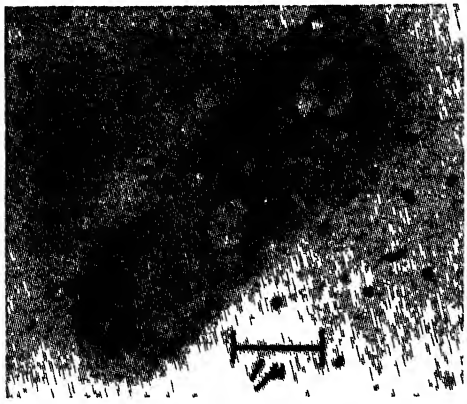

FIG. 234.—Electron micrograph of *Escherichia coli* + phage (γ), 23 minutes contact × 20,500 Ghost cell from which all content has been liberated and surrounded by bacteriophage particles and protoplasmic granules. (*After Luria, Delbrück, and Anderson*)

**Antigenicity of Bacteriophage.**—Bacteriophages are antigenic, being capable of stimulating antibody production when introduced into an animal body. Such antisera also contain antibodies against the bacterial substance present in filtrates of lysed bacteria. Bacteriophage activity is suspended on the addition of the homologous antiserum. Phages are serologically heterogeneous but may be placed into a number of distinct groups.

**Therapeutic Value of Bacteriophage.**—When bacteriophages were first discovered, it was thought that they would be of great value therapeutically. Since phage particles lyse organisms readily in vitro, it was believed that they would be capable of doing the same in vivo. A few years ago, it was a common practice for physicians to prescribe phages for many types of infections. Unfortunately, clinical observations have not borne out this claim, and it is probable that phages have no appreciable effect upon the outcome of infections. Their use for this purpose has been largely discontinued.

For additional information, see Baylor, Severens, and Clark (1944), Delbrück (1945*a*,*b*,*c*, 1946), Demerec and Fano (1945), Dubos, Straus, and Pierce (1943), Edwards and Wyckoff (1947), Hershey, Kalmanson, and Bronfenbrenner (1943*a*,*b*), Luria and Anderson (1942), Luria, Delbrück, and Anderson (1943), Morton and Engley (1945*a*,*b*), Morton and Perez-Otero (1945), Mudd (1944), Mudd and Anderson (1944), and Pijper (1945).

## HYPERSENSITIVITY

The term "allergy" includes all types of reactions of hypersensitiveness except anaphylaxis. The antigen may be protein or nonprotein. In man, there is little tendency to generalized shock. Typical anaphylactic shock has been occasionally observed, but the tendency is for localization of manifestations, especially in the respiratory and gastrointestinal tracts and in the skin. Typical allergic conditions include hay fever, asthma, atopic dermatitis, urticaria (hives), angioneurotic edema, drug allergy, contact dermatitis, migraine, and bacterial allergy.

The term "anaphylaxis" is generally used loosely but, strictly speaking, it is defined as a hypersensitiveness to substances that are antigenic, *i.e.*, capable of stimulating the production of antibodies, when injected.

A guinea pig may be inoculated with a large dose of foreign antigen without any ill effect being noted. The animal is then said to be sensitized. If, after an incubation period of 10 days or more, the animal is given a minute injection of the same antigen (intoxicating dose), violent symptoms may occur and lead to the death of the animal. If the animal recovers, it is usually refractory to another injection of the same antigen. If a guinea pig is given repeated injections of a foreign antigen, at brief intervals of 7 days or less, it does not become hypersensitive and usually shows only a mild reaction. The animal is then said to be desensitized to further injections of the same antigen.

All animal species are not equally susceptible to anaphylaxis. Guinea pigs are more sensitive than any other laboratory animal. Death is due to a bronchial spasm followed by acute asphyxia and death. Man, on the other hand, usually shows only skin lesions (rash).

The immune bodies responsible for anaphylaxis are probably identical with precipitin and complement-fixing antibodies. Anaphylaxis is believed to be a cellular reaction, taking place within the cells in which the antibody is fixed. Some of the antibodies are released from the cells and may be demonstrated in the blood stream. There is considerable evidence to support the statement that the intoxicating dose is followed by the release of histamine, heparine, and possibly choline, which are responsible for the symptoms of anaphylactic shock.

The symptoms are due to a contraction of the smooth or nonstriated muscle tissue. This may be shown by removing a piece of smooth muscle from a sensitized guinea pig and placing it in a solution of the specific antigen. Contraction immediately takes place.

Anaphylaxis is essentially a laboratory phenomenon. Guinea pigs are extremely easy to kill under conditions of anaphylaxis, but it is not very likely that many of them under natural conditions ever die of ana-

phylactic shock. Artificial conditions are set up in the laboratory that probably seldom or never occur in nature.

**Skin Test for Allergy.**—Skin tests may be used to determine if an individual is sensitive to certain foods, pollens, or other proteins. The test is performed by introducing extracts of the various protein substances into the skin of the arm and noting the results. A positive test is indicated by the appearance of a large, localized, inflamed area, or urticarial wheal, surrounding the site of injection. Many different extracts may be tested at the same time and the reaction appears after a few minutes.

For more information, see Dragstedt (1941, 1945).

## BLOOD GROUPS

Landsteiner (1900) discovered that, when the red blood cells of certain individuals were mixed with the serum of others, a clumping or agglutination of the red cells occurred. This is an example of isoagglutination. He found that there were present two antigenic components, *A* and *B*, in red blood cells and two components in serum that agglutinated them. It was subsequently shown that all human blood could be placed into four groups on the basis of the presence or absence in the erythrocytes of the two antigenic components *A* and *B*.

According to Landsteiner, the four blood groups may be represented as shown in Table 52. It may be seen that the red blood cells of the first group do not contain either the *A* or the *B* component; the cells of the second group contain only *A*; the cells of the third group contain only *B*; and those of the fourth group contain both *A* and *B*. Persons in group *A* do not have serum antibodies capable of agglutinating *A* cells but do have antibodies that agglutinate *B* cells; those in group *B* do not have antibodies capable of agglutinating *B* cells but do have antibodies that agglutinate *A* cells; those in group *AB* do not have either type of antibody; and those in group *O* have both types of antibodies.

TABLE 52.—THE FOUR BLOOD GROUPS OF LANDSTEINER

| Group | Antigen in cells | Agglutinin in serum | Reaction* with serum of type | | | | Percentage of occurrence in adults, U.S. |
|---|---|---|---|---|---|---|---|
| | | | *O* | *A* | *B* | *AB* | |
| *O* | – | Anti-*A* + anti-*B* | – | – | – | – | 45 |
| *A* | *A* | Anti-*B* | + | – | + | – | 39 |
| *B* | *B* | Anti-*A* | + | + | – | – | 12 |
| *AB* | *AB* | – | + | + | + | – | 4 |

* + = agglutination.

For blood typing, only antisera for groups *A* and *B* are required. Three microscope slides are cleaned and labeled *A*, *B*, and *C* (control).

One drop of *A* serum is placed in the center of the slide labeled *A*; one drop of *B* serum is placed in the center of the slide labeled *B*; and a drop of normal salt solution is placed in the center of the slide labeled *C*. To each slide is added an equal volume of the unknown blood cell suspension (5 per cent suspension of blood in salt solution). The slides are rocked for about 5 min., then examined under the low-power objective.

If no agglutination occurs on slides *A* and *B*, the blood is of group *O*; if agglutination occurs on both slides *A* and *B*, the blood is of group *AB*; if agglutination occurs on slide *A* but not *B*, the blood is of group *B*;

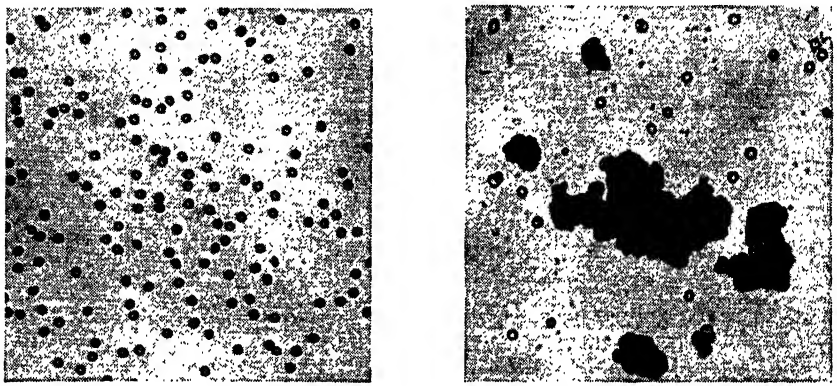

FIG. 235.—Blood grouping. Left, group *A* blood cells mixed with group *A* typing serum. Negative agglutination, indicating compatibility. Right, group *A* blood cells mixed with group *B* typing serum. Positive agglutination, indicating incompatibility.

and if agglutination occurs on slide *B* but not *A*, the blood is of group *A* (Fig. 235).

These four groups are of tremendous importance in blood transfusions, in cases of disputed parentage, and in the identification of persons.

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# CHAPTER XXV

# BACTERIAL AND VIRUS DISEASES OF PLANTS

## BACTERIA

The first recorded observations on a bacterial disease of plants dates back to the work of Burrill (1881) who discovered the causative organism of pear blight. This work was confirmed by Waite (1891) who isolated the etiological agent and proved its pathogenicity. Since then, approximately 300 species of bacterial plant pathogens have been described. It is safe to assume that there are as many bacterial diseases of plants as of man and animals.

Before an organism can be stated definitely to be the causative agent of a plant disease, it must be isolated from the plant tissue and its pathogenicity proved beyond doubt. Koch (1883) postulated certain requirements that should be met before an organism can be said to be the cause of a specific disease. These requirements have been generally accepted by both plant and animal pathologists. Koch's postulates are as follows:

1. An organism must be consistently associated with the disease in question.
2. The organism must be isolated in pure culture and accurately described.
3. The organism in pure culture, when inoculated into healthy plants, must be capable of reproducing the disease.
4. The organism must be reisolated from the diseased plant tissue and shown to be identical with the original species.

The bacterial diseases of plants may be placed into five groups on the basis of the location and character of the lesions produced: (1) soft rots, (2) vascular diseases or wilts, (3) blights, (4) intumescence diseases, and (5) local lesions or spots.

## SOFT ROTS

Organisms responsible for soft rots reduce the plant tissue to a soft, very moist, pulpy mass. The condition may be better recognized as a state of rottenness. The bacterial attack may or may not be due to a specific organism.

The organisms producing soft rots differ from the other forms found in the soil in that they have the power to attack healthy plant tissue by the secretion of an extracellular enzyme. The enzyme dissolves the pectin or cement-like material that binds together the plant cells. The action is probably hydrolytic, resulting in the liberation of soluble sugars, which are

utilized by the bacteria for food. The result is that the plant is reduced to a mass of separate cells, which become converted later into a slimy, pulpy material.

In most cases, the specific organism is accompanied or closely followed by many saprophytic soil bacteria and fungi. These organisms find a favorable environment in the exposed cells and produce relatively large quantities of ammonia by the deaminization of the amino acids present in the proteins of dead plant tissue. The ammonia produces a destruction of the neighboring plant cells and rapidly reduces the plant to a slimy,

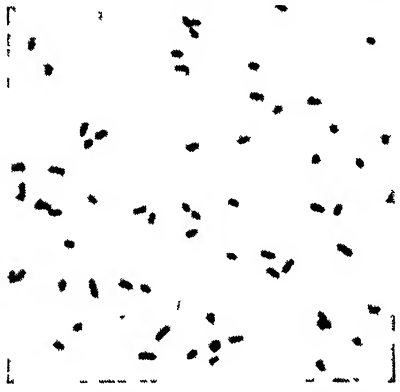

FIG. 236.—*Erwinia carotovora*, the cause of soft rot in carrots and other plants

FIG. 237.—*Erwinia phytophthora*, the cause of stem rot in potato and other plants

pulpy, foul-smelling mass. The unpleasant odor is due to the secondary invaders Plants decayed by pure cultures of the specific disease organisms do not give off an objectionable odor.

The group of organisms causing soft rots includes four species: (1) *Erwinia carotovora*, which produces a rapid soft rot of roots, rhizomes, fruits, and the fleshy stems of carrot, cabbage, celery, cucumber, eggplant, iris, muskmelon, hyacinth, onion, parsnip, pepper, potato, radish, tomato, turnip, and other plants (Fig. 236); (2) *E. phytophthora*, which produces a stem rot of potato, cucumbers, and other vegetables (Fig. 237); (3) *E. atroseptica*, the cause of soft rot of potato and other vegetables; and (4) *E. aroideae*, which produces a soft rot of calla lily, potato, eggplant, cauliflower, radish, cucumber, cabbage, parsnip, turnip, and tomato. Two other little-known species are sometimes included: *E. flavida*, the cause of soft rot of sugar cane; and *E. erivanensis*, the cause of root rot of cotton.

Much evidence has accumulated to the effect that the group is composed of only one variable species, namely, *E. carotovora*.

The organisms causing soft rots are usually small, motile, nonspore-

forming rods with peritrichous flagella, and grow readily on the commonly employed laboratory media. They vary in color from white to yellow and are Gram-negative.

For additional information on soft rots, see Elrod (1941*a*).

### VASCULAR DISEASES OR WILTS

Some organisms multiply and accumulate in large numbers in the vascular system, causing an interruption in the flow of sap in the plant. Infections of this type are grouped under the vascular diseases. A complete interruption in the flow of sap results in a rapid wilting of the plant. A partial interruption results in the growth of a sickly plant, which makes poor headway and finally dies. In many cases, death is due to the action of secondary invaders. The most important organisms causing vascular diseases include (1) *Erwinia tracheiphila*, which produces wilt of cucumber, cantaloupe, muskmelon, pumpkin, and squash; (2) *Bacterium stewartii*, which produces a wilt of corn; (3) *Pseudomonas solanacearum*, which produces a brown rot of potato, tomato, and tobacco; (4) *Corynebacterium michiganense*, the cause of canker of tomato; and (5) *C. flaccumfaciens*, the cause of wilt disease of bean.

The organisms causing vascular diseases or wilts are small rods, being yellow or white in color, motile or nonmotile. Most species are Gram-negative.

### BLIGHTS

Organisms producing blight diseases are capable of penetrating considerable distances between the cells, leaving the neighboring tissue intact. The bacteria grow in the plant juices without producing any digestion of the tissues. The rods usually produce a discoloration of the leaves and branches. Death is probably due to an interference with the flow of the plant sap.

A partial list of the organisms producing blights includes (1) *Erwinia amylovora*, the cause of fire blight or pear blight (Fig. 238); (2) *E. lathyri*, the organism responsible for streak disease of sweet pea and clover; (3) *Xanthomonas phaseoli*, which attacks the bean, lupine, and other plants; (4) *X. juglandis*, the etiological agent of walnut blight (Fig. 239); (5) *Pseudomonas mori*, the cause of mulberry blight; (6) *P. medicaginis*, the cause of stem blight of alfalfa; and (7) *P. pisi*, the cause of stem blight of field and garden peas (Fig. 240).

The characteristics of the organisms are similar to those in the preceding group.

For more information on *E. amylovora*, see Elrod (1941*b*).

### INTUMESCENCE DISEASES

Some bacteria have the power to produce galls or tumors on plants. These excrescences or abnormal growths are due to the action of organisms

on the meristematic tissue of the plants. Tissues infected in this manner are grouped under the intumescence diseases.

In some infections, the galls remain small; in others, they may assume large proportions. Sugar beets have been known to carry tumors larger than the original plants. The bacteria are believed to elaborate some

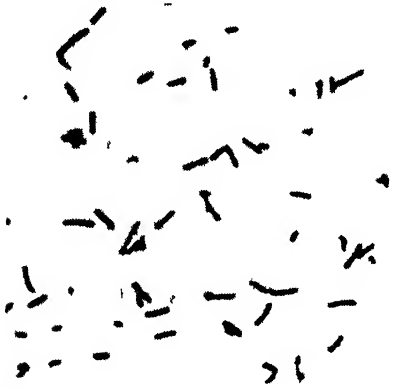

FIG. 238.—*Erwinia amylovora*, the cause of blight on pear and apple trees.

FIG. 239.—*Xanthomonas juglandis*, pathogenic for the English walnut.

FIG. 240.—*Pseudomonas pisi*, the causative agent of stem blight in field and garden peas

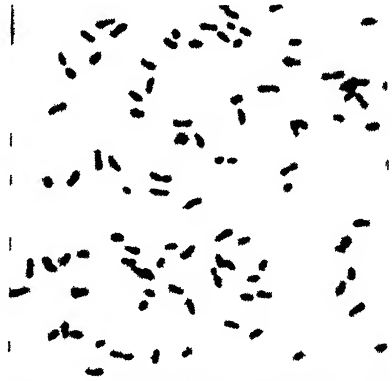

FIG 241.—*Agrobacterium tumefaciens*, the cause of crown gall of plants.

irritating metabolic product which causes rapid division of the neighboring plant cells. Some believe that tumor infections in plants are similar to cancerous growths in man and animals. Intracellular organisms are not necessary for the development of the characteristic lesions.

The important organisms producing intumescence diseases include (1) *Pseudomonas savastanoi*, the cause of olive knot; (2) *P. tonelliana*, the etiological agent of galls on the oleander plant; (3) *Agrobacterium tumefaciens*, the causative agent of crown galls on Paris daisy, cross-inoculable on many other plants (Fig. 241); (4) *A. gypsophilae*, the organism responsi-

ble for the development of galls on *Gypsophila paniculata* and related plants; and (5) *Bacterium pseudotsugae*, the cause of galls on the Douglas fir in California.

The organisms are small, white, motile or nonmotile, nonspore-forming, Gram-negative rods. The optimum temperature for growth is about 25°C.

For more information, see Felt (1940) and Riker, Spoerl, and Gutsche (1946).

## LOCAL LESIONS OR SPOTS

In many plant diseases, the attack is restricted to a small area around the point of entry. These diseases are grouped under local lesions or spots. The organisms responsible for leaf-spot diseases produce a vigorous attack on the plant tissue with the result that the cells become heavily infected and strongly discolored. The discolored areas dry up and frequently fall out, leaving holes in the leaves.

FIG. 242.—*Xanthomonas begoniae*, the cause of leaf spot of begonia.

Some of the organisms causing leaf-spot diseases are (1) *Xanthomonas cucurbitae*, the cause of leaf spot of squash and related plants; (2) *X. malvacearum*, the cause of angular leaf spot, stem lesion, and boll lesion of cotton; (3) *X. ricinicola*, the causative agent of leaf spot of castor bean; (4) *X. vesicatoria*, the cause of spotted tomato fruits in South America; (5) *X. begoniae*, the cause of leaf spot of begonia (Fig. 242); (6) *Pseudomonas angulata*, the agent responsible for angular leaf spot of tobacco; (7) *P. maculicola*, the organism responsible for cauliflower spot; and (8) *P. mellea*, the cause of brown rusty spots on Wisconsin tobacco.

The organisms responsible for local lesions or spots are similar morphologically to those producing galls.

## MODE OF INFECTION

The mode of entry of bacteria into the plant is usually through wounds. Roots, leaves, and stems are easily injured mechanically by means of agricultural implements, by animals, etc. Plants become easily infected following injury to the roots, whereas sound plants remain free from bacterial attack. Hailstones are known to produce injury to plants and make them vulnerable to infection. However, the usual cause of plant injury is through the bite of various insects. Sometimes the insects carry the etiological agent on their mouth parts, making it possible to injure and infect the plant in one operation.

In many of the leaf and fruit infections, the organisms gain entry through natural openings known as stomata. The organisms pass from the stomata into the intercellular spaces. The bacteria greatly reduce the resistance of the cells, by suffocation or poisoning, and make it possible for the etiological agent to enter the affected plant cells.

Bacteria may enter plants by way of the hydathodes or organs for the excretion of water. An excessive elimination of water results in the collection of considerable moisture on the plant surface. Bacteria readily collect in the water droplets making it possible for some to gain entrance to the plant.

Lenticels are also unprotected openings, which may offer bacteria a path for invasion of the plant. These organs are cortical pores in the stems of woody plants through which air penetrates to the interior.

Many insects are responsible for plant infections. Their proboscides or legs act as carriers of bacteria that are capable of attacking the plant. This is especially true of those plants which produce nectars designed to attract bees and other insects for the fertilization of flowers.

For additional reading on bacterial diseases of plants, consult Elliott (1930, 1943), Elrod and Braun (1942), Fawcett (1936), Heald (1933), Snieszko and Bonde (1943), Starr (1946), Stevens and Hall (1933), Walker (1939), and Wormald (1939).

## PLANT VIRUSES

A virus may be defined as a disease-producing agent, often referring to one too small to be seen with the usual light microscope. Viruses show the following characteristics:

1. They are very small, being generally below microscopic visability. However, they may be seen by the electron microscope.
2. They maintain themselves only within certain specific living cells.
3. They produce typical and similar disease in suitable hosts in unbroken series.
4. They are antigenic, being capable of stimulating antibody production when introduced into an animal body.
5. They show great capacity for variation.

Iwanowski (1892) was probably the first to report the existence of ultramicroscopic particles capable of producing disease. He showed that the agent causing mosaic disease of tobacco passed through a filter which retained all the bacteria then known. Since that time, many filter-passing agents causing diseases of plants, animals, and bacteria have been discovered.

There are probably more plant diseases caused by viruses than by bacteria. They are the causes of some of the most destructive diseases of

agricultural crops. It is safe to say that almost all cultivated plants are affected by at least one virus. It is not uncommon to encounter plants affected by two or more viruses. For example, the potato is susceptible to at least 25 viruses and the tobacco to at least 12 virus infections. Because of these multiple infections, it is often very difficult, if not impossible, to identify a virus by the symptoms produced.

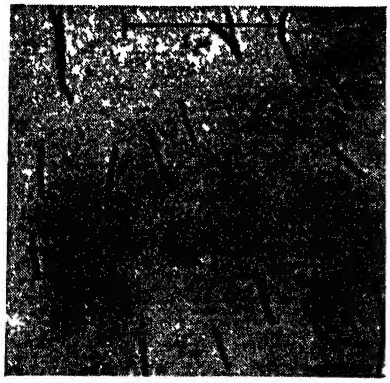

FIG 243.—Electron micrograph of crystalline tobacco-mosaic virus ×22,100 (*After Stanley and Anderson*)

**Chemical Structure of Viruses.**—Stanley (1935) isolated from Turkish tobacco plants diseased with tobacco mosaic, a high-molecular-weight crystallizable nucleoprotein that possessed the properties of tobaccomo-saic virus (Fig 243) Similar crystals were also obtained from unrelated species of plants diseased with tobacco mosaic. Tobacco-mosaic virus activity has not been demonstrated in the absence of the characteristic nucleoprotein. The reports of Stanley on tobacco mosaic have stimulated work on other agents causing plant diseases, with the result that a number of viruses have been obtained in crystalline form (Fig 244)

Many strains of tobacco-mosaic virus have been recognized in nature. The virus nucleoproteins isolated from these strains were found to be similar in physical and chemical properties, yet different from each other and from ordinary tobacco-mosaic virus. A virus may become modified after cultivation in an unnatural host. Such a modified virus is probably accompanied by a change in the physical and chemical properties of the specific nucleoprotein. Therefore, typical symptoms of a plant virus disease are produced only by a nonmutated strain inoculated into a natural host plant.

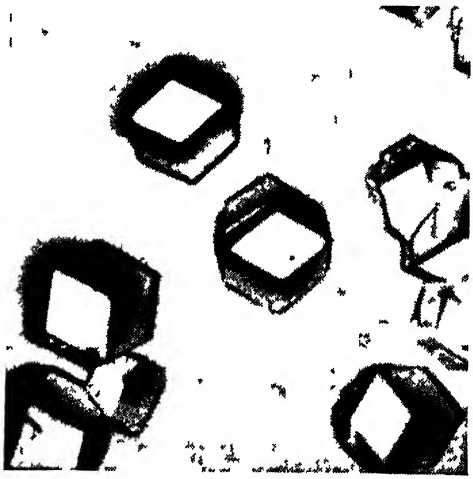

FIG. 244.—Crystalline bushy stunt virus ×168. (*After Stanley*)

The exact chemical structure of a single protein is not yet known. This is equally true of the nucleoproteins. Proteins have large molecular weights

ranging from thirty to several hundred thousand. On the other hand tobacco-mosaic virus was found to have a molecular weight of about 40 million Tobacco-mosaic virus is a conjugate protein, being composed of protein in combination with nucleic acid.

Ross (1942) hydrolyzed tobacco-mosaic virus, and identified the presence of 13 different amino acids Four amino acids were found to be absent. Loring (1939) reported that the nucleic acid present in tobacco-mosaic virus was similar to, if not identical with, ribonucleic acid (see page 587).

The composition of tobacco-mosaic virus, according to Ross, is given in Table 53

TABLE 53 —COMPOSITION OF TOBACCO-MOSAIC VIRUS

| Hydrolytic Product | Per Cent by Weight |
|---|---|
| Glycocoll (glycine) | 0 0 |
| Alanine | 2 4 |
| Valine | 3 9 |
| Leucine | 6 1 |
| Serine | 6 4 |
| Cysteine | 0 7 |
| Methionine | 0 0 |
| Threonine | 5 3 |
| Tyrosine | 3 9 |
| Phenylalanine | 6 0 |
| Arginine | 9 0 |
| Lysine | 0 0 |
| Histidine | 0 0 |
| Aspartic acid | 2 6 |
| Glutamic acid | 5 3 |
| Tryptophane | 4 5 |
| Proline | 4 6 |
| Amide nitrogen (as $NH_3$) | 1 9 |
| Nucleic acid | 5 8 |
| Total | 68 4 |

Knight and Stanley (1941) made analyses of the tyrosine, tryptophane, phenylalanine, arginine, and phosphorus contents of a number of strains of tobacco mosaic isolated from different host plants and two strains of cucumber virus. The results showed that distinct differences existed in the amounts of the amino acids present in the virus strains. Serological tests were also performed, and the results correlated closely with the chemical findings of the strains examined. It may be concluded that mutated strains of tobacco-mosaic virus do show differences in their make-up which may be detected both chemically and serologically. It seems unlikely that such changes could be brought about in fully formed virus molecules, but that these changes occur during the synthesis of new virus molecules within the cells of the host plant.

## AGENTS RESPONSIBLE FOR THE DISSEMINATION OF VIRUSES

Probably all virus diseases are disseminated by insects. Other methods are also capable of transferring viruses to plants but these are of secondary importance. Chief among these may be mentioned (1) wind, (2) water, (3) soil, (4) seed, and (5) pollen.

**Wind.**—Wind plays a minor role in the dissemination of plant viruses. However, there are a few exceptions. The tobacco-mosaic virus, for example, is very resistant to desiccation and may be spread by wind in the form of dried, crumbled plant tissue. The virus is capable of readily infecting healthy plants through slight wounds.

**Water.**—Water appears to be a minor factor in the spread of the great majority of plant virus diseases. Here again, there are a few notable exceptions. It is possible to infect healthy plants with tobacco necrosis by bathing the roots in water containing the virus. The virus can infect healthy plants without the aid of artificial wounding.

**Soil.**—The soil itself is not an agent for the transmission of virus diseases. Infection may occur through roots and other underground parts of plants by soil water or by insects working in the soil. Since it is very difficult to observe underground parts of plants, the mechanism for infection by this route remains obscure. However, it has been definitely shown that wheat-mosaic virus may be transmitted underground through roots, or the crown, or both. The virus is capable of surviving in the soil for some time and is difficult to remove from soil by thorough washing. The mechanism for the entrance of the virus into the plants is not known.

**Seed.**—Seed transmission of plant diseases does occur for some viruses. Bean-mosaic virus disease occurs by this route to the extent of about 50 per cent of the plants under experimental conditions. The results are usually inconsistent. A plant may show both healthy and infected seeds. Plants of the family *Leguminosae* appear to be more susceptible to infection by this route than plants of other families.

**Pollen.**—It has been shown that virus infections may be transmitted to seeds by pollen from infected plants. In the plant *Datura stramonium*, or Jimson weed, up to 79 per cent of the seeds may become infected. Bean seeds may also be infected in this manner.

**Insect Transmission.**—The most important agents for disseminating plant viruses are insects. Many of them are known to transmit virus diseases. Some insects transmit the virus mechanically; others transmit the infection biologically. The former method usually occurs in those insects which have chewing mouth parts. The latter method occurs only in the sucking insects, but transmission by these insects is not always biological.

According to Leach (1940), biological transmission of plant viruses by insects usually has one or more of the following attributes:

1. An apparent multiplication or increase of the virus in the insect's body.
2. An incubation period in the body of the insect, *i.e.*, a necessary period after feeding on infected plants before the insect becomes infective or viruliferous.
3. A degree of specificity between the insect and the virus that it transmits.
4. An obligatory relationship.
5. A relation between the age or life stage of the insect and its ability to transmit the virus.
6. Congenital transmission of the virus from one generation to the next.

See page 674 for more information on viruses. For additional reading on plant viruses, consult Atkinson (1944), Bawden (1939), Bawden and Pirie (1937), Best and Lugg (1944), Cohen (1942), Holmes (1939), Miller (1942*a*,*b*), Miller and Stanley (1941, 1942), Rawlins (1942), Rawlins, Roberts, and Utech (1946), Seiffert (1944), Smith (1937), Stanley (1936*a*,*b*, 1938, 1943), and Storey (1939).

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## CHAPTER XXVI

## BACTERIAL, VIRUS, AND RICKETTSIAL DISEASES OF MAN

Diseases of man and animals are caused not only by bacteria but also by other classes of organisms such as protozoa, yeasts, molds, viruses, and rickettsias.

**Bacteria.**—Thousands of bacterial types have been studied but only a very small number of these are capable of producing infections in man and animals. The important kinds of disease-producing bacteria capable of infecting man probably do not greatly exceed 100 species. Some organisms are quite specific in that they attack only one host; others are less specific, being capable of naturally infecting more than one host. Scarlet fever occurs naturally only in man. The organism that produces glanders in horses attacks also goats, sheep, dogs, cats, rabbits, guinea pigs, and man. The anthrax organism attacks not only cattle, sheep, and horses, but also man.

**Protozoa.**—A large number of important diseases of man and animals are produced by protozoa. Among these may be mentioned malaria, being produced by *Plasmodium vivax*, *P. malariae*, and *P. falciparum;* African sleeping sickness, caused by *Trypanosoma gambiense* and *T. rhodesiense;* amoebic dysentery, produced by *Endamoeba histolytica;* kala azar, produced by *Leishmania donovani;* and schistosomiasis, caused by at least three species of *Schistosoma.*

**Yeasts.**—A few species of yeasts or yeast-like organisms are known to be parasitic on man and animals. *Candida albicans* produces generally an ulcerative condition of the mouth and throat, which may later become localized in some internal organ, such as the lungs, or produce a generalized infection. When the disease becomes confined to the mucous membranes of the mouth and throat, it is referred to as "thrush".

American blastomycosis is an ulcerative infection of the skin and subcutaneous tissues caused by *Blastomyces dermatitidis.* The organism sometimes invades the internal organs, such as lungs, spleen, and kidneys, and the bones.

Coccidioidal granuloma is produced by the yeast-like organism *Coccidioides immitis.* The disease manifests itself in so many forms that no general description can be given. Bronchial or pulmonary lesions are almost always present. The skin and subcutaneous tissues are usually involved. The lesions consist of firm or soft nodules, abscesses, ulcers,

sinus infections, etc. The bones and joints may also be involved. The organisms appear in the tissues as large, round, or spherical cells with thick cell walls. They are sometimes spoken of as double-contoured bodies. Spores appear in the larger cells. On maturity, the spore-filled cells rupture, releasing the spores. Each spore increases to full size and then repeats the cycle.

**Molds.**—The molds and mold-like organisms produce several very important infections in man.

Probably the best known parasitic molds are those producing dermatophytosis in man. Several genera and species are involved. These organisms produce superficial infections of the keratinized epidermis, the hair, hair sheaths, nails, and skin. Infections by this group of molds are generally referred to as ringworm. When the infection is confined to the feet, it is called "athlete's foot." Genera of organisms usually producing ringworm and athlete's foot include *Microsporum, Trichophyton, Epidermophyton, Achorion,* and *Endodermophyton.*

Sporotrichosis is produced by the mold *Sporotrichum schenckii.* The organism produces four types of disease in man: (1) lymphangitic type, (2) disseminated type, (3) local lesions, and (4) allergic form. The lymphangitic type produces a cutaneous granuloma on the skin known as a sporotrichic chancre. A chain of cutaneous and subcutaneous nodules then develops along the lines of the regional lymphatics. The infection runs a mild course. The disseminated type produces subcutaneous nodules resembling gummata, which develop in scattered areas on the body. The nodules usually break down and ulcerate. Large abscesses are sometimes produced. Localized lesions occur, most frequently in the joints, bones, and tendon sheaths. The allergic form manifests itself as papular cutaneous lesions due to sensitization.

## BACTERIAL DISEASES OF MAN AND ANIMALS

The number of pathogenic bacteria is so large that it is beyond the scope of a textbook on fundamentals to give a detailed discussion of each disease and its specific etiological agent. For this reason, only a brief outline of the more important diseases of man and animals is included for convenient reference. Several excellent textbooks on the disease organisms are listed at the end of this section and may be consulted by those desiring additional information.

### MICROCOCCI

**The micrococci are most commonly found in boils, furuncles, abscesses, and other suppurative processes. They are normally present on the skin, and their entrance into a cut or scratch may lead to an infection. It has been shown that, under some conditions, especially during periods of weakened tissue resistance, the organisms may invade the unbroken skin.**

These organisms were formerly placed under the genus *Staphylococcus*, but in the new classification this name has been abandoned in favor of *Micrococcus* (see page 405)

**Micrococcus pyogenes var. aureus.**—Spheres 0 8 to 1 0 $\mu$ in diameter, occurring singly, in pairs in short chains, and in irregular clusters resembling bunches of grapes Produces a golden-yellow insoluble pigment Nonmotile and Gram-positive

*Disease Produced* —Found on skin and mucous membranes The causative organism of boils, furuncles, abscesses, and suppuration in wounds (Figs 245 and 246) Pus consists of an accumulation of bacteria and polymorphonuclear leucocytes in the in-

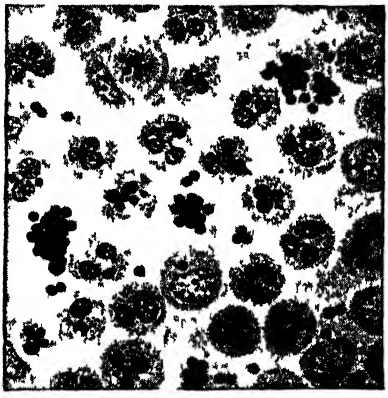

FIG 245 *Micrococcus pyogenes* var *aureus* Smear of pus showing organisms and white blood cells (*From Muir, Bacteriological Atlas E and S Livingstone Edinburgh, Scotland*)

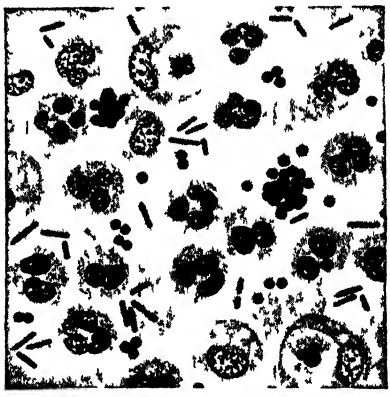

FIG 246—Micrococci and *Pseudomonas aeruginosa* Smear of pus showing the presence of organisms and white blood cells The two organisms are frequently found together in pyogenic infections (*From Muir Bacteriological Atlas E and S Livingstone Edinburgh, Scotland*)

fected area The organism rarely produces septicemia but may be a secondary invader in peritonitis, pyemia, cystitis, and meningitis

Some pathogenic strains secrete several kinds of toxins Culture filtrates are capable of (1) hemolyzing red blood cells (hemolysin), (2) destroying leucocytes (leucocidin), (3) causing death of animals when injected intravenously (endotoxin), (4) producing food poisoning when ingested (enterotoxin), and (5) dissolving or digesting human fibrin (fibrinolysin)

*Source of Infection* —Pus, skin, air, contaminated clothing, food, water, etc

*Mode of Transmission* —Transmitted by entrance of organisms into a cut or break in the skin, or even the unbroken skin

*Immunity* —Stock polyvalent bacterins, autogenous bacterins, and other heat-killed preparations have been used with some success However, very little immunity is produced

*Prevention and Control* —Aseptic surgery, destruction of soiled dressings by burning, oral and skin cleanliness

**Micrococcus pyogenes var. albus.**—This organism is similar to *M pyogenes* var *aureus* in morphological, cultural, and physiological characteristics It differs from the latter in that it produces a white pigment, is less pathogenic, and does not produce so much toxin It is agglutinated by *M pyogenes* var *aureus* antiserum

**Micrococcus aerogenes.**—Spheres 0.6 to 0.8 $\mu$ in diameter, occurring in pairs, in short chains, and in irregular clusters. Nonmotile and Gram-positive.

*Disease Produced.*—Found in natural cavities of the body, especially in the tonsils and in the female genital organs. Causes puerperal fever.

*Source of Infection.*—Discharges from infected tonsils and body cavities, air, food, contaminated clothing, etc.

*Mode of Transmission.*—Transmitted by contact; by entrance of organisms into a cut or break in the skin.

*Immunity.*—Very little immunity is produced.

*Prevention and Control.*—Aseptic surgery; destruction of soiled dressings by burning; oral and skin cleanliness.

## GAFFKYA

The members of the genus *Gaffkya* are also spherical cells and related to the micrococci. They divide in two directions of space and tend to grow in clusters of fours, known as tetrads. The most important species is *G. tetragena.*

**Gaffkya tetragena.**—The cells are spheres, measuring 0.6 to 0.8 $\mu$ in diameter, tend to appear in clusters of fours known as tetrads, and are surrounded by a capsule when growing in body fluids. Nonspore-forming. Nonmotile, Gram-positive.

*Disease Produced.—G. tetragena* is generally believed to be a normal inhabitant of sputum or saliva. It is found in tuberculous sputum, in the blood in cases of septicemia, in the pus of abscesses, and in the spinal fluid in meningitis. It appears to be a secondary invader of low virulence, invading the tissues only when weakened by some other infectious organism. Pathogenic for Japanese mice (Fig. 247).

## STREPTOCOCCI

The streptococci are found associated with a variety of pathological conditions, including erysipelas, septicemia, puerperal fever, focal infections, sore throat, rheumatic fever, scarlet fever, tonsillitis, arthritis, and vegetative endocarditis. The organisms divide in one plane producing short or long chains. The cells are nonmotile, Gram-positive, aerobic and facultative anaerobic, and bile-insoluble. This latter property is used to differentiate streptococci from pneumococci, which are bile-soluble.

**Classification of Streptococci.**—The streptococci are among the most difficult groups of bacteria to classify. One of the earliest classifications is that proposed by Brown (1919) who divided the organisms into three groups according to their effect on blood agar:

1. Alpha streptococci, producing a greenish coloration (methemoglobin formation) of the medium and partial hemolysis in the immediate vicinity of the colonies.
2. Beta streptococci, producing completely hemolyzed clear, colorless zones around the colonies.
3. Gamma streptococci, having no effect on blood agar.

The most important contribution to methods for the classification of the streptococci is the serological technique (precipitin test) proposed by Lancefield (1933). On the basis of this method, the streptococci may be placed into the following groups:

Group A. *S. pyogenes.* Under this species are placed those organisms causing scarlet fever, erysipelas, tonsillitis, puerperal fever, septicemia, and sore throat. They are hemolytic, liquefy fibrin, do not curdle milk or hydrolyze sodium hippurate.

Group B. *S. agalactiae.* This species has been isolated from mastitis in cows and occasionally from human sources. It curdles milk, hydrolyzes sodium hippurate, and does not liquefy fibrin. Most strains are hemolytic.

Group C. This group includes three rather clearly defined biochemical groups: (1) *S. equi*, the cause of "strangles" in horses, (2) the "animal pyogenes" *Streptococcus*, and (3) the "human C" *Streptococcus*. Some of these have been isolated from animals; others are of human origin.

Group D. This group includes both hemolytic and nonhemolytic types. The most important member is *S. zymogenes*. Other members are *S. faecalis* and *S. liquefaciens*. Because of their close relationships, some have considered *S. zymogenes* and *S. liquefaciens* as varieties of *S. faecalis*.

Group E. This group includes nonpathogenic streptococci isolated from milk. They are hemolytic, do not liquefy fibrin or hydrolyze sodium hippurate.

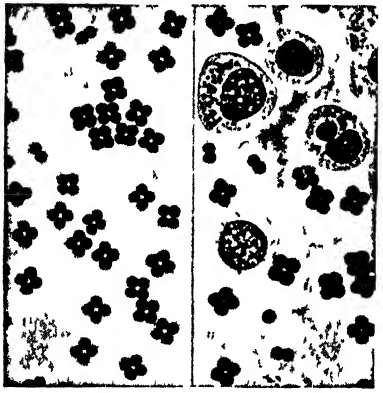

FIG. 247.—*Gaffkya tetragena.* Left, smear from a 24-hr. agar slant culture; right, smear of sputum from a case of chronic tuberculosis. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

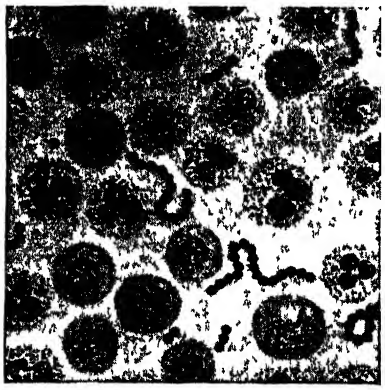

FIG. 248.—*Streptococcus pyogenes.* Smear from human pus showing the typical appearance of hemolytic streptococci together with polymorphonuclear and mononuclear leucocytes. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

Group F. This organism is generally present in normal throats and is sometimes referred to as the "minute hemolytic streptococcus." On blood agar plates, the organism produces extremely small pin-point colonies, frequently barely visible, but surrounded by a zone of true hemolysis.

Group G. This is a heterogeneous group of hemolytic streptococci which have been isolated from the normal human throat and nose, vagina, skin and feces. They are not believed to be of any importance in producing disease in humans.

**Streptococcus pyogenes.**—Cells spheres or oval-shaped, 0.6 to 1.0 $\mu$ in diameter, occurring in pairs or in chains. Capsule formation variable. Nonmotile and Gram-positive.

*Disease Produced.*—Found in human mouth, throat, respiratory tract, inflammatory exudates; produces septic sore throat, septicemia, erysipelas, scarlet fever, puerperal fever, and many other infections (Fig. 248). Some believe that each disease is caused by a distinct species, but it is generally held that the organisms are all strains of *S. pyogenes*.

*Source of Infection.*—Organisms spread by contaminated milk, pus, sputum, nasal discharges, droplet infection from the mouth and nose, etc.

*Mode of Transmission.*—Organisms transmitted by direct contact; by inhaling

droplets expelled during coughing, sneezing, or talking; consumption of contaminated milk, etc.

*Immunity.*—Very little, and of temporary duration. The exception is immunity to scarlet fever. This organism secretes an extracellular toxin against which an antitoxin is produced.

*Prevention and Control.*—Pasteurization of milk; avoid contact with infected individuals or carriers; care in treating cuts and abrasions; disinfection of dressings, discharges, clothing, etc.

Culture filtrates of typical strains are capable of hemolyzing red blood cells. The soluble toxin is called a "hemolysin." Two types of hemolysin are elaborated: one being oxygen-sensitive, and the other oxygen-stable. On a blood agar plate, the organisms produce a type of hemolysis generally referred to as $\beta$-hemolysis and possessing considerable diagnostic importance.

**Scarlet Fever.**—Scarlet fever is an acute febrile disease of the throat accompanied by a scarlet rash. Invasion of other parts of the body may occur, resulting in infections of the middle ear, kidneys, etc.

The scarlet rash is due to the elaboration by the organisms of an extracellular erythrogenic toxin. Immunity to the disease is an immunity to the toxin rather than to the organisms.

An antitoxin may be prepared by immunizing animals against culture filtrates of the scarlet fever strain of *Streptococcus pyogenes.* Administration of the antitoxin in cases of scarlet fever produces a favorable result on the outcome of the infection. The antitoxin neutralizes the damaging effect of the toxin and, in so doing, decreases the duration of the rash, changes the character and extent of desquamation, and reduces the number of complications.

Scarlet fever is diagnosed by its clinical symptoms and by the isolation of the specific organisms from the throat. For susceptibility to the disease, the Dick test may be used. This test consists of the intradermal injection of 0.1 cc. of a known strength of toxin and the reaction read after 24 hr. A positive test manifests itself as a bright red area from 1.5 to 3 cm. or more in diameter, with swelling and tenderness of the skin.

For more information, see Lancefield (1943), Lancefield and Stewart (1944), Watson and Lancefield (1944), Stewart, Lancefield, Wilson, and Swift (1944).

## PNEUMOCOCCI

The pneumococcus (*Diplococcus pneumoniae*) is the causative agent of lobar pneumonia. The disease is nearly always due to this organism, although other bacteria are occasionally involved.

**Diplococcus pneumoniae.**—Cells 0.5 to 1.25 $\mu$, occurring in pairs, sometimes in chains; more rarely in tetrads or small clumps. Young cells are Gram-positive. Usually grow poorly or not at all on artificial media. Aerobic species are bile-soluble; anaerobic species not bile-soluble (Figs. 249 and 250).

The pneumococci are generally classified according to types. The cells contain two types of antigens: (1) the so-called "somatic antigen" and (2) the "polysaccharide hapten" or soluble specific substance (SSS). The somatic antigen is probably a nucleoprotein and is found to react with all pneumococci regardless of types. The carbohydrate hapten is type specific and serves to differentiate the various types. In other words, by means of immunological reactions, the various types of pneumococci can be distinguished from one another by the composition of the polysaccharide comprising the capsular material. These polysaccharides belong to a group of substances called

"haptens," or partial antigens. They are not antigenic in themselves but may become so when combined with protein (see page 602). At least 40 different serological types of pneumococci are now recognized.

*Typing of Pneumococci.*—A number of methods are employed for the determination of pneumococcal types:

1. Precipitin Test.—A sample of sputum (about 1 cc) from a suspected case of pneumonia is injected into the peritoneal cavity of a mouse. The animal will appear sick in about 8 hr. The mouse is usually killed after this period of time, the peritoneal cavity opened, and the exudate washed into a Petri dish with the aid of about 2 cc.

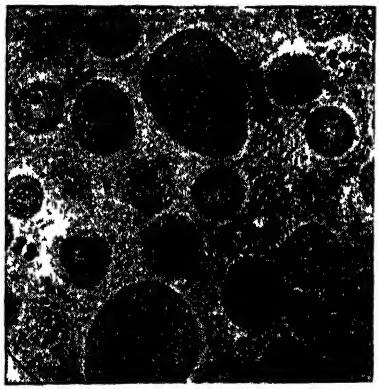

FIG. 249.—*Diplococcus pneumoniae.* Smear of sputum from a case of lobar pneumonia (*From Muir, "Bacteriological Atlas," E and S. Livingstone, Edinburgh, Scotland.*)

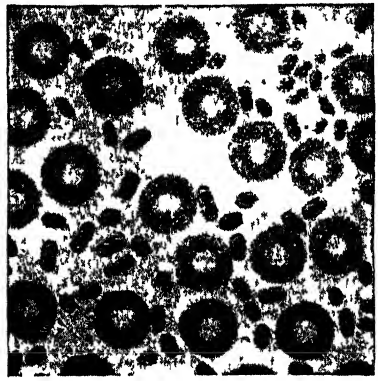

FIG. 250.—*Diplococcus pneumoniae.* Smear prepared from the heart blood of an infected mouse. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland*)

of saline solution. The washings are centrifugated and the supernatant liquid used for the precipitin test.

2. Microscopic Agglutination Test.—The peritoneal exudate is prepared as given under (1), and small drops of the washings are placed on a slide. The various type sera are added to the drops and spread out in thin films. The films are allowed to dry, then stained, and examined under the microscope for the presence of clumps. The type of serum producing clumps indicates the type of organism present in the peritoneal exudate.

3. Quellung Test.—This test is based on the observation that the capsules of pneumococci become swollen when placed in contact with specific immune serum. A loopful of undiluted immune serum is placed on a slide and mixed with a fleck of sputum. A loopful of dilute methylene blue solution is then added and the mixture examined under the microscope. If the mixture is homologous, the capsules will appear swollen with very distinct outlines (see Fig. 33). The test is made with many types of antisera and as many flecks of sputum.

The typing of pneumococci is of great importance because efficient antisera may be prepared against some types, especially type 1, which are of great therapeutic value.

*Disease Produced.*—The commonest cause of lobar pneumonia, the incidence being as high as 95 per cent. The organism is present in the alveoli and bronchioles of the lung, in the lymph channels, and sometimes in the blood. The organism may also

produce pericarditis, arthritis, meningitis, otitis media, mastoiditis, endocarditis, rhinitis, tonsillitis, conjunctivitis, septicemia, osteomyelitis, and peritonitis.

*Source of Infection.*—Sputum, blood, and exudates in pneumonia; cerebrospinal fluid in meningitis; mastoiditis; saliva from respiratory tract of normal individuals.

*Mode of Transmission.*—Direct contact with infected person or carrier; inhalation of droplets expelled from the throat during coughing or sneezing.

*Immunity.*—Immunity relatively slight and of short duration. Prophylactic immunization by the use of a vaccine prepared by chemical treatment (capsular substance) produces good results.

*Prevention and Control.*—Isolation of suspected cases of pneumonia. Injections of the proper therapeutic antiserum; employment of drugs of the sulfonamide type, especially sulfapyridine, sulfathiazole, and sulfadiazine; the use of antibiotics, especially penicillin. The mortality rate has been reduced to an insignificant figure since the employment of these chemotherapeutic agents.

## KLEBSIELLA

*K. pneumoniae* was first isolated by Friedländer and is usually referred to as Friedländer's bacillus. It is not infrequently found associated with upper respiratory infections in man. In most instances, the organism appears to be present as a secondary invader. Less than 1 per cent of pneumonias are caused by this organism.

**Klebsiella pneumoniae.**—Short rods, somewhat plump, with rounded ends, usually occurring singly. Produces a heavy capsule in the mucoid phase. Nonmotile and Gram-negative. Aerobic, growing well on ordinary culture media (Fig. 251).

*Disease Produced.*—Encountered in the respiratory, intestinal, and genitourinary tracts of man. Isolated from the lungs in lobar pneumonia. Associated with pneumonia and other inflammations of the respiratory tract. May also produce otitis media, empyema, pericarditis, meningitis, and septicemia.

*Source of Infection.*—Buccal and nasal discharges of infected persons or carriers; articles contaminated with such discharges.

*Mode of Transmission.*—Direct contact with infected person or carrier.

*Immunity.*—Immunity relatively slight and of short duration.

*Prevention and Control.*—Isolation of infected persons; concurrent disinfection.

## GRAM-NEGATIVE COCCI

The Gram-negative cocci are grouped under the genus *Neisseria*. The genus includes *N. gonorrhoeae* (gonococcus), the cause of gonorrhoea, and *N. intracellularis* (meningococcus), the cause of epidemic meningitis. The gonococcus shows a predilection for the urethral membranes, the meningococcus, a selective action for the meninges of the brain and spinal cord. Another member *N. catarrhalis* is commonly present in the nasopharynx, saliva, and respiratory tract and is frequently associated with other organisms in certain inflammatory conditions of the mucous membranes. Its presence is probably of minor importance.

**Neisseria gonorrhoeae.**—Cells spherical, 0.6 to 1.0 $\mu$ in diameter, occurring singly and in pairs. Paired spheres are flattened where they come in contact and are usually described as coffee bean-shaped. The organism is parasitic only for mammals. The organism is nonmotile and Gram-negative.

*Disease Produced.—N. gonorrhoeae* is the causative agent of gonorrhoea in man (Fig. 252). The infection is in almost every case transmitted by direct contact from individual to individual The organism attacks chiefly the human urethra in both males and females, producing an acute catarrhal condition. There is a marked tendency

for the infection to spread, producing in the male epididymitis, prostatitis, cystitis, and other inflammatory conditions. In the female, the entire genitourinary tract may be involved, including the cervix. The organism may also invade the blood stream and be carried to various parts of the body. The organism shows a predilection for the synovial membranes of the joints, producing gonorrhoeal rheumatism; and for the heart valves, causing endocarditis. The organisms may persist for many years and are probably never completely eliminated.

*Ophthalmia neonatorum.*—This is an inflammation of the conjunctiva in the newborn which is a consequence of maternal infection. If neglected, this is probably the

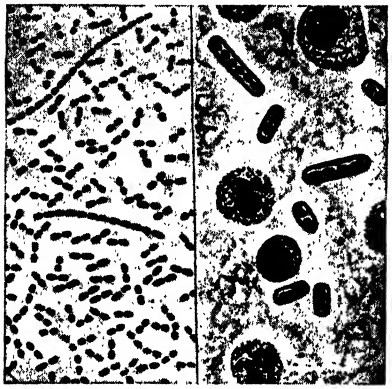

FIG. 251.—*Klebsiella pneumoniae* (Friedländer's bacillus). Left, smear from a culture; right, smear of sputum. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

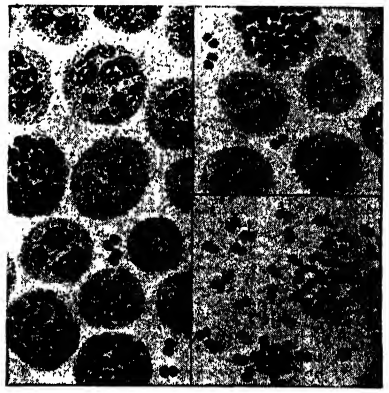

FIG. 252.—*Neisseria gonorrhoeae*, the cause of gonorrhea. Left, a smear of pus showing the presence of the organism within the polymorphonuclear leucocytes; upper right, same but stained differently; lower right, smear from a pure culture. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

most common cause of blindness. Approximately 10 per cent of all cases of blindness are due to gonorrhoeal infections. This is easily prevented by instillation of one or two drops of 2 per cent silver nitrate in the eyes of the newborn.

*Diagnosis.*—Direct smears are prepared from the urethral or conjunctival discharges and stained by the Gram method. The smears are examined for the presence of Gram-negative, intracellular diplococci slightly flattened at their adjacent surfaces. The organisms may be cultivated by streaking some of the urethral pus over the surface of a blood agar plate and incubating the plate in an atmosphere containing 10 per cent of carbon dioxide. This may be approximated by placing the plate in a screw-cap jar with a lighted candle and replacing the cover.

*Oxidase Test.*—The gonococcus produces an oxidase which causes a 1 per cent solution of dimethyl-paraphenylenediamine to turn first pink, then rose, magenta, and finally black. The test is made by flooding a 24-hr. agar plate culture with the reagent and noting the development of the above series of colors.

*Source of Infection.*—Purulent venereal discharges; blood, and pus from infections of the conjunctiva and joints.

*Mode of Transmission.*—Transmissible by direct contact; sexual intercourse.

*Immunity.*—An attack of gonorrhoea produces very little, if any, immunity.

*Prevention and Control.*—Treatment usually consists in the use of germicides, vaccines, and diathermy. Modern treatment consists in the administration of sulfa drugs and penicillin, especially the latter, which has proved of inestimable value in the control of the disease. Public-health clinics have been established in most cities to treat infected individuals.

**Neisseria intracellularis.**—Cells spherical, 0.6 to 0.8 $\mu$ in diameter, occurring singly, in pairs, and occasionally in tetrads. Paired spheres are flattened at their adjacent surfaces, giving the organisms the appearance of a coffee bean. Nonmotile and Gram-negative.

The meningococci are generally classified into four main types on the basis of the agglutination reaction, designated, I, II, II*a*, and IV. Most strains may be placed into one or another of these four types. As is true with the pneumococci, the presence of carbohydrate capsular substances is believed to be responsible for the immunological specificities of the various meningococcal types.

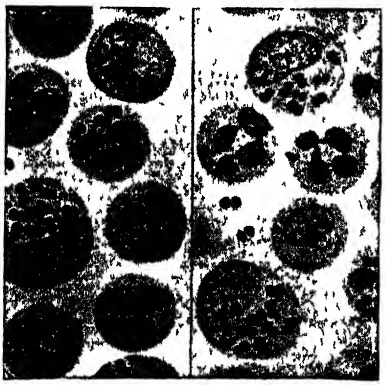

FIG. 253.—*Neisseria intracellularis*, the cause of meningococcus meningitis. Left, smear of sediment from a centrifugalized specimen of spinal fluid; right, same but stained differently. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

*Disease Produced.*—*N. intracellularis* is the cause of epidemic meningitis. The organism attacks the base and cortex of the brain and the surfaces of the spinal cord. The organism is present in the spinal fluid. If growth is heavy, the fluid may be turbid. The organisms may appear both free and within the leucocytes. *N. intracellularis* is frequently present in the blood stream of meningitis patients. The organism has also been isolated from cases of arthritis, pericarditis, and from the nasopharynx in cases of rhinopharyngitis.

*Diagnosis.*—The meningococcus is present in large numbers in the spinal fluid of positive cases and may be diagnosed by centrifugating the spinal fluid and examining stained smears of the sediment. The typical picture is the presence of Gram-negative intracellular diplococci. Typical smears of the meningococcus and gonococcus are indistinguishable. They may be distinguished by their cultural reactions.

*N. intracellularis* may be cultivated by streaking some sediment from spinal fluid over the surface of blood agar or heated blood agar. The plate is then incubated in an atmosphere containing about 10 per cent carbon dioxide.

As in the case of the gonococcus, this organism also produces an oxidase that is capable of oxidizing dimethyl-paraphenylenediamine to a black color. The test is made by flooding a 24-hr. plate culture with a 1 per cent solution of the reagent and noting the development of a black color in colonies of the meningococcus.

*Source of Infection.*—The organisms are found in the nasopharynx, blood, cerebrospinal fluid, conjunctiva, pus from joints, etc., of persons suffering from the disease (Fig. 253).

*Mode of Transmission.*—The disease is disseminated by direct contact and by droplet infection during coughing and sneezing. The organism is frequently found in the nasopharynx of healthy persons. Such individuals are referred to as carriers because they are able to spread the disease to others. Organisms are very easily killed when outside of the body and probably never reach a new individual except by direct contact. Epidemics generally develop during periods of overcrowding as occurs in army camps.

*Immunity* —Agglutinins against the organism are demonstrable in the blood stream after an infection, but the duration of the immunity is uncertain

*Prevention and Control* — Preventive measures consist of the isolation of infected individuals and the prevention of overcrowding The disease is treated by injections of polyvalent antimeningococcus serum into the cerebrospinal canal after first removing the purulent spinal fluid The use of polyvalent antiserum is a necessity in the treatment of the disease

Sulfa drugs have been reported to be of some value, especially when combined with serum treatment

## CORYNEBACTERIUM GROUP

The members of this group are generally Gram-positive Sometimes young cells and sometimes old cells are Gram-negative With few exceptions, the cells are nonmotile

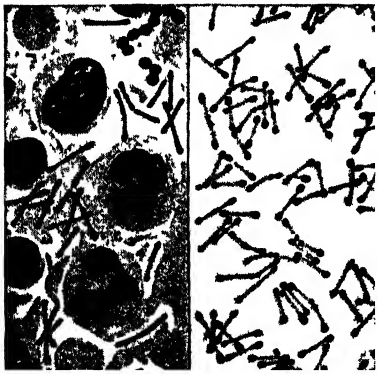

FIG 254 —*Corynebacterium diphtheriae* Left smear prepared from a throat swab taken from a case of diphtheria, right smear from a 12-hr culture on Loeffler's blood serum medium (*From Muir, 'Bacteriological Atlas E and S Livingstone Edinburgh Scotland*)

FIG 255 —*Corynebacterium diphtheriae* Smear prepared from a 5-day old agar slant culture showing involution or club-shaped forms frequently encountered in old cultures (*From Muir, "Bacteriological Atlas," E and S Livingstone, Edinburgh Scotland*)

The species are quite aerobic but some may be microaerophilic or even anaerobic This group is widely distributed in nature The best known species are parasitic and pathogenic on man and domestic animals Some species are well-known plant pathogens, others are found in dairy products, water, and soil The most important member of this group is *C diphtheriae*, the cause of diphtheria in man

**Corynebacterium diphtheriae.**—Cells are rod-shaped and vary greatly in size, ranging from 0 3 to 0 8 by 1 0 to 8 0 $\mu$, and occurring singly Rods are straight or slightly curved, frequently club-shaped at one or both ends Snapping division produces angular and palisade (picket-fence) arrangements of cells Stained rods show the presence of metachromatic granules Nonmotile and Gram-positive The cells are often vacuolated or segmented, giving the rods a barred appearance (Figs 254 to 256)

*Disease Produced* —*C diphtheriae* is the cause of diphtheria in man The organism localizes in the throat where it produces a powerful extracellular toxin, which is absorbed into the blood stream and may cause death unless neutralized by antibodies known as antitoxin

The symptoms of diphtheria are sore throat, fever, and leucocytosis. The throat

becomes considerably inflamed, especially the fauces, where a grayish false membrane is formed. The membrane may eventually spread to the entire respiratory tract. The toxic action of the organism results in the destruction of the superficial layer of cells. This is followed by the exudation of a plasma-like fluid which clots and covers the surface of the injured mucous membrane with a tough elastic network of fibrin in which are embedded dead cells and bacteria. The toxin elaborated by the organisms produces an injurious action on the kidneys and muscles of the heart. Injury to the heart is probably the most important action of the toxin.

*Carriers.*—Convalescents usually harbor the organism for 3 or 4 weeks, after which

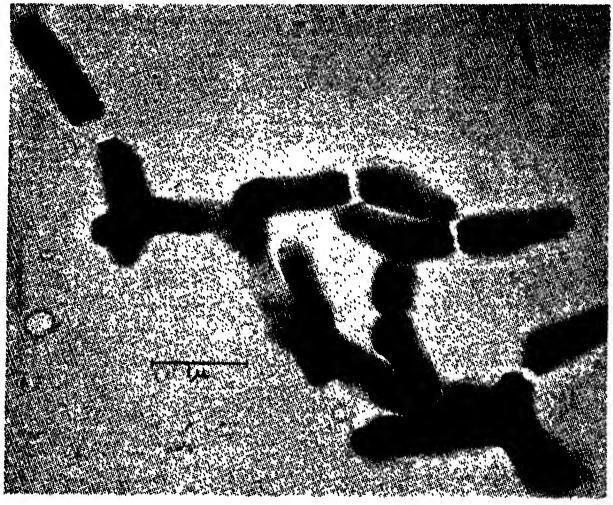

FIG. 256.—*Corynebacterium diphtheriae.* Discoidal dense granules can be seen near ends of the cells. The protoplasm is shrunken away from the cell walls of several cells. Reduced from an electron micrograph with a magnification of 26,500 diameters. (*After Morton.*)

the bacteria gradually disappear. Three negative throat examinations are generally required before a convalescent is released from quarantine. However, studies on the bacterial flora of normal throats have revealed the presence of a surprisingly high percentage of persons who habitually have the diphtheria organisms in their throats. These are chronic carriers and of no importance unless the organisms are of the virulent type. Whether they are or not can be determined in the laboratory by performing virulence tests on animals.

*Diagnosis.*—A sterile swab is rubbed over the tonsillar region of the throat or the pharynx and then streaked over the surface of coagulated serum known as Loeffler's medium. The swab is also rubbed over the surface of a glass slide and stained with methylene blue. The culture is incubated at 37°C. for 24 hr. and the slide is examined under the microscope for the presence of characteristic barred and granular rods. The slide examination gives a preliminary idea of what to expect from the culture.

Since the culture on blood serum will show the presence of many kinds of organisms, it is necessary to streak some of the mixed growth over the surface of a solid medium contained in a Petri dish to obtain *C. diphtheriae* in pure form. An excellent preparation for this purpose is known as cystine-tellurite medium. Typical colonies of *C. diphtheriae* on this medium are opaque and dark gray or black in color. Characteristic colonies are

transferred to tubes of Loeffler's coagulated serum medium, incubated at 37°C. for 24 hr., and then tested for virulence.

*Virulence Test.*—The method generally employed is the intracutaneous test, performed as follows: Two guinea pigs are used, one of them being the control. The abdomens of the animals are shaved. The control pig is given about 250 units of diphtheria antitoxin intraperitoneally. The growth from a 24-hr. culture of the organism to be tested is emulsified in about 20 cc. of salt solution and 0.15 cc. injected intracutaneously into each guinea pig. If the culture contains virulent diphtheria bacilli, it will produce toxin which will have no effect on the immunized pig but will produce a definite local inflammatory lesion in 24 hr. and becomes necrotic in 48 to 72 hr.

*Schick Test.*—This test is employed to determine the susceptibility of an individual to diphtheria. The test is performed as follows: Diphtheria toxin is diluted so that 0.1 cc. contains $\frac{1}{50}$ of the minimum lethal dose (M.L.D.) required to kill a 250-gm. guinea pig in 96 hr. One-tenth cubic centimeter of toxin so diluted is injected intracutaneously on the arm. The same amount of heated toxin is injected on the other arm as a control. The injected areas are examined daily for several days. Usually the fourth day gives the most reliable readings. The reactions may be recorded as follows:

1. A positive test is indicated by the presence of a slightly raised area of redness from 1 to 2 cm. in diameter which appears in from 24 to 36 hr. and reaches a maximum in from 48 to 72 hr. The reaction persists for about 1 week, then gradually fades, and finally disappears. The control arm shows no reaction.
2. A negative test does not show an area of redness in the test or control arm.

*Source of Infection.*—Discharges from diphtheritic lesions of the pharynx, larynx, trachea, nose, conjunctiva, and vagina; secretions from the healthy pharynx and nose of carriers.

*Mode of Transmission.*—Disease spread from person to person by fingers, or articles, such as eating utensils, toys, pencils, handkerchiefs containing nasal discharges and saliva, and by inhalation of droplets expelled from the throat during coughing and sneezing.

*Immunity.*—One attack of diphtheria usually confers immunity for life. Resistance to the disease increases with age. This is believed to be due to continued exposure to the disease.

*Prevention and Control.*—Isolation of suspected cases of diphtheria. Persons showing a positive Schick test may be susceptible to the disease. Active immunization of susceptible individuals may be practiced by the use of two types of preparations: (1) toxoid and (2) alum-precipitated toxoid.

*Toxoid.*—Toxoid is prepared by treating toxin with formaldehyde to destroy its toxic properties without affecting its ability to stimulate the production of antitoxin (see page 605). Usually three doses at intervals of 1 month are necessary to give a negative Schick test.

*Alum-precipitated Toxoid.*—It has been found that toxoid precipitated with alum is superior to ordinary toxoid as an immunizing agent. The precipitate is insoluble and remains in the tissue for a longer period of time before it is completely absorbed. This affords a more prolonged antigenic response. Usually two injections are necessary to render Schick-positive individuals negative.

*Therapy.*—Passive immunization with diphtheria antitoxin in clinical cases. The antitoxin is administered as early in the disease as possible. The dosage is about 10,000 units in mild cases and 30,000 to 50,000 units in severe cases. The antitoxin neutralizes the damaging effect of the toxin (see page 604). Passive immunization lasts usually from 2 to 4 weeks. If infection still persists, the dose should be repeated.

## MYCOBACTERIUM

This genus includes a number of organisms that differ from the great majority of bacteria in containing a high content of waxy or fatty substances. This material is stained with difficulty but, when once stained, is not decolorized by acid. Because of this fact, they are called "acid-fast" organisms. The organisms produce diseases characterized by the presence of nodules or tubercles in various organs. The most important members are *M. tuberculosis* var. *hominis*, the cause of human tuberculosis; *M. tuberculosis* var. *bovis*, the cause of tuberculosis in cattle; *M. paratuberculosis*, the cause of Johne's disease in cattle and sheep, and *M. leprae*, the etiological agent of leprosy.

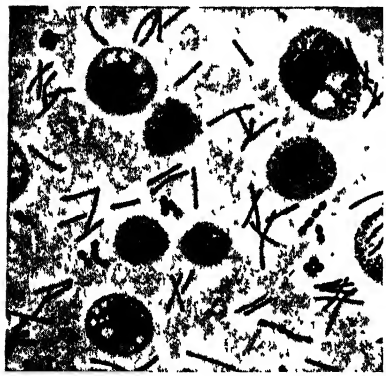

FIG. 257.—*Mycobacterium tuberculosis* var. *hominis* Smear of putum from a case of pulmonary tuberculosis (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh Scotland*).

**Mycobacterium tuberculosis var. hominis.**—Slender filaments, straight or slightly curved rods, 0.3 to 0.6 by 0.5 to 4.0 $\mu$, frequently irregular in form with only slight and occasional branching. Rods may stain solidly or show a banded or beaded appearance. Conidia not formed. Growth on media slow for most species. Aerobic, nonmotile, Gram-positive, and acid-fast.

*Disease Produced.*—The cause of tuberculosis in man, monkey, dog, and parrot. Experimentally very pathogenic for guinea pigs, but not for rabbits, cats, goats, oxen, or domestic fowls (Fig. 257).

*Diagnosis.*—Suspected material, such as sputum, urine, feces, cerebrospinal fluid, or stomach contents, is examined for the presence of tubercle bacilli.

Sputum may be smeared on a slide and stained by the Ziehl-Neelsen acid-fast technique. The presence of typical organisms is usually indicative of infection with the tubercle bacillus.

It is usually better to concentrate the tubercle bacilli before making laboratory tests. This may be performed as follows: The infected material is treated with 3 per cent sodium hydroxide to digest the sputum, pus, or other material with which the organisms are mixed. The digested material is then neutralized with acid and centrifugated. Only the sediment is retained. The sediment may be used for the (1) preparation of smears for staining and direct microscopic examination, (2) injection into animals, or (3) inoculation of culture media.

*Animal Inoculation.*—A guinea pig is inoculated into the groin or the muscle of the thigh with some of the sediment. Enlargement of the regional lymphatics occurs in 2 or 3 weeks, and the animal usually dies in about 6 to 8 weeks. On autopsy, the animal shows necrotic areas in the liver and spleen, and enlarged lymph nodes filled with caseous material. The lungs and kidneys are rarely attacked.

*Inoculation of Culture Media.*—Some of the sediment may be used for the inoculation of appropriate culture media. A variety of media may be used, consisting largely of egg, glycerin, and some dye to kill or inhibit the growth of contaminating organisms. The media are tubed, slanted, then sterilized by heat. During the heating process, the egg albumin is coagulated, producing a solid medium.

*Use of Tuberculins.*—A large number of tuberculins are available. They are pre-

pared in different ways but consist of filtrates of liquid cultures of the tubercle bacillus. They contain certain products liberated after the death and disintegration of the organisms.

The first tuberculin preparation, known as "Koch's old tuberculin", is prepared as follows: The organisms are cultivated in a slightly alkaline 5 per cent glycerin peptone broth for 6 to 8 weeks. The culture is concentrated in a water bath, heated to 80°C., until reduced to one-tenth of its original volume. The culture is then filtered to remove bacterial debris. The clear filtrate contains the tuberculin.

Tuberculins are used to test the sensitiveness of persons or animals to proteins of

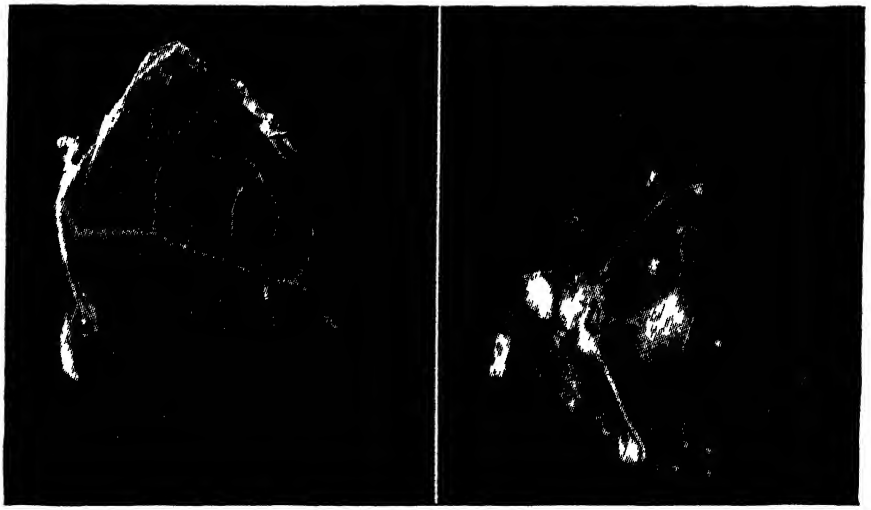

FIG. 258.—Differentiation of virulent from nonvirulent strains of *Mycobacterium tuberculosis*. Left, membrane after inoculation with a nonvirulent strain but showing no abnormality; right, membrane after inoculation with a virulent strain showing discrete and conglomerate tubercles. (*After Emmart and Smith.*)

the tubercle bacillus. By injecting tuberculin intradermally, a positive test appears in 6 to 8 hr., reaches a maximum in 24 to 48 hr., and generally subsides in 6 to 10 days. It is characterized by a reddening of the skin about 1 cm. in diameter. The reaction is positive in those having active or healed lesions. Since most individuals have healed tuberculous lesions, the test is of limited value.

*Differentiation of Virulent from Avirulent Strains.*—Emmart and Smith (1941, 1943) employed the chorioallantoic membrane of the developing chick as a medium for testing the virulence of various strains of tubercle bacilli (Fig. 258). They found that within 6 days after inoculation 96 per cent of the membranes inoculated with virulent strains showed the presence of tubercles, whereas only 18 per cent positives were obtained with the avirulent strains. These results correlated quite closely with guinea pig tests. They concluded that the chorioallantoic membrane of the chick embryo can be successfully used to determine differences in virulence between closely related strains of tubercle bacilli.

*Source of Infection.*—Discharges from lesions, or articles freshly soiled with discharges, the most important being sputum. Of less importance are discharges from intestinal and genitourinary tracts, or from lesions of the lymph nodes, bone, and skin.

*Mode of Transmission.*—Transmitted through discharges of the respiratory tract,

less frequently through discharges of the digestive tract; by inhaling droplets expelled during coughing, sneezing, talking, or singing; by kissing; by the use of contaminated eating and drinking utensils; by contaminated dust, flies, etc. Infection usually results from continued contact with an infected individual.

*Immunity.*—Natural immunity to disease generally negative. Resistance to disease increased by improved living conditions. Susceptibility is highest in children under three years of age, lowest from three to twelve years, and relatively high for the remainder of life. Disease more prevalent in the undernourished, neglected, and fatigued, than in the well-fed and well-cared for.

*Prevention and Control.*—Avoid overcrowding. Improve living and working conditions. Pasteurization of all milk. Slaughtering of tuberculous cattle. Separation of newborn from tuberculous mothers. Education of public in regard to the dangers of tuberculosis and mode of transmission. Tuberculous patients should be prohibited from handling foods. Fresh air, sunshine, and nourishing food play a very important role in building up a resistance to the disease.

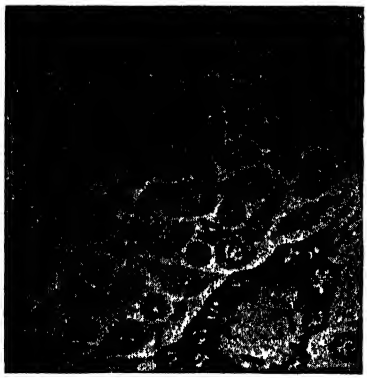

FIG. 259.—*Mycobacterium tuberculosis* var. *bovis*. Section of udder of cow suffering from chronic tuberculosis. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

Isolation of positive cases, preferably in a sanitorium for proper treatment. Concurrent disinfection of sputum and articles likely to be soiled with sputum, such as handkerchiefs, towels, paper, eating and drinking utensils. Cure based chiefly on rest, good food, fresh air, sunshine, and freedom from worry.

**Mycobacterium tuberculosis var. bovis.**—Rods shorter and more plump than the human variety, ranging from 1.0 to 1.5 $\mu$. Short rods often mixed with longer forms. Cells may stain solidly or show a banded or beaded appearance. When freshly isolated, grows poorly or not at all on egg, glycerin egg, glycerin potato, and glycerin broth media whereas the human type grows well on such media. More virulent for rabbits than the human type. Causes generalized lesions in calves whereas the human type produces only local lesions or, at most, a spreading to the nearest lymph node. Gram-positive. Acid-fast.

*Disease Produced.*—The cause of tuberculosis in cattle. May be transmitted to man and domestic animals. Pathogenic for ox, man, monkey, goat, sheep, pig, cat, parrot, cockatoo, and other birds. Experimentally highly pathogenic for rabbit and guinea pig. More highly pathogenic for animals than the human type (Fig. 259).

*Source of Infection.*—Tuberculous cows may eliminate the organisms in the feces, urine, and milk. Milk from infected udders may contain enormous numbers of the organisms.

*Mode of Transmission.*—Infection spread to healthy cows through milk, urine, and feces of diseased cows. Children, particularly those under five years of age, may become infected by drinking contaminated milk from diseased cows.

*Immunity.*—Natural immunity to disease generally negative. Susceptibility is highest in children under five years of age and decreases with increasing age.

*Prevention and Control.*—Pasteurization of all milk. Slaughtering of tuberculous cattle to prevent spread to healthy animals. Education of public in regard to dangers of tuberculosis and mode of transmission.

Vaccination of healthy animals with a special vaccine known as B.C.G. (Bacille Calmette-Guérin). This is a bovine strain rendered avirulent by cultivating for many generations on a bile-glycerol-potato medium. This change appears to be permanent, the loss of virulence not being restored when the organisms are again transferred to ordinary media.

**Mycobacterium paratuberculosis.**—Plump rods 1.0 to 2.0 $\mu$ in length. The plump rods stain uniformly; the longer forms show alternate stained and unstained segments. Nonmotile. Gram-positive. Acid-fast. The organism grows poorly on laboratory media.

*Disease Produced.*—The cause of Johne's disease, a chronic diarrhea in cattle and sheep. The disease is characterized by emaciation, running a long course of several months, ending usually in death of the animal. Lesions limited to the intestinal tract. The mucosa of the small intestine is greatly thickened and shows a wrinkled or corrugated appearance. The surface of the corrugations shows the presence of hemorrhages and occasionally small nodules. The bacilli may be isolated from the enlarged, edematous, and pigmented mesenteric glands.

*Source of Infection.*—Organisms found in feces of infected animals, in contaminated water, food, pastures, and bedding.

*Mode of Transmission.*—Natural infection occurs from consuming infected food, or water and grass containing fecal material.

*Immunity.*—Natural immunity to disease generally negative.

*Prevention and Control.*—Isolation or preferably destruction of diseased animals. Thorough disinfection of all buildings. Dung and fodder should be burned. Contaminated pastures should be plowed under and allowed to rest for at least a year. No specific treatment.

**Mycobacterium leprae.**—Cells rod-shaped, 0.3 to 0.5 by 1.0 to 8.0 $\mu$, with parallel sides and rounded ends. Cells may stain solidly or show a banded or beaded appearance. Sometimes curved forms may be present. Organisms usually arranged in parallel bundles. Cells may appear club-shaped, single, double, coccoid, and in chains. Nonmotile. Gram-positive. Acid-fast. The organism has not been successfully cultivated on laboratory media.

*Disease Produced.*—The cause of leprosy in man. Leprosy is a chronic disease which occurs in three forms: (1) anesthetic (nerve) type, (2) nodular (muscle) type, and (3) mixed type (combination of both). Death rarely due to leprosy but to some secondary invader. Disease shows a low rate of infectivity. Intimate and prolonged contact with infected individuals necessary for infection to occur. Workers in leper colonies seldom contract the disease. Incubation period of the disease is not known, although some claim that it is from 1 to 7 years.

*Diagnosis.*—The disease may be recognized by lesions of the skin and mucous membranes, and by neurological manifestations. Diagnosis by microscopic examination is usually possible in the nodular and mixed types, but more difficult, or even impossible, in the neural type. The nodular type may be diagnosed by making a snip in the skin, preparing a smear of the fluid that oozes out, and staining by the acid-fast technique.

*Source of Infection.*—Discharges from lesions.

*Mode of Transmission.*—Disease transmitted by intimate and prolonged contact with lepers. Nodules on skin may liquefy, ulcerate, and discharge great masses of the bacilli. Patients showing the presence of acid-fast organisms in smears, even though ulcers are not present, are potentially open cases. It is generally believed that the disease is the result of living under filthy, unsanitary conditions, but there is very little evidence to substantiate this statement. Children contract the disease more easily than adults. Babies of lepers rarely, if ever, become infected if separated at birth.

*Immunity.*—No racial immunity.

*Prevention and Control.*—Leprosy may be contracted in adult life but is usually acquired in childhood. Positive cases should be isolated in leprosaria until the disease has been arrested. Usually three negative bacteriological tests at intervals of 6 months are required before a patient is released. Paroled individuals should be reexamined every 6 months thereafter.

Discharges and articles soiled with discharges should be disinfected. Living premises of patient should be thoroughly cleaned. Treatment based chiefly on rest, good food, fresh air, sunshine, and freedom from worry. No specific treatment known.

## PASTEURELLA GROUP

This group includes a number of small, Gram-negative, rod-shaped organisms that exhibit bipolar staining. The two most important members attacking humans are (1) *P. pestis*, the cause of plague and (2) *P. tularensis*, the etiological agent of tularemia.

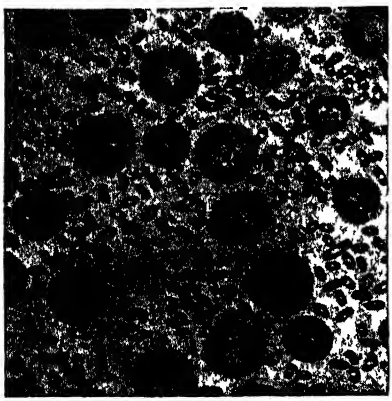

FIG. 260.—*Pasteurella pestis*, the cause of bubonic, pneumonic, and septicaemic plague in man. Smear prepared from a bubo. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

**Pasteurella pestis.**—Cells short, plump rods, with rounded ends, slightly thicker in the middle, occurring singly, and measuring 1.0 by 2.0 $\mu$. Organisms exhibit bipolar staining. Nonmotile. Nonspore-forming. Gram-negative.

*Disease Produced.*—The cause of plague in man, rats, ground squirrels, and other rodents. Transmissible experimentally to mice, guinea pigs, and rabbits.

Human plague may be of three types: (1) bubonic, (2) pneumonic, and (3) septicemic.

In bubonic plague the bacteria are carried through the blood and lymph vessels to the lymph glands in the groin, armpits, neck, etc. The bacteria multiply, produce pus, and cause an enlargement of the glands. The glands may ulcerate and discharge their contents. The enlarged glands are referred to as buboes and the infection as bubonic plague.

In the pneumonic type, the organism gives the picture of a virulent septic pneumonia. The lungs become engorged and hemorrhages appear under the pleura. Bacteria are found in large numbers in the peribronchial lymph spaces and in the adjoining alveoli.

The disease may be mild, or it may take an acute septicemic form which generally produces rapid death.

*Diagnosis.*—Blood cultures prove positive in about 30 per cent of cases. Direct smears can be prepared from open buboes, or such material can be inoculated into culture media. If the material is contaminated, it can be purified by inoculating a guinea pig and isolating the plague bacillus from the heart's blood. In the pneumonic type, the organisms are usually present in large numbers in sputum and may be recognized by direct smear.

*Source of Infection.*—Organisms found in buboes, blood, pleural effusion, spleen, and liver of infected persons and rodents. In addition, organisms present in the sputum in cases of pneumonic plague (Fig. 260). The infection usually reaches man through contact with diseased rats.

*Mode of Transmission.*—Transmitted from rat to rat and from rat to man by infected rat fleas, the most important of which are *Xenopsylla cheopis* and *Ceratophyllus fasciatus*. The flea becomes infected by feeding on a diseased rat. The flea next feeds on a person and at the same time deposits feces and possibly some regurgitated blood. The bacilli are then rubbed into the skin by scratching.

*Immunity.*—Susceptibility to disease is general. Lasting acquired immunity results following recovery from the infection. The administration of plague vaccine may produce active immunity which usually lasts about 6 months.

*Prevention and Control.*—Rat extermination, ratproofing, and other necessary measures. Isolation of infected individuals. Disinfection of sputum and articles soiled with sputum in pneumonic type. Use of vaccine and immune serum has proved of some value.

**Pasteurella tularensis.**—Morphologic units include globi and globules, flat and cylindrical bacillary forms, coccoid forms, delicate filaments, and minimal reproductive units. Nonmotile. Nonspore-forming. Gram-negative.

*Disease Produced.*—The cause of tularemia in man. Organism is infectious for rabbits, guinea pigs, rats, gray mice, and ground squirrels. Onset of disease in man is sudden, with pains and fever. Patient usually prostrated and confined to bed. Fever may last for 3 or 4 weeks followed by slow convalescence. Lymph glands may become swollen and tender and suppurate in 50 per cent of infections.

*Diagnosis.*—Disease may be diagnosed by (1) animal inoculation, (2) isolation of organism from lesions or discharges, and (3) performing an agglutination test with serum from patient.

*Source of Infection.*—Transmitted to man by contact with infected animals such as wild rabbit, hare, woodchuck, coyote, muskrat, opossum, tree squirrel, quail, skunk, cat, deer, dog, fox, hog, sage hen, and bull snake, or by the bite of the horsefly (*Chrysops discalis*), wood tick (*Dermacentor andersoni* and *D. variabilis*), and possibly other biting insects. Hunters, cooks, and butchers may contract the disease during rabbit-hunting season.

*Mode of Transmission.*—Transmitted by bites of infected flies and ticks and by inoculation through handling of diseased animals, especially in skinning rabbits during the hunting season. Ingestion of insufficiently cooked meat from a diseased animal, also from drinking contaminated water.

*Immunity.*—Disease attacks persons of all ages. Recovery from an attack followed by permanent immunity. An immune person exposed to the disease may develop a local infection through a break in the skin but this does not cause any constitutional disturbance. Disease most prevalent during the hunting season.

*Prevention and Control.*—Disinfection of discharges from ulcer, lymph glands, or conjunctiva of infected individuals. Avoidance of bites or handling of flies and ticks during the bloodsucking season. Use of rubber gloves in the handling and dressing of rabbits or in performing autopsies on animals likely to be infected. Meat from wild rabbits and other susceptible animals should be thoroughly cooked before eating. Water from infected areas should not be used unless first boiled or disinfected.

For more information on the morphology of *P. tularensis*, see Hesselbrock and Foshay (1945).

## HEMOPHILUS GROUP

In this group are included several important disease-producing species: *H. pertussis*, the cause of whooping cough in man; *H. influenzae*, present in influenza infections; *H. duplex*, the etiological agent of subacute infectious conjunctivitis; and *H. ducreyi*, the cause of soft chancre or chancroid. The various species grow best (or only) in the

presence of hemoglobin and various growth accessory substances. Because of this fact, they are generally referred to as the hemoglobinophilic organisms.

**Hemophilus pertussis.**—Cells are minute and oval rods, measuring 0.2 to 0.3 by 0.5 to 2.0 $\mu$, occurring singly and in pairs, and show a tendency to pleomorphism. Organisms frequently show bipolar staining. Nonmotile. Nonspore-forming. Gram-negative.

Various forms of the organism have been isolated, including smooth, rough, and intermediate types. The smooth forms are pathogenic whereas the rough and intermediate forms are not. The organisms may exist in four phases on the basis of serological reactions. Freshly isolated or phase I strains are encapsulated, virulent for laboratory animals, hemolytic, and require the presence of the *X* and *V* factors (see page 251). The phase I properties are lost on artificial cultivation, the organisms changing to phases II, III, or IV. Only virulent organisms in phase I are suitable for the production of vaccines.

*Disease Produced.*—Believed to be the cause of whooping cough. The organism is sometimes referred to as the Bordet-Gengou bacillus after the names of its discoverers. Whooping cough is an acute, specific, infectious disease of the trachea and bronchi. It is characterized by a cough typical of the disease and lasts from 1 to 2 months. The disease starts as a catarrhal condition followed by an irritating cough. The cough becomes paroxysmal after a period of 1 to 2 weeks. The paroxysms consist of a repeated series of violent coughs often followed by a characteristic long-drawn whoop during inhalation. Paroxysms are sometimes followed by vomiting. The period of communicability probably does not last longer than 3 weeks after the cough appears.

Whooping cough shows its greatest incidence in children under five years of age, and the death rate is highest in those under one year of age. Children suffering from the disease show a predisposition to infections by micrococci, streptococci, pneumococci, and tubercle bacilli.

*Diagnosis.*—The organism may be recovered by the cough-plate method. This consists of exposing a Petri dish, containing an appropriate medium, before a patient's mouth during a cough in the early paroxysmal stage of the infection. The plate is then incubated and characteristic colonies isolated.

*Source of Infection.*—Mucous discharges from larynx and bronchi of infected persons.

*Mode of Transmission.*—Contact with an infected person or with the discharges of an infected person. Disease easily spread among children by personal contact. There is no evidence of a carrier state.

*Immunity.*—One attack of the disease confers a definite immunity but not for life; second attacks are known to occur. Passive immunity may be conferred by the use of immune or convalescent serum.

Vaccines prepared from suspensions of heat-killed organisms have been employed in the treatment of the disease with favorable results. The vaccines are generally prepared from a number of freshly isolated smooth strains of *H. pertussis*, killed by exposure to phenol, and standardized to contain approximately 15 billion bacteria per cubic centimeter. When used therapeutically, the vaccine is said to reduce the number and severity of the paroxysms and to shorten the duration of the disease.

*Prevention and Control.*—Isolation of infected individuals especially from children. Disinfection of discharges from nose and throat of patient and articles soiled with such discharges. Prophylactic vaccination of those exposed to the disease has been practiced with apparent good results. As a therapeutic agent, the vaccine has been claimed to lessen the number and severity of the paroxysms and to shorten the duration of the disease.

**Hemophilus influenzae.**—Cells are minute rods, measuring 0.2 to 0.3 by 0.5 to 2.0 $\mu$, occurring singly, in pairs, occasionally in short chains, and sometimes in the form

of long threads. Organisms show a marked tendency to bipolar staining. Some strains are encapsulated. Nonmotile. Nonspore-forming. Gram-negative.

*H. influenzae* is an obligate parasite and grows only in the presence of hemoglobin and other body fluids. It will not grow in the absence of the *X* and *V* factors present in blood (see page251 ). On the basis of serological reactions (precipitin tests), six types of *H. influenzae* have been recognized.

The various types are capsulated and may be separated from each other by means of the capsule-swelling (Quellung) reaction as employed for the typing of pneumococci.

FIG. 261.—*Hemophilus influenzae* Smear of sputum from a case of influenzal pneumonia The oval-shaped cells are *Diplococcus pneumonae.* (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

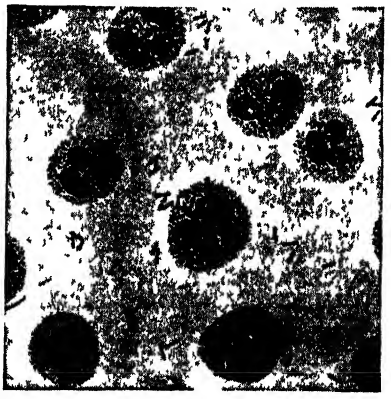

FIG. 262.—*Hemophilus influenzae.* Smear of pus from a case of acute conjunctivitis. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

The use of type specific immune serum has proved of value in determining the type of organism involved. On the basis of the capsular swelling method, Zepp and Hodes (1943) reported that four strains of type VI and three strains of type XXIX pneumococcus appeared to have one or more capsular antigens in common with seven strains of type B *H. influenzae*, which were recovered from the spinal fluid or blood of infants.

*Disease Produced.*—*H. influenzae* was at one time believed to be the etiological agent of influenza, but evidence now points to the fact that the disease is caused by a filterable virus (see page 679). The organism is commonly present in the normal nose and throat and has been found to be a secondary invader in a number of bacterial and virus infections, including scarlet fever, measles, chicken pox, and whooping cough. It is also believed to be responsible for cases of endocarditis, sinusitis, meningitis, bronchopneumonia, and acute infectious conjunctivitis or "pink eye" (Figs. 261 and 262). Type B organisms have been found to be the cause of severe throat infections in children.

*Source of Infection.*—Discharges from nose and throat or articles soiled by such discharges.

*Mode of Transmission.*—Contact with an infected person; the use of towels or other freshly contaminated articles; the inhalation of droplets expelled during coughing, sneezing, or talking.

*Immunity.*—Doubtful.

*Prevention and Control.*—Isolation of infected individuals; avoidance of overcrowding; disinfection of articles soiled by discharges from nose and throat.

**Hemophilus duplex.**—Short rods 0.4 to 0.5 by 2.0 $\mu$, occurring singly, in pairs, and in short chains. Nonspore-forming. Nonmotile. Gram-negative.

*Disease Produced.*—The cause of subacute infectious conjunctivitis or angular conjunctivitis. Isolated from conjunctiva (Fig. 263).

*Source of Infection.*—Discharges from conjunctivae.

*Mode of Transmission.*—Contact with an infected person or with articles freshly soiled with discharges from such person.

*Immunity.*—No acquired immunity.

*Prevention and Control.*—Isolation of patient; disinfection of conjunctival discharges and articles soiled by such discharges.

**Hemophilus ducreyi.**—Small rods 0.5 by 1.5 to 2.0 $\mu$, with rounded ends, occurring singly and in short chains. Nonspore-forming. Nonmotile. Gram-negative.

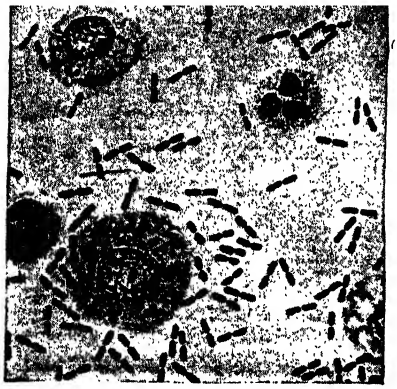

FIG. 263.—*Hemophilus duplex.* Smear of lachrymal secretion from a case of chronic conjunctivitis. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

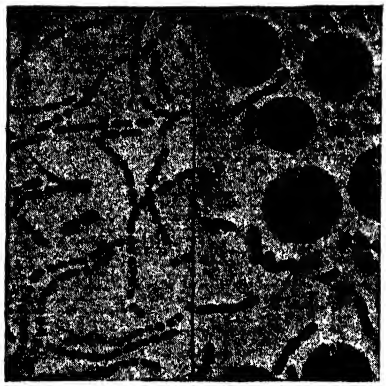

FIG. 264.—*Hemophilus ducreyi*, the cause of soft chancre or chancroid. Left, smear from a young blood agar culture; right, smear from a soft chancre. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

*Disease Produced.*—The cause of soft chancre or chancroid, an acute inflammatory lesion that occurs upon the genitals or, less frequently, the skin surrounding the genitals. The lesion starts as a small pustule which eventually ruptures to form an open ulcer. The infection easily spreads to other areas. This is a purely local process, never producing a generalized infection.

*Source of Infection.*—Discharges from ulcerated lesions (Fig. 264).

*Mode of Transmission.*—Transmitted chiefly by sexual intercourse, or by articles soiled with discharges from ulcerated lesions. The organism quickly loses its viability outside of the body and soon dies.

*Immunity.*—Probably no immunity.

*Prevention and Control.*—Chancroid is spread largely by sexual contact. It is not a serious disease and yields readily to local treatment.

## ENTERIC GROUP

The enteric organisms are grouped under the genera *Escherichia, Salmonella,* and *Shigella.* These organisms are sometimes, and probably more commonly, referred to as members of the colon, typhoid, and dysentery subgroups.

The various species are practically indistinguishable on microscopic examination. They are small rods, Gram-negative, do not produce spores, and may be present in the intestinal tract of normal persons or after an infection. Fermentation reactions and agglutinin absorption tests are of value in the differentiation of the various species.

**Escherichia coli.**—Cells short, plump rods, occurring singly, in pairs, and in short chains, measuring 0.5 by 1.0 to 3.0 $\mu$. Ferment glucose and lactose with the production of acid and gas. Carbon dioxide and hydrogen produced in approximately equal volumes from glucose. Do not produce spores. Usually motile. Gram-negative (Fig. 184).

*Disease Produced.—E. coli* is a normal inhabitant of the intestinal tract of man and other vertebrate animals. It is generally nonpathogenic; in certain instances, it has been found to overcome the defense mechanisms of the body to produce septicemia, peritonitis, inflammation of the liver and gall bladder, cystitis, meningitis, and other infections. Since the organism is found in the intestinal contents, its presence in water and foods generally means contamination with fecal material.

**Salmonella typhosa.**—Cells short, plump rods, occurring singly, in pairs, and occasionally in short chains, measuring 0.6 to 0.7 by 2.0 to 3.0 $\mu$. Facultative anaerobic. Motile with peritrichous flagella. Nonspore-forming. Gram-negative.

*Disease Produced.*—The cause of typhoid fever, an acute infectious disease transmitted by water, milk, food, and flies. The disease causes an irritation of the walls of the gastrointestinal tract with the formation of ulcers and the production of diarrhea. The infection produces an enlargement of the spleen, rose spots on the trunk, and a variety of severe constitutional disturbances. *S. typhosa* may be recovered from the feces and, during the first week of the infection, the organism is generally present in the blood stream. The disease may be transferred to laboratory animals by inoculation (Figs. 265 and 266).

FIG. 265.—*Salmonella typhosa.* Smear from a 24-hr. agar slant culture. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

*Carriers.*—Typhoid fever may be transferred from person to person by individuals known as carriers. A carrier is one who has recovered from the disease but still continues to discharge the bacilli in the intestinal contents. These organisms are no longer pathogenic to the carrier but are capable of producing typhoid fever when they reach the intestinal tracts of other persons. Carriers generally harbor the organisms in their gall bladders, which is believed to be the reservoir of the bacillus. Removal of the gall bladder appears to be the best method for the treatment of some carriers.

*Diagnosis.*—Motile species of *S. typhosa* contain two antigenic components, referred to as the flagellar or *H* antigen and the somatic or *O* antigen (see page 603). Agglutinins may be produced against both antigenic components. During infection, both kinds of agglutinins are present in the blood serum. Typhoid fever may be diagnosed by testing for the presence of agglutinins in the blood stream.

*Widal Reaction.*—This is a specific agglutination test for the diagnosis of typhoid fever. The test is performed by mixing gradually increasing dilutions of the patient's serum with a suspension of typhoid bacilli and observing for the presence of clumping or agglutination of the organisms. Since the reaction of the patients' serum does not become positive until during the third week of the infection, the test is of no value in the early days of the disease.

During the first few days of the disease, it is better to make a diagnosis by isolating

the organism from the feces, preparing a suspension, and testing for agglutination against a specific immune serum.

*Source of Infection.*—Feces and urine of infected persons or carriers.

*Mode of Transmission.*—Transmitted through direct contact with patients or carriers. Foods contaminated by fingers of typhoid patients or carriers. The most common source of typhoid outbreaks is through milk contaminated by a dairy worker. Oysters and shellfish, grown in sewage-polluted waters, may harbor the organism. Water-borne epidemics, due to sewage contamination, sometimes occur.

*Immunity.*—Natural immunity exists to some extent in adults. Permanent acquired

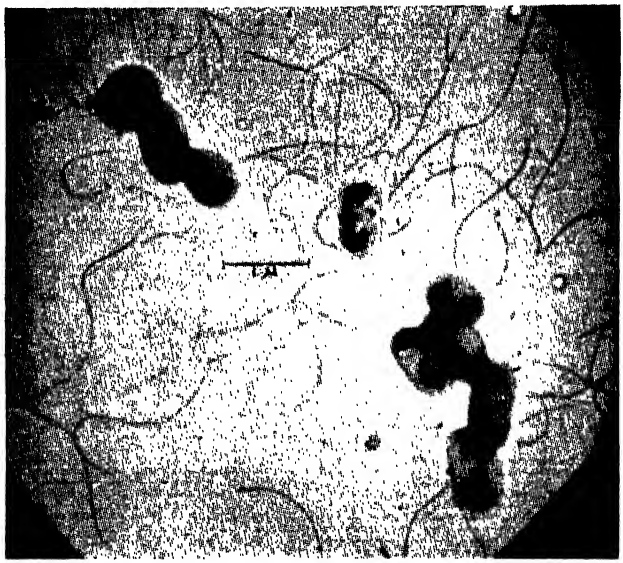

FIG. 266.—*Salmonella typhosa*, reduced from an electron micrograph with a magnification of 26,000 diameters. Note the presence of peritrichous flagella. (*After Mudd, Polevitzky, and Anderson.*)

immunity usually follows the disease. Active artificial immunity of about 2 years' duration developed after vaccination.

The vaccine is prepared by growing a freshly isolated, smooth strain on an appropriate medium, suspending the growth in saline, standardizing the suspension to contain about 1 billion organisms per cubic centimeter, killing the organisms at a temperature of 53°C. for 1 hr., then preserving with 0.25 per cent tricresol. Three injections of the vaccine in doses of 500 million, 1 billion, and 1 billion at intervals of 7 to 10 days are generally sufficient for establishing a satisfactory active artificial immunity.

*Prevention and Control.*—Isolation of infected individuals. Disinfection of all bowel and urinary discharges, and articles contaminated with such discharges. Vaccination of susceptible members in the family or household of the patient.

Other measures include efficient sewage disposal system; pure water supply; pure milk supply; sanitary control of foods, especially milk products and shellfish; periodic examination of individuals who handle foods for public consumption; extermination of flies.

**Salmonella schottmuelleri.**—Cells short, plump rods, occurring singly and in pairs, measuring 0.6 to 0.7 by 2.0 to 3.0 $\mu$. Motile. Nonspore-forming. Gram-negative.

*Disease Produced.*—The cause of paratyphoid fever in man. Also responsible for cases of food poisoning (see page 541). The disease is not naturally found in animals. It is characterized by continued fever, involvement of the lymphoid tissues of the intestines, enlargement of the spleen, sometimes rose spots on the trunk, and usually accompanied by a diarrheal condition. The organism may be present in the feces, urine, and blood and may be identified by fermentation and serological reactions.

*Source of Infection.*—Feces and urine of infected persons or carriers; water or foods contaminated with discharges of infected persons or healthy carriers.

*Mode of Transmission.*—Transmitted by direct contact with infected persons, or by articles soiled with the discharges of infected persons; through water, food, and milk contaminated with the discharges of infected persons or carriers; and by insects.

*Immunity.*—Natural immunity believed to exist in some persons. Acquired immunity is usually permanent after recovery from the disease. Active artificial immunity of about 2 years' duration developed after vaccination.

*Prevention and Control.*—Isolation of infected individuals. Disinfection of feces and urine of infected persons and carriers, or articles soiled with discharges from such persons. Food, water, and milk sanitation. Efficient sewage disposal system. Periodic examination of individuals who handle foods for public consumption. Extermination of flies.

**Salmonella paratyphi.**—Cells short, plump rods, occurring singly, measuring 0.6 by 3.0 to 4 $\mu$. Motile. Nonspore-forming. Gram-negative.

*Disease Produced.*—The cause of paratyphoid fever in man. Also responsible for cases of food poisoning (see page 541). The organism is probably not a natural pathogen for animals. The disease is characterized by continued fever, involvement of the lymphoid tissues of the intestines, enlargement of the spleen, sometimes rose spots on the trunk, and usually accompanied by a diarrheal condition. The organism may be present in the feces, urine, and blood and may be identified by fermentation and serological reactions.

*Source of Infection.*—Feces and urine of infected persons or carriers; water or foods contaminated with discharges of infected persons or healthy carriers.

*Mode of Transmission.*—Transmitted by direct contact with infected persons, or by articles soiled with the discharges of infected persons; through water, food, and milk contaminated with the discharges of infected persons or carriers; and by flies.

*Immunity.*—Natural immunity believed to exist in some persons. Acquired immunity is usually permanent after recovery from the disease. Active artificial immunity of about 2 years' duration developed after vaccination.

*Prevention and Control.*—Isolation of infected individuals. Disinfection of feces and urine of infected persons and carriers, or articles soiled with discharges from such persons. Food, water, and milk sanitation. Efficient sewage disposal system. Periodic examination of individuals who handle foods for public consumption. Extermination of flies.

**Salmonella typhimurium.**—Cells short, plump rods, occurring singly, measuring 0.5 by 1.0 to 1.5 $\mu$. Motile. Nonspore-forming. Gram-negative.

*Disease Produced.*—Outbreaks of food poisoning have sometimes been traced to this species (see page 541). It is a natural pathogen for mice, rats, guinea pigs, sheep, pigs, parrots, chickens, ducks, turkeys, pigeons, and canaries.

*Source of Infection.*—Feces and urine of infected persons or carriers; water or foods contaminated with discharges of infected persons or healthy carriers.

*Mode of Transmission.*—Transmitted by direct contact with infected persons, or by articles soiled with the discharges of infected persons or carriers; through water, food, and milk contaminated with the discharges of infected persons or carriers; and by flies.

*Immunity.*—Natural immunity believed to exist in some persons. Acquired immunity is usually permanent after recovery from the disease. Active artificial immunity of about 2 years' duration developed after vaccination.

*Prevention and Control.*—Isolation of infected individuals. Disinfection of feces and urine of infected persons and carriers, or articles soiled with discharges from such persons. Food, water, and milk sanitation. Efficient sewage disposal system. Periodic examination of individuals who handle foods for public consumption. Extermination of flies.

**Salmonella enteritidis.**—Cells rod-shaped, occurring singly, in pairs, and sometimes in short chains, measuring 0.6 to 0.7 by 2.0 to 3.0 $\mu$. Motile. Nonspore-forming. Gram-negative.

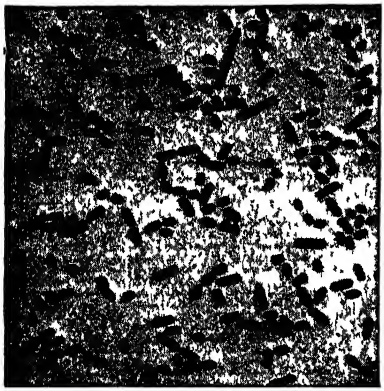

FIG. 267.—*Shigella dysenteriae.* Smear from a 24-hr. culture. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

*Disease Produced.*—One of the organisms responsible for food poisoning in man (see page 541). Organism produces natural infections in domestic and wild animals.

*Source of Infection.*—Feces and urine of infected persons or carriers; water or foods contaminated with discharges of infected persons or healthy carriers.

*Mode of Transmission.*—Transmitted by direct contact with infected persons, or by articles soiled with the discharges of infected persons or carriers; through water, food, and milk contaminated with the discharges of infected persons or carriers; and by flies.

*Immunity.*—Natural immunity believed to exist in some persons. Acquired immunity is usually permanent after recovery from the disease. Active artificial immunity of about 2 years' duration developed after vaccination.

*Prevention and Control.*—Isolation of infected individuals. Disinfection of feces and urine of infected persons and carriers, or articles soiled with discharges from such persons. Food, water, and milk sanitation. Efficient sewage disposal system. Periodic examination of individuals who handle foods for public consumption. Extermination of flies.

**Shigella dysenteriae.**—Cells rod-shaped, occurring singly, measuring 0.4 to 0.6 by 1.0 to 3.0 $\mu$. Nonmotile. Nonspore-forming. Gram-negative. This organism is differentiated from the **Escherichia** and **Salmonella** in being nonmotile.

*Disease Produced.*—The cause of bacillary dysentery in man and monkeys (Fig. 267). The disease is characterized by an acute onset accompanied by diarrhea, sometimes fever in severe cases, tenesmus, and frequent stools containing blood and mucus.

*S. dysenteriae* produces a powerful exotoxin. When inoculated into rabbits, the toxin produces typical paralysis and severe nerve lesions after an incubation period of a few hours to 4 days. It is believed that the lesions produced in the gastrointestinal tract of persons suffering from the disease are the result of the action of the toxin rather than the direct action of the organisms.

*Carriers.*—Dysentery may be transferred from person to person by carriers. A carrier is one who has recovered from the disease but still continues to discharge the bacilli in the intestinal contents. These organisms are no longer pathogenic to the carrier but are capable of producing dysentery when they reach the intestinal tracts of other persons.

*Source of Infection.*—Bowel discharges of infected persons and carriers. Healthy carriers are common.

*Mode of Transmission.*—Transmitted by direct contact; by eating contaminated foods; by articles soiled with discharges from infected persons or carriers; by drinking contaminated water; and by flies. The disease is most prevalent in the summer months.

*Immunity.*—Slight acquired immunity of relatively short duration after recovery from the disease.

Vaccine has been used for prophylaxis, but the results have not been encouraging. In some cases, the reactions have been severe.

*Prevention and Control.*—Isolation of infected persons. Disinfection of feces and urine of infected persons and carriers, or articles soiled with discharges from such persons.

Other measures include efficient sewage disposal system; protection and purification of water supplies; pasteurization of milk supplies; sanitary control of foods; periodic examination of individuals who handle foods for public consumption; extermination of flies.

**Shigella paradysenteriae.**—Cells rod-shaped, occurring singly, measuring 0.4 to 0.6 by 1.0 to 3.0 $\mu$. Nonmotile. Nonspore-forming. Gram-negative.

*Disease Produced.*—A cause of dysentery in man and summer diarrhea in children. Disease characterized by an acute onset accompanied by diarrhea, sometimes fever, tenesmus, and frequent stools containing blood and mucus. Symptoms are generally milder than in infections in which *S. dysenteriae* is involved.

*Carriers.*—Disease may be transferred from person to person by carriers. A carrier is one who has recovered from the disease but still continues to discharge the bacilli in the intestinal contents. The organisms are no longer pathogenic to the carrier but may produce dysentery when they reach the intestinal tracts of other persons.

*Source of Infection.*—Bowel discharges of infected persons and carriers.

*Mode of Transmission.*—Transmitted by direct contact; by eating contaminated foods; by articles soiled with discharges from infected persons or carriers; by drinking contaminated water; and by flies. The disease is most prevalent in the summer months.

*Immunity.*—Slight acquired immunity of relatively short duration after recovery from the disease.

Vaccine has been used for prophylaxis, but the results have not been encouraging.

*Prevention and Control.*—Isolation of infected persons. Disinfection of feces and urine of infected persons and carriers, or articles soiled with discharges from such persons.

Other measures include efficient sewage disposal system; protection and purification of water supplies; pasteurization of milk supplies; sanitary control of foods; periodic examination of individuals who handle foods for public consumption; extermination of flies.

**Shigella sonnei.**—Cells rod-shaped. Nonmotile. Gram-negative.

*Disease Produced.*—A cause of mild dysentery in man and summer diarrhea in children. Disease characterized by an acute onset accompanied by diarrhea, sometimes fever, tenesmus, and frequent stools. Symptoms milder than in infections in which *S. dysenteriae* is involved.

*Carriers.*—Disease may be transferred from person to person by carriers (see page 660).

*Source of Infection.*—Bowel discharges of infected persons and carriers.

*Mode of Transmission.*—Transmitted by direct contact; by eating contaminated foods; by articles soiled with discharges from infected persons or carriers; by drinking contaminated water; and by flies. The disease is most prevalent in the summer months.

*Immunity.*—Slight acquired immunity of relatively short duration after recovery from the disease.

*Prevention and Control.*—Isolation of infected persons. Disinfection of feces and urine of infected persons and carriers, or articles soiled with discharges from such persons.

Other measures include efficient sewage disposal system; protection and purification of water supplies; pasteurization of milk supplies; sanitary control of foods; periodic examination of individuals who handle foods for public consumption; extermination of flies.

## BRUCELLA GROUP

This group includes a number of small pathogenic species that produce abortion in animals and Malta fever or undulant fever in man. They invade animal tissue, producing infections of the genital tract, the mammary gland or lymphatic tissues, and the intestinal tract. The genus *Brucella* is named after Bruce (1887) who was the first to isolate the organism of undulant fever from the spleens of fatal cases of the disease on the island of Malta in the Mediterranean.

**Brucella abortus.**—Cells short, oval rods, occurring singly, in pairs, and occasionally in chains, measuring 0.3 to 0.4 $\mu$ in length. Organism requires 10 per cent $CO_2$ for isolation; becomes aerobic after several transfers. Nonmotile. Microaerophilic. Gram-negative.

*Disease Produced.*—The cause of contagious abortion in cattle. The same effects are produced in mares, sheep, rabbits, and guinea pigs. Causes undulant fever or brucellosis in man.

Undulant fever is a general infection with gradual or insidious onset and characterized by irregular fever usually of prolonged duration, sweating, chills, pain in the joints and muscles.

*Diagnosis.*—Since brucellosis is generally accompanied by a septicemia, a diagnosis can usually be made on the basis of a blood culture.

Agglutinins and complement-fixing antibodies are present in the serum of patients suffering from the disease. In the event the blood culture is negative, it is generally desirable to test the patient's serum for the presence of agglutinins. An agglutination titer of 1:500, or above, is positive evidence of undulant fever.

*Source of Infection.*—Tissues, blood, milk, and urine of infected animals, especially goats, swine, and cattle. Laboratory infections are quite common.

*Mode of Transmission.*—Drinking milk from infected animals and by direct contact with infected animals or animal products.

*Immunity.*—Most persons have some natural immunity or have acquired partial immunity by ingestion of small doses of the organism. Duration of immunity not known. One attack of undulant fever usually protects against a second attack.

*Prevention and Control.*—Source of infection should be ascertained. Pasteurization of all milk supplies whether from cows or goats. Animals should be tested by the agglutination technique and positive reactors isolated or slaughtered. Animals have been vaccinated with stock vaccines, but the results have been inconclusive. Autogenous vaccines have been used in humans with some success. The disease is seldom fatal.

**Brucella melitensis.**—Cells short, oval rods, occurring singly, in pairs, and occasionally in chains, measuring 0.3 to 0.4 $\mu$ in length. Nonmotile. Microaerophilic. Gram-negative.

*Disease Produced.*—The cause of abortion in goats. May infect cows and be excreted in milk. Infectious for all domestic animals. Causes undulant fever or brucellosis in man (Fig. 268).

Undulant fever is a general infection with gradual or insidious onset and charac-

terized by irregular fever usually of prolonged duration, sweating, chills, pain in the joints and muscles.

*Diagnosis.*—Since brucellosis is generally accompanied by a septicemia, a diagnosis can usually be made on the basis of a blood culture. Blood cultures are positive in about 80 per cent of the cases after the second day and may continue in the septicemic form for a number of months.

Agglutinins and complement-fixing antibodies are present in the serum of patients suffering from the disease. In the event the blood culture is negative, it is generally desirable to test the patient's serum for the presence of agglutinins. Agglutinins generally occur in the serum about the tenth day of the fever. An agglutination titer of 1:100, or above, is positive evidence of undulant fever. Since antiserum for *B. melitensis* will cross agglutinate with *B. abortus* and *B. suis*, agglutinin absorption tests are necessary for diagnosis.

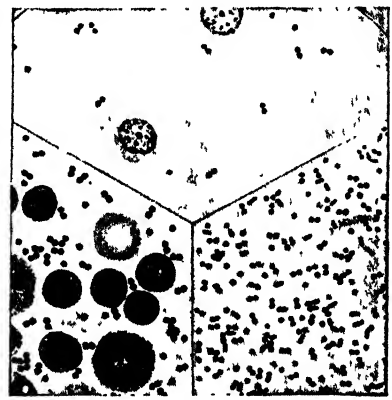

FIG. 268.—*Brucella melitensis.* Upper, smear of milk from an infected goat; lower left, spleen smear from a case of Malta fever; lower right, smear prepared from a young culture. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

*Source of Infection.*—Tissues, blood, milk, and urine of infected goats. Disease spread to man through the milk of such animals. The disease in both goats and man is a septicemia. The organism attacks other animals, especially cattle and swine. Laboratory infections are quite common.

*Mode of Transmission.*—Drinking milk from infected animals and by direct contact with infected animals or animal products.

*Immunity.*—Most persons have some natural immunity or have acquired partial immunity by ingestion of small doses of the organism. Duration of immunity not known. One attack of undulant fever usually protects against a second attack.

*Prevention and Control.*—Source of infection should be ascertained. Pasteurization of all milk supplies whether from cows or goats. Animals should be tested by the agglutination technique and positive reactors isolated or slaughtered. Animals have been vaccinated with stock vaccines, but the results have been inconclusive. Autogenous vaccines have been used in humans with some success. The disease is seldom fatal.

**Brucella suis.**—Cells short, oval rods, occurring singly, in pairs, and occasionally in chains, measuring 0.3 to 0.4 $\mu$ in length. Nonmotile. Gram-negative.

*Disease Produced.*—The cause of abortion in swine. May also infect horses, dogs, cows, monkeys, and laboratory animals. Causes undulant fever or brucellosis in man.

Undulant fever is a general infection with gradual or insidious onset and characterized by irregular fever usually of prolonged duration, sweating, chills, pain in the joints and muscles.

*Diagnosis.*—Since brucellosis is generally accompanied by a septicemia, a diagnosis can usually be made on the basis of a blood culture. Blood cultures are positive in about 80 per cent of the cases after the second day and may continue in the septicemic form for a number of months.

Agglutinins and complement-fixing antibodies are present in the serum of patients suffering from the disease. In the event the blood culture is negative, it is generally desirable to test the patient's serum for the presence of agglutinins. Agglutinins gen-

erally occur in the serum about the tenth day of the fever. An agglutination titer of 1:100, or above, is positive evidence of undulant fever. Since antiserum for *B. suis* will cross-agglutinate with *B. melitensis* and *B. abortus*, agglutinin absorption tests are necessary for diagnosis.

*Source of Infection.*—Tissues, blood, milk, and urine of infected animals. Disease spread to man through the milk of such animals. The disease in both animals and man is a septicemia. Laboratory infections are quite common.

*Mode of Transmission.*—Drinking milk from infected cows and by direct contact with infected animals or animal products.

*Immunity.*—Most persons have some natural immunity or have acquired partial

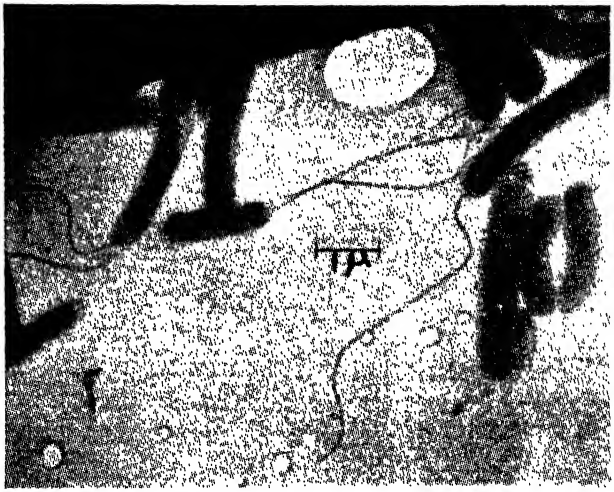

FIG. 269.—*Vibrio comma*, slightly reduced from an electron micrograph with a magnification of 9,300 diameters. Note the single polar flagellum, which seems to traverse the cell wall to join the bacterial protoplasm. (*After Mudd and Anderson.*)

immunity by ingestion of small doses of the organism. Duration of immunity uncertain. One attack of undulant fever usually protects against a second attack.

*Prevention and Control.*—Source of infection should be ascertained. Pasteurization of all milk supplies. Animals should be tested by the agglutination technique and positive reactors isolated or slaughtered. Animals have been vaccinated with stock vaccines, but the results have been inconclusive. Autogenous vaccines have been used in humans with some success. The disease is seldom fatal.

## VIBRIO GROUP

The genus *Vibrio* includes a number of species characterized as small, curved rods, the most important being *V. comma*, the cause of Asiatic cholera. The genus also includes several nonpathogenic species.

**Vibrio comma.**—Cells short, slightly curved rods, occurring singly and in spiral chains, measuring 0.4 to 0.6 by 1.5 to 3.0 $\mu$. Motile by means of one or two polar flagella. Gram-negative. Organisms grown on culture media for several generations tend to become less curved. Old cultures exhibit involution forms.

*Disease Produced.*—The cause of Asiatic cholera in man (Fig. 269). In mild cases, the disease may produce only a diarrhea. In more severe, or typical cases, the symptoms

may include, in addition to diarrhea, vomiting, "rice-water" stools, and general symptoms of dehydration accompanied by thirst, abdominal pain, and coma. The organisms penetrate the mucosa of the intestines and accumulate in layers next to the submucosa. The organisms may be present in large numbers in the stools. The disease runs a short course, terminating in death sometimes within 12 hr. after the appearance of symptoms. The organisms may be present in large numbers in the stools.

*Diagnosis.*—*V. comma* may be isolated from the feces of both infected individuals and carriers. The intraperitoneal inoculation of guinea pigs with pure cultures results in the death of the animals within 24 hr.

*Carriers.*—Patients convalescing from the disease usually continue to eliminate the organisms in the feces for about 7 to 14 days after recovery. Healthy carriers may also be found who excrete the cholera vibrio without exhibiting any signs of the disease. Both convalescent and healthy carriers play an important role in the dissemination of the disease.

*Source of Infection.*—Intestinal contents and vomitus of infected persons and feces of convalescent or healthy carriers. Also from food and water.

*Mode of Transmission.*—By water and foods; by contact with infected persons or carriers, or articles soiled with discharges from such persons; by flies.

*Immunity.*—Active artificial immunity for about 6 to 12 months may be produced by the use of vaccines. Recovery from an attack is said to confer immunity for life.

*Prevention and Control.*—Isolation of persons suffering from the disease. Disinfection of stools and vomitus, and articles soiled by such discharges. Food left by patient should be destroyed by burning. Room occupied by patient should be thoroughly cleaned and disinfected. Carriers should also be isolated.

Boiling of all water whether for drinking purposes or for washing dishes and food. Careful supervision of food and drink. During outbreaks, only cooked foods should be used. Destruction of flies and their breeding places.

## ANTHRAX BACILLUS

The anthrax organism belongs to the genus *Bacillus*, the members of which are capable of forming heat-resistant spores. Almost all the species are Gram-positive. With the exception of the anthrax bacillus, all the members are saprophytic and usually not pathogenic. *B. subtilis* has been known to become pathogenic at times, but this is the exception rather than the rule. The members are typically aerobic, but some can grow in the almost complete absence of oxygen.

Spores are not produced under anaerobic conditions. On the other hand, the anaerobic spore-producing species do form spores under anaerobic conditions. This fact offers a means for the separation of the aerobic from the anaerobic spore-formers. By the application of heat to a mixed culture, the vegetative cells are destroyed and leave only the anaerobic spores which are capable of germinating into vegetative cells under favorable conditions.

Members of the genus *Bacillus* are universally distributed in soil and water. Spores and vegetative cells of such species are easily carried into the air by gentle air currents. This explains why viable spores of such organisms are universally present in air and are responsible for many laboratory contaminations of culture media and cultures.

The genus *Bacillus* includes over 100 members. Some of the better known species, in addition to *B. anthracis*, are *B. subtilis*, *B. mycoides*, *B. mesentericus*, and *B. megatherium*.

**Bacillus anthracis.**—Cells rod-shaped, with square or concave ends, occurring singly but usually in long chains, measuring 1 to 1.25 by 5 to 10 $\mu$. Spores measure from 0.7

to 0.8 by 1.5 to 1.8 $\mu$, being central and ellipsoidal in shape, do not cause a bulging of the cell, and are produced only under aerobic conditions. They are not formed in the animal body. Nonmotile. Gram-positive.

*Disease Produced.*—The cause of anthrax, an acute specific disease of cattle, sheep, and swine, sometimes occurring in workers handling wool and hides of animals affected with the disease (Fig. 270). Usually occurs as a febrile disease of animals that runs a rapid course and terminates in a septicemia. Mortality rate may run as high as 80 per cent. The infection causes a marked enlargement of the spleen in which may be found enormous numbers of bacilli.

Two forms occur in man: cutaneous (malignant pustule) and internal anthrax. Cutaneous anthrax is produced by direct inoculation through a cut or abrasion in the skin. This type occurs most frequently in persons working with livestock. It is characterized by the appearance of a small furuncle within 12 to 24 hr. after entrance of the organisms. The furuncle ulcerates and discharges a seropurulent exudate, which may heal and disappear, or gangrene may set in followed by a septicemia. This usually terminates fatally in about 5 days.

FIG. 270.—*Bacillus anthracis.* Left, smear from the liver of an experimentally inoculated guinea pig; right, smear from a 72-hr. agar slant culture. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

The internal or pulmonary type is contracted by inhalation or by swallowing spores of *B. anthracis.* The disease is characterized by a pneumonia that generally terminates fatally. Before death, it is possible to isolate the organism from the sputum. The organism may also be recovered from the blood and spinal fluid.

*Diagnosis.*—In the skin type, smears may be prepared from the seropurulent exudate and stained by Gram's method. The presence of large, Gram-positive, encapsulated organisms without spores is strong evidence for the presence of *B. anthracis.* The organism may be confirmed by guinea pig inoculation. The animals usually die in from 12 hr. to 3 days with a septicemia.

In the pneumonic type, sputum and blood are examined by the Gram technique. Cultures may be prepared by inoculating blood into broth and examining for characteristic organisms after an incubation period of 24 hr. A confirmation test may be made by guinea pig inoculation as given above.

*Source of Infection.*—Hair, hides, wool, flesh, and feces of infected animals.

*Mode of Transmission.*—Inhalation of spores; ingestion of insufficiently cooked food; mechanically by flies; accidental inoculation by wounds or scratch.

*Immunity.*—Man is less susceptible to the disease than the herbivora but more so than the carnivora. Immunity may develop after recovery from the disease. Active, artificial immunity produced in animals by the use of a vaccine. This is not practiced in human beings.

*Prevention and Control.*—Isolation of infected individuals until lesions have healed. Disinfection of discharges from lesions and of articles soiled by such discharges.

Hides, wool, hair, and bristles from areas not known to be free from anthrax should be examined bacteriologically. Human beings handling hides, wool, and hair should report immediately any skin abrasion. All hair, wool, and bristles should be disinfected

if obtained from sources not known to be free from anthrax. Infection has occurred from the use of shaving brushes and toothbrushes made from unsterilized bristles.

Animals suspected of having the disease should be isolated in care of a veterinarian. Animals proved positive should be killed and disposed of, preferably by incineration. Animals exposed to the disease should be promptly vaccinated. Milk from infected animals should never be used.

## ANAEROBIC SPORE-FORMING GROUP

The members of this group belong to the genus *Clostridium*. These organisms are characterized as obligate anaerobes, produce spores, actively attack proteins and carbohydrates, and are Gram-positive. The group includes many soil species, some of which are pathogenic for man. The presence of *C. tetani* in wounds may result in tetanus; the presence of certain others may produce gas gangrene. Many of the clostridia are employed industrially in various types of fermentations. The important disease-producing species include *C. histolyticum*, *C. perfringens*, *C. tetani*, *C. botulinum*, *C. botulinum* type C, and *C. parabotulinum*.

FIG. 271.—*Clostridium tetani*. Smear from a 72-hr. glucose agar stab culture. The spores are round and situated at one end of the rod. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

**Clostridium histolyticum.**—Cells rod-shaped with rounded ends, occurring singly and in pairs, measuring 0.5 to 0.7 by 3.0 to 5.0 $\mu$. Spores oval, subterminal, and cause a bulging of the rods. Motile with peritrichous flagella. Anaerobic. Gram-positive.

*Disease Produced.*—Isolated from war wounds. Actively proteolytic producing necrosis of muscle tissue. Action due to elaboration of a cytolytic exotoxin. Intra-muscular injection of small amounts of culture into guinea pigs produces rapid digestion of muscle tissue. Not toxic on feeding. Pathogenic for small laboratory animals.

*Source of Infection.*—Organism found in soil.

*Mode of Transmission.*—Disease produced by entrance of the organism into the broken skin where it multiplies and produces an extracellular cytolytic toxin.

*Immunity.*—Passive immunity may be produced by the use of antitoxin.

*Prevention and Control.*—The exotoxin can be neutralized by the injection of the homologous antitoxin.

**Clostridium tetani.**—Cells rod-shaped with rounded ends, occurring singly, in pairs, and in chains, measuring 0.4 to 0.6 by 4.0 to 8.0 $\mu$. Spores spherical, terminal, and cause a bulging of the rod. Motile with peritrichous flagella. Anaerobic. Gram-positive.

*Disease Produced.*—The cause of tetanus in man (Fig. 271). The organism produces two kinds of toxins: (1) tetanospasmin, which causes the symptoms of tetanus, and (2) tetanolysin, which causes hemolysis of red blood cells of horses, rabbits, goats, and other animals.

Tetanospasmin is an extracellular toxin and may be obtained in crude form by filtering a culture of the organisms. The inoculation of horses and other animals with culture filtrates results in the development of a powerful antitoxin.

Symptoms in man develop after the toxin reaches the central nervous system. This may vary from about 1 to 4 or 5 weeks. The symptoms include headache, difficulty

in swallowing and opening the mouth owing to spasms of the masseter muscles. This is accompanied by a slight stiffness of the neck and spasm of the cheek muscles. The spasms spread to the trunk and back. Swallowing becomes increasingly more difficult. The organisms only rarely invade tissues but remain localized in the wound where they secrete a powerful toxin, tetanolysin.

*Diagnosis.*—Disease may be diagnosed by preparing smears from infected materials, staining by Gram's method, and examining under the microscope for typical cells with spores.

*Source of Infection.*—Soil, street dust, human and animal feces, especially the latter.

*Mode of Transmission.*—Disease due to entrance of organism into the broken skin or wound where it multiplies and secretes a potent exotoxin. Toxin intensely toxic on injection but not on feeding.

FIG. 272—*Clostridium perfringens.* Upper, smear of the exudate from a case of human gas gangrene; lower left, smear from a young agar culture; lower right, another smear from a case of gas gangrene. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

*Immunity.*—Active artificial immunity may be produced by the use of tetanus toxoid (see page 605). Three doses of 1 cc. each at intervals of 3 weeks are generally given and are considered capable of producing a high concentration of antitoxic antibodies. The immunity is said to last for about 5 years. Toxoid precipitated by alum and given in two injections of 1 cc. each at intervals of 2 to 4 weeks has been reported to produce a greater and more rapid immunity than toxoid.

*Prevention and Control.*—The use of tetanus antitoxin is an effective protection against the disease. The injection of about 1500 U.S.A. units of antitoxin in slight injuries and about 2000 to 3000 U.S.A. units in more severe injuries will prevent tetanus. Since antitoxin tends to disappear, an additional injection should be given within 10 days.

After disease has developed, large doses of antitoxin are injected by spinal puncture. At the same time, an intravenous injection of about 10,000 units should be administered. The intraspinal injections should be repeated every 24 hr. until three doses have been given. If intraspinal doses cannot be given, larger intravenous injections should be administered.

**Clostridium perfringens.**—Cells rod-shaped, short and plump, occurring singly, in pairs, and less frequently in short chains, measuring 1.0 to 1.5 by 4.0 to 8.0 $\mu$. Spores oval, central to excentric, and do not cause a bulging of the rods. Capsules produced. Anaerobic. Nonmotile. Gram-positive.

Four types of the organism are recognized, designated as types I, II, III, and IV. They are based on fermentation reactions. Antitoxin from any one type will neutralize the toxin from the other types.

*Disease Produced.*—Isolated from wounds. This anaerobe is the most frequent cause of gas gangrene in man (Fig. 272). The organism produces extensive necrosis and considerable gas in tissues. The gas bubbles cause an expansion in the tissues accompanied by pressure, which results in cutting off the blood supply. This causes the tissues to die. Organisms may be recovered from liver and heart blood.

*C. perfringens* produces a powerful exotoxin which may be obtained in crude form by filtering a culture of the organisms. The exotoxin aids in weakening the patient.

The injection of animals with culture filtrates results in the development of a powerful antitoxin.

*Source of Infection.*—Soil, street dust, human and animal feces.

*Mode of Transmission.*—Disease due to entrance of organism into the broken skin or wound where it multiplies and secretes a potent exotoxin.

*Immunity.*—Passive immunity by means of antitoxin may be used for prophylaxis.

*Prevention and Control.*—Antitoxic sera have been used both for prophylactic and therapeutic administration, and beneficial results have been reported.

For additional information on gas gangrene, see Reed and Orr (1943) and Turner and Rodwell (1943).

**Clostridium botulinum (Type A), C. parabotulinum (Type B), and C. botulinum (Type C).**—Cells rod-shaped with rounded ends, occurring singly, in pairs, and in short to occasionally long chains, measuring 0.5 to 0.8 by 3.0 to 8.0 $\mu$. Spores oval-shaped and located centrally, terminally or subterminally. Motile. Gram-positive.

*Disease Produced.*—The cause of botulism in man and limberneck in chickens. Pathogenic for monkeys, rabbits, guinea pigs, cats, and other animals. Produce powerful exotoxins which are neurotoxic both on injection and on feeding. Symptoms develop suddenly with gastrointestinal pain, headache, diarrhea or constipation, prostration, several types of paralyses of the central nervous system which are produced by the extracellular neurotropic toxin.

*Source of Infection.*—Contaminated smoked, pickled, or canned foods improperly processed (see page 541).

*Mode of Transmission.*—Consumption of smoked, pickled, or canned foods containing the exotoxin.

*Immunity.*—Passive immunity with specific antitoxin is of value before symptoms have developed.

*Prevention and Control.*—Inspection of commercial processing of canned and preserved foods. Education of housewives in methods for the safe processing of home-canned foods. Since the soluble toxin is destroyed on boiling, all home-canned foods should be boiled before serving.

Specific antitoxin will neutralize the toxin and is of value before symptoms of the disease have developed.

## ACTINOMYCES

The actinomycetes are members of the order *Actinomycetales* which have characteristics intermediate between the true bacteria and the molds. They grow as filaments that show a definite tendency to branch.

A number of species of the genus *Actinomyces* are pathogenic for man and animals. *A. bovis* produces actinomycosis or lumpy jaw of cattle and occasionally of man. *A. madurae* produces a serious infection of the feet and is usually referred to as madura foot. Other parts of the body may also be involved. *A. hominis* is the cause of actinomycosis in man.

**Actinomyces hominis.**—Rods grow in the form of a much-branched mycelium, which may break up into segments and function as conidia. In pus, *A. hominis* appears in the form of small granular bodies, gray or pale yellow in color, like sulfur granules. The granules appear in the form of rosettes with opaque, dense centers composed of a closely meshed network of filaments. Radial striations around the margin are found in the characteristically club-shaped bodies. Nonmotile. Anaerobic. Gram-positive.

*Disease Produced.*—The cause of actinomycosis in man. A local or general, acute or chronic, suppurative infection combined with growth of connective tissue and characterized by the presence of colonies of *A. hominis* in the lesions.

*Diagnosis.*—The organism may be identified by microscopic examination of discharges from lesions.

*Source of Infection.*—Not known but believed to be grains, grasses, air, saliva, pus, nasal discharges.

*Mode of Transmission.*—Among cattle, principally by grains, grasses, and other cattle feed, stable bedding contaminated with discharges from lesions, the organisms entering through abrasions or wounds of oral cavity or body surface. Not known how disease is transmitted to man.

*Immunity.*—Acquired immunity does not follow recovery from the disease.

*Prevention and Control.*—Disinfection of discharges from lesions. Care should be taken to prevent contact with lesions. No specific treatment known.

**Actinomyces madurae.**—Mycelium and hyphae straight and branched. A few open spirals occasionally formed. Filaments 1.0 to 1.5 $\mu$ in thickness. The ends break up into ovoid conidia. Club-shaped forms appear in lesions. Aerobic. Nonmotile. Gram-positive.

*Disease Produced.*—The cause of madura foot, a chronic, suppurative, granulomatous disease of man. Disease usually affects the feet, but other parts of the body may be involved. It is especially common in tropical countries. The infection is characterized by the presence of pus in which are present large masses of growth containing a core of closely packed filaments, the tips of which enlarge and become club-shaped. These masses of filaments or mycelial growth are yellow in color and are usually called "sulfur granules."

*Diagnosis.*—The disease may be diagnosed by crushing some of the mycelial growth between two glass slides, staining with an appropriate dye, and examining under the microscope for the presence of radial structures of hyphae.

*Source of Infection.*—Common in tropical countries, particularly where people walk barefooted. Organism probably present in soil.

*Mode of Transmission.*—Organism probably present in soil and enters through an abrasion or wound on the foot.

*Immunity.*—Acquired immunity does not follow recovery from the disease.

*Prevention and Control.*—Disinfection of discharges from lesions. Care should be taken to prevent contact with lesions. No specific treatment known.

## BORRELIA

Members of this genus are classified under the order *Spirochaetales* which have characteristics intermediate between the true bacteria and the protozoa. The organisms possess small, flexible, spiral filaments 8 to 16 $\mu$ in length, with three to five large wavy spirals. The group includes the following species: (1) *B. vincentii* which is associated with *Fusobacterium plauti-vincenti* in Vincent's angina and related infections, (2) *B. duttoni*, the cause of West African relapsing fever, (3) *B. recurrentis*, the etiological agent of European relapsing fever, (4) *B. novyi*, the cause of American relapsing fever, (5) *B. kochii*, the causative agent of African relapsing fever, and (6) *B. carteri*, the cause of relapsing fever in India.

**Borrelia vincentii.**—Organism spiral-shaped, measuring 0.3 by 12 to 25 $\mu$.

*Disease Produced.*—*B. vincentii* occurs in association with *F. plauti-vincenti* in Vincent's angina, an acute infection of the tonsils, or neighboring parts, and characterized by the appearance of a pseudomembranous inflammation followed by ulceration. The disease is sometimes called "trench mouth" (Fig. 273).

The cells of *F. plauti-vincenti* are rod-shaped, occurring in pairs, in short curved chains, or in long spirillum-like threads, measuring from 0.5 to 1.0 by 8 to 16 $\mu$. Organ-

isms that occur in pairs have blunt ends together and outer ends pointed. Rods show from two to six deeply staining granules. Anaerobic. Nonmotile. Gram-negative.

*Diagnosis.*—The disease may be diagnosed by preparing smears direct from the deeper ulcerated areas, staining by the Gram technique, and examining under the oil-immersion objective. A characteristic smear shows the presence of spirochaetes and bacilli in large numbers.

*Source of Infection.*—Found in deposit on teeth; the oral cavity.

*Mode of Transmission.*—The disease is not ordinarily communicable. Under unusual conditions of crowding, such as may prevail among soldiers, the infection may become transmissible. The disease appears to be associated with a state of lowered

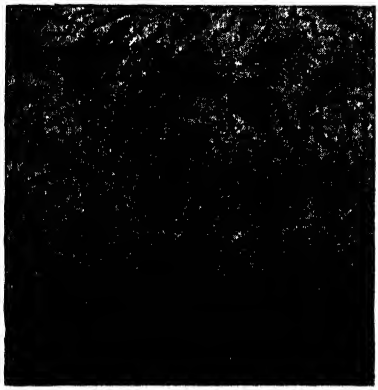

FIG. 273.—*Borrelia vincentii* and *Fusobacterium plaut-vincenti* growing in association. Smear prepared from a throat swab, taken from a case of Vincent's angina. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

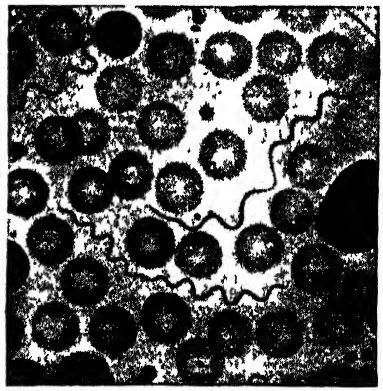

FIG. 274.—*Borrelia duttonii.* Blood smear from a case of West African relapsing fever. It is sometimes referred to as African tick fever. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

resistance. The tonsillar ulceration occurs often in individuals whose resistance has been lowered by diseases such as measles, tuberculosis, diabetes, and scarlet fever.

*Immunity.*—Acquired immunity does not follow recovery from the disease.

*Prevention and Control.*—Disease usually yields to local treatment. The most effective drug appears to be salvarsan, an arsenic compound which was at one time largely employed in the treatment of syphilis.

For additional information, see Dean and Singleton (1945).

**Borrelia duttoni.**—Cells spiral-shaped, measuring 0.2 to 0.5 by 14 to 16 $\mu$. Organisms show a long, curved, delicate projection at each extremity. Anaerobic.

*Disease Produced.*—The cause of West African relapsing fever (Fig. 274). Pathogenic for rats and mice.

*Source of Infection.*—Organisms present in the blood stream of infected individuals.

*Mode of Transmission.*—Disease transmitted by the bite of the bloodsucking tick *Ornithodoros moubata.*

*Immunity.*—Active immunity produced during course of the disease which is sufficiently powerful to overcome the blood infection, resulting in the disappearance of the spirochaetes from the circulation.

*Prevention and Control.*—Disease easily cured by injection of salvarsan or of sodium potassium bismuth tartrate.

**Borrelia recurrentis.**—Cells spiral-shaped, cylindrical or slightly flattened, measuring 0.35 to 0.5 by 8 to 16 $\mu$. Ends pointed. Distance between curves is 1.5 $\mu$. Terminal spiral filament present.

*Disease Produced.*—The cause of European relapsing fever. Can be transmitted to man, monkeys, mice, and rats (Fig. 275).

*Source of Infection.*—Organisms present in the blood stream of infected individuals.

*Mode of Transmission.*—Disease believed to be transmitted by the bite of the tick *Ornithodoros moubata* and the common bedbug *Cimex lectularius.*

*Immunity.*—One attack of disease confers immunity for life. Agglutinins may be demonstrated in the blood stream. Infection is never fatal.

*Prevention and Control.*—Destruction of bedbugs and other bloodsucking insects. Disease cured by the injection of salvarsan or of sodium potassium bismuth tartrate.

## LEPTOSPIRA

The members of this genus are the smallest of the spirochaetes. They are chiefly saprophytic organisms being found in water and sometimes in the normal mouth.

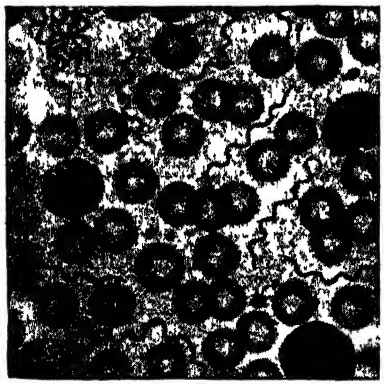

FIG. 275.—*Borrelia recurrentis.* Blood smear from a case of European relapsing fever. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

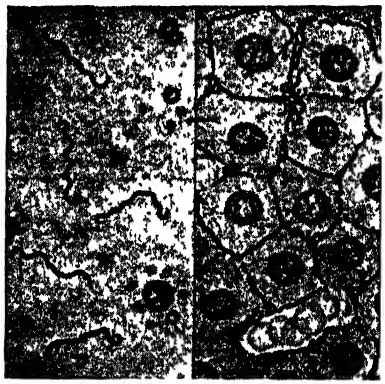

FIG. 276.—*Leptospira icterohaemorrhagiae*, the cause of Weil's disease or infectious jaundice in man. Left, smear of urine; right, section of liver from an infected rat. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

The most important member producing disease is *L. icterohaemorrhagiae*, the cause of infectious jaundice or Weil's disease. The disease-producing forms are indistinguishable from the saprophytic or harmless species.

**Leptospira icterohaemorrhagiae.**—Cells finely coiled, measuring 0.25 to 0.3 by 6 to 9 $\mu$, with pointed ends. Spirals 0.3 $\mu$ in depth and 0.4 to 0.5 $\mu$ in amplitude. One or more wavy curves throughout the length of the organism. One or both ends bent into a hook. Nonflagellated. Highly motile end portion well developed in the last six or eight spirals. Transverse division. Aerobic.

*Disease Produced.*—The cause of infectious jaundice or Weil's disease in man. Found also in the blood of dogs and wild rats (Fig. 276). The disease is characterized by malaise, prostration, gastrointestinal symptoms, muscular pains, and fever at the start followed by jaundice. Relapses may occur. In severe cases hemorrhages may

occur at various sites and kidney damage may be marked. The organism is found in blood and urine of patients.

*Diagnosis.*—Disease diagnosed by inoculating blood or urine from a patient into a guinea pig.

*Source of Infection.*—Urine and feces of rats, dogs, cats, mice, and other animals. Water and soil become contaminated with discharges of infected animals.

*Mode of Transmission.*—Infection in man probably occurs through rubbing contaminated soil into the skin, eyes, and nose, or from swallowing contaminated water.

*Immunity.*—A refractory state develops following recovery. Immune bodies may be demonstrated for a considerable period after recovery. Urine may show the presence of the organism for months after convalescence.

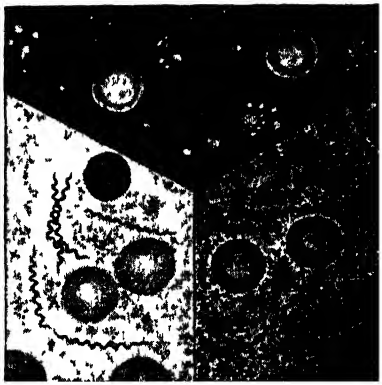

FIG. 277.—*Treponema pallidum*, the cause of human syphilis. Upper, exudate from a primary sore, viewed by dark ground illumination; lower left, smear of material from a chancre; lower right, same but stained by a different method. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

*Prevention and Control.*—Disinfection of urine and feces of patients. Drainage of infected areas and the suppression or exclusion of rats. Vaccine has been employed with promising results. Most promising treatment appears to be the use of horse antiserum.

## TREPONEMA

The members of this genus are small, slender, spiral organisms also classified under the *Spirochaetales*. They are difficult to stain but may be easily seen by dark-ground illumination. Some are parasitic; others are pathogenic. The most important species producing disease in man is *T. pallidum*, the cause of syphilis.

**Treponema pallidum.**—Cells cylindrical, with pointed ends, measuring 0.25 to 0.3 by 6 to 14 $\mu$. Amplitude of spiral 1 $\mu$ and regular. Depth of spiral 0.5 to 1 $\mu$ and constant. Cell appears to consist of a spirally wound axial filament. Terminal spiral filament present and easily seen in cultures. Multiplication transverse or possibly longitudinal also. Anaerobic.

*Disease Produced.*—The cause of syphilis in man (Figs. 277 and 278). Syphilis is acquired almost entirely by sexual contact. In an acquired infection, disease first manifests itself as a primary lesion. This starts as a papule at the site of infection, increases in size, and ulcerates. The ulcer is generally referred to as a chancre. This is followed by constitutional symptoms and lesions of the skin and mucous membranes. These secondary lesions eventually heal and may reappear during the first 5 years after infection. Later manifestations may include disturbances of the cardiovascular and central nervous systems.

*Diagnosis.*—Disease in primary stage may be diagnosed by examining the serous exudate from a chancre under dark-ground illumination. Presence of spirochaetes indicates a syphilitic infection.

Disease in later stages may be diagnosed by serological reactions (see page 612).

*Source of Infection.*—Discharges from lesions of the skin and mucous membranes, from blood of infected individuals, only rarely from articles freshly soiled with discharges.

*Mode of Transmission.*—Direct personal contact with syphilitic individual, chiefly by sexual intercourse, occasionally by kissing; by dental instruments; only rarely

through articles freshly soiled with discharges. Transmitted by syphilitic mother to offspring (congenital syphilis).

*Immunity.*—Recovery is said to confer some immunity, although reinfections do occur.

*Prevention and Control.*—Disease confirmed by dark-ground illumination and by serologic reactions. Treatment should be instituted immediately after infection.

Patients in communicable stage should be isolated and not permitted to engage in

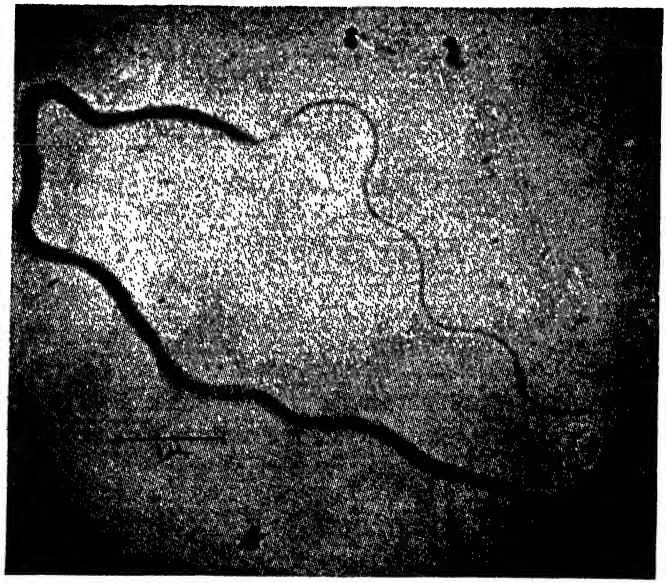

FIG. 278.—Electron micrograph of *Treponema pallidum.* ×14,500. The cell wall is continued beyond the protoplasm of the cell as an end filament. Flagella are seen arising from the side of the cell. (*After Mudd, Polevitzky, and Anderson.*)

any occupation in which he or she may infect others. Sexual intercourse should be prohibited until infected individual is rendered noncommunicable by treatment.

Disinfection of discharges from open lesions and of articles soiled by such discharges.

Education in personal and sexual hygiene.

For more information on the pathogenic bacteria, consult American Public Health Association (1945), Gay (1935), Holmes (1944), Jordan and Burrows (1946), Zinsser and Bayne-Jones (1939), and Topley and Wilson (1946).

## VIRUS DISEASES OF MAN

The etiological agents of disease discussed in the preceding section can be seen with the aid of a light microscope. The viruses, with few exceptions, are invisible under the highest power of the ordinary type of microscope. Their presence can be demonstrated by examination under an electron microscope or by the inoculation of the virus-containing material into a susceptible animal or plant. They pass through filters capable of retaining bacteria. Viruses are found only in cellular material; like-

wise multiplication occurs only in the presence of living cells. In neutral or slightly alkaline suspensions, most viruses show a negative electrical charge. In this respect they behave like bacteria.

**Nature of Viruses.**—The exact nature of viruses is not clearly understood. Some believe that viruses are living organisms of infinitesimal size and develop by multiplication of preexisting forms. In support of this hypothesis is the work of Paschen (1906) who demonstrated the presence of elementary bodies in material from smallpox and vaccinia that he believed to be the etiological agent of the disease. Since that time, elementary bodies have been shown to be present in other virus diseases.

Only a few viruses have been analyzed chemically. Hoagland, Smadel, and Rivers (1940), Hoagland, Ward, Smadel, and Rivers (1942), and Smadel and Hoagland (1942) made chemical analysis on purified elementary bodies (Paschen bodies) of vaccinia and found that they contained organic phosphorus, nitrogen, $\alpha$-amino nitrogen, carbon, lipid (cholesterol, phospholipid, neutral fat), cysteine and reducing sugar after hydrolysis, and the enzymes phosphatase, catalase, and lipase. Approximately 5.6 per cent of the virus particles consisted of desoxyribonucleic acid, which agreed closely with the figure obtained by calculation from the amount of organic phosphorus present in the elementary bodies. From the foregoing, it may be concluded that Paschen bodies are probably living organisms, differing from spherical bacterial cells in being smaller and in lacking certain essential enzymes. The fact that multiplication of the virus particles occurs may be explained on the assumption that the Paschen bodies utilize the necessary enzymes present in the cells of the host. This may possibly explain the specificity of viruses in that they attack only those cells which have the necessary enzymes or other specific mechanisms.

Work on the viruses of influenza, rabbit papilloma, and equine encephalomyelitis indicates that they are predominantly nucleoprotein in composition.

Others believe that viruses are not living organisms but autocatalytic bodies capable of producing an injurious action in a susceptible animal or plant cell with the result that the cell is stimulated to produce more virus of the same kind. The cell is eventually destroyed after which the autocatalytic agent spreads to other cells of the host. Supporting this belief is the work of Stanley (1935) who isolated the virus of tobacco-mosaic disease from infected tobacco and cucumber plants in the form of fine needle-shaped rods or crystals in a high state of purity which were capable of reproducing the disease in healthy plants (see page 630). This preparation was found to consist entirely of nucleoprotein. Repeated recrystallizations failed to change the infectivity of the crystals. The protein could be denatured or changed in other ways resulting in a partial or complete loss of infectivity. Since that time, other workers have isolated crystalline

viruses from a number of diseased plants and have confirmed the findings of Stanley. The virus crystals have been shown to be composed of pure nucleoprotein molecules.

Perhaps both theories are correct. Available evidence at present seems to point to the fact that the animal viruses are living whereas some, or possibly all, of the plant viruses are nonliving, crystalline, protein molecules. All the crystalline viruses have been obtained from plant cells. The successful crystallization of a virus from an animal cell has not yet been realized.

**Size of Viruses.**—Viruses show considerable variation in size, ranging from 10 to 300 *mu* (Fig. 279). Some are as large as the smallest bacteria; others approach the dimensions of the smallest protein molecules. Most of the viruses responsible for animal diseases are more or less spherical in form. Available evidence seems to point to the fact that the virus of poliomyelitis assumes the shape of slender rods.

It is difficult to understand how a virus particle consisting of only one molecule can be considered to be a living organism capable of multiplication. It is equally difficult to understand how a particle of psittacosis virus can be so large as to be visible microscopically and yet be considered a protein molecule or an autocatalytic, enzyme-like molecule manufactured by a living cell.

**Cultivation of Viruses.**—Living cells of plant or animal tissue are required for the successful propagation of plant or animal viruses. Although viruses multiply only in the presence of living cells, it does not follow that all viruses can be cultivated in this manner. Many viruses, especially those of animal origin, have not been successfully cultivated. Some animal viruses may be cultivated in minced embryo medium, on the chorioallantoic membrane of the developing chick, and in living tissue fragments embedded in plasma (tissue culture). Plant viruses cannot be propagated in animal tissues. They require plant tissues for successful multiplication. Root-tip cultures are usually used for this purpose.

**Resistance of Viruses.**—Viruses are heat-labile and sensitive to the same agents that destroy bacteria but they are, in general, more resistant. The addition of 0.5 per cent phenol to vaccine virus does not destroy its infectivity. Glycerin, in a concentration of 50 per cent, gradually destroys bacteria but has no appreciable effect on viruses. On the other hand, viruses are susceptible to oxidative destruction. This may be largely prevented by adding a suitable reducing agent, such as cysteine, to the virus suspension.

**Immunity in Virus Diseases.**—Recovery from a virus disease usually confers a strong, almost lasting immunity. Second attacks of such virus diseases as smallpox, mumps, measles, poliomyelitis, and chickenpox rarely occur. On the other hand, there are some virus infections in which the immunity developed is of short duration. Some of these are influenza,

COMPARATIVE SIZES OF VIRUSES

| | Molecular weight X $10^{-6}$ (Particle weight X 6.06 X $10^{-17}$) | Diam or length X width in $m\mu$ |
|---|---|---|
| Red blood cells* | 173 000 000 | 7 500 |
| Bacillus prodigiosus* | 173 000 | 750 |
| Rickettsia* | 11 100 | 300 |
| Psittacosis* | 8 500 | 275 |
| Vaccinia* | 4 300 | 225 |
| Myxoma* | 4 300 | |
| Canary pox* | 4 300 | |
| Pleuro-pneumonia organism* | 1 400 | 150 |
| Pseudo rabies | 1 400 | |
| Ectromelia | 1 400 | |
| Herpes simplex | 1 400 | |
| Rabies fixe* | 800 | 125 |
| Borna disease | 800 | |
| Influenza | 400 | 100 |
| Vesicular stomatitis | 400 | |
| Staphylococcus bacteriophage† | 300 | 90 |
| Fowl plague | 300 | |
| $C_{16}$ bacteriophage | 173 | 75 |
| Chicken tumor I* | 142 | 70 |
| Tobacco mosaic* | 35 | 280 X 15 |
| Cucumber mosaics 3 and 4* | 35 | |
| Gene (Muller's est of max. size)* | 33 | 125 X 20 |
| Latent mosaic of potato* | 26 | 430 X 9.8 |
| Rabbit papilloma (Shope)* | 25 | 40 |
| Equine encephalitis | 23 | 38 |
| Megatherium bacteriophage | 23 | |
| Rift valley fever | 11 | 30 |
| Tomato bushy stunt* | 8 | 26 |
| Hemocyanin molecule (Busycon)* | 6.7 | 22 |
| Yellow fever | 4.3 | 22 |
| Tobacco ring spot* | 3.4 | 19 |
| Louping ill | 2.8 | 19 |
| Hemocyanin molecule (Octopus)* | 2.8 | 20 |
| Alfalfa mosaic* | 2.1 | 16.5 |
| Poliomyelitis | 0.7 | 12 |
| Staphylococcus bacteriophage† | 0.4 | 10 |
| Foot-and-mouth disease | 0.4 | |
| Hemoglobin molecule (Horse)* | 0.069 | 15 X 3 |
| Egg albumin molecule* | 0.040 | 9 X 3 |

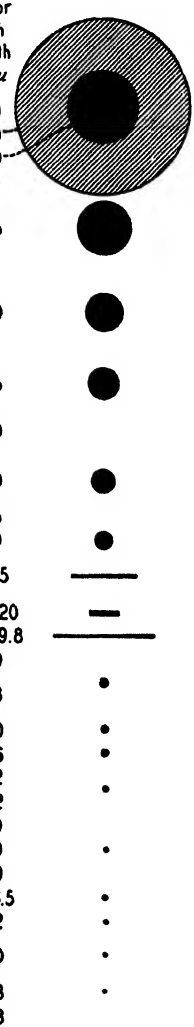

FIG. 279.—Relative sizes of viruses, including bacteriophages, as compared to red blood cells, *Serratia marcescens*, *Rickettsia*, and protein molecules. Particles known to be asymmetric are so indicated and the estimated length and width and the molecular weight in accordance with the asymmetry are given. The asterisk indicates that evidence regarding shape is available. (*After Stanley.*)

common cold, and herpes simplex (cold sores, fever blisters). Various kinds of immune substances have been identified in the circulating blood, including agglutinins, precipitins, complement-fixing, and neutralizing antibodies.

Recovery from some virus diseases does not mean necessarily that the etiological agent is entirely eliminated from the host. The virus may persist in the recovered individual for life. This does not mean that the immune person is capable of spreading the virus because the agent is believed to be stored in certain living cells where it cannot come in contact with circulating antibodies. Since viruses are believed to multiply and carry on their activities only in living cells, they cannot be attacked by circulating antibodies and destroyed. Consequently, if the host cells are not destroyed by the virus, the two can live together without the infecting agent ever coming in contact with the humoral antibodies.

The important virus diseases of man include the following:

Acute anterior poliomyelitis or infantile paralysis.
Common cold.
Epidemic influenza.
Herpes simplex or fever blisters.
Herpes zoster or shingles.
Infectious encephalitis.
Infectious parotitis or mumps.
Psittacosis or parrot fever.
Rabies or hydrophobia.
Rubella or German measles.
Rubeola or measles.
Varicella or chickenpox.
Variola or smallpox.
Verruca or warts.
Yellow fever.

**Smallpox (Variola, Vaccinia, Alastrim).**—Virus capable of passing through most filters, resistant to low temperatures, to 50 per cent glycerol and 0.5 per cent phenol, but sensitive to heat, being destroyed at 55°C. or over. Paschen bodies are believed to be the virus particles causing the disease and may be demonstrated in vesicular fluid (Fig. 280).

*Disease Produced.*—Smallpox is an acute, specific, infectious disease characterized by sudden onset, usually with severe chill, with rapidly rising temperature, followed by an eruption passing through papular, vesicular, and pustular stages. Permanent scars frequently remain. Eruption most abundant and earliest on the face, next on forearms, wrists, and hands, favoring the limbs more than the trunk. Lesions more abundant on shoulders and chest than on loins or abdomen.

Vaccinia and alastrim are milder forms of the disease, presumably caused by the same virus, which has become altered in virulence.

*Source of Infection.*—Lesions of mucous membranes and skin of infected persons.

*Mode of Transmission.*—Contact with diseased persons or by articles soiled with discharges from such persons.

*Immunity.*—Susceptibility to infection is universal but not every individual exposed to virus contracts the disease. Permanent active immunity usually follows recovery from disease.

According to Smadel and Rivers (1942), Shedlovsky and Smadel (1942), and Rivers (1943), vaccinia contains two soluble antigens designated as the *L* and *S* components. The *L* antigen is heat-labile and the *S* antigen is heat-stable. These two antigens occur as a complex consisting of a single protein substance with two serologically active parts, each of which may be degraded independently of the other. This single complex *L–S*

antigen precipitates in equal titers with optimal amounts of *L* and *S* antibodies and is completely removed from solution by absorption with either antibody

Vaccine-immune serum has been shown to contain agglutinating, precipitating, complement-fixing, and neutralizing antibodies.

*Prevention and Control.*—Isolation in screened wards of infected persons. Disinfection of articles soiled with discharges from infected individuals. Thorough cleaning and disinfection of premises.

General vaccination in early childhood. This affords protection for one or several

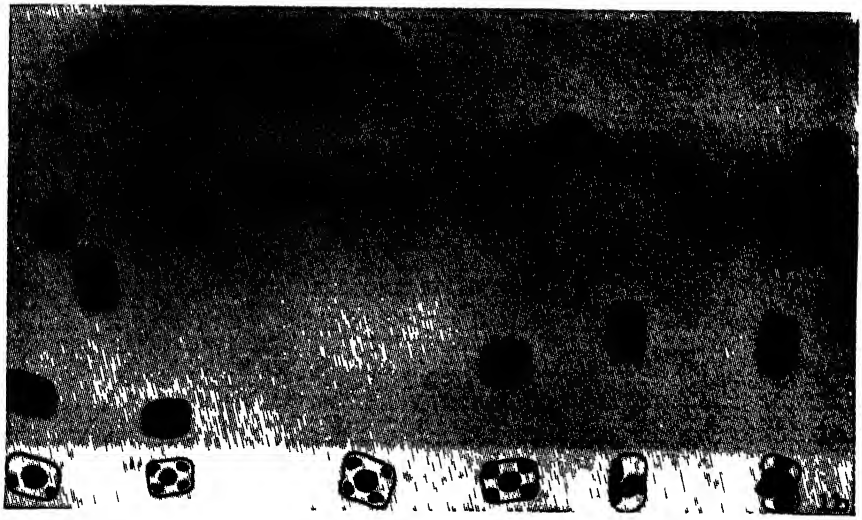

FIG. 280.—Elementary bodies of vaccinia. (*After Smadel and Hoagland.*)

years. Children on entering school should be revaccinated, and the entire population should be so treated when disease appears in severe form.

Vaccine most generally employed is prepared from calf lymph, obtained by rubbing the virus into the scarified abdomen of calves six months old. After 5 days, the scarified areas are scraped under aseptic conditions. The harvested pulp is mixed with twice its weight of water and passed through a sieve. The emulsion is preserved by the addition of glycerin and phenol to give a final concentration of 50 per cent of the former and 0.5 per cent of the latter. The preserved pulp is stored at 10°C.

Another vaccine that has been extensively employed is prepared by growing the virus in a medium composed of minced chick embryo tissue suspended in Tyrode's solution. This is a living tissue medium.

**Epidemic Influenza (La Grippe).**—Virus capable of passing through most filters, resists freezing for about 2 weeks, and retains its potency in 50 per cent glycerin for the same length of time. When dried from the frozen state (lyophilized), the virus retains its potency for at least 6 weeks in the refrigerator. The virus is capable of infecting mice, ferrets, and possibly swine intranasally.

Knight and Stanley (1944) found that the virus was sensitive to strong oxidizing agents such as iodine, salts of heavy metals, Mercurochrome, formaldehyde, and the wetters Phemerol, Roccal, and sodium dodecyl sulfate. On the other hand, the virus was only slightly affected by reducing agents, sulfathiazole, dilute phenol solutions, glucose, ammonium sulfate, calcium chloride, and sodium thiosulfate.

*Disease Produced.*—Epidemic influenza was formerly believed to be due to the organism *Hemophilus influenzae*, but it has now been shown to be caused by a filterable virus. In typical, severe cases, *H. influenzae* is also present, but it is probably of secondary importance (see page 654).

Influenza is an acute infection characterized by sudden onset, fever of 1 to 7 days' duration, catarrh of the respiratory tract (sometimes alimentary tract), pains in the head and muscles, coryza, sore throat, bronchitis, and a tendency to pneumonic complications. The disease produces marked prostration.

Two distinct types of influenza virus, designated as type A and type B, have been identified. Type A is the older and more widely distributed; Type B has usually been found in smaller and more localized outbreaks. Some outbreaks do not appear to be caused by either A or B, which would indicate that at least one more type is involved (Figs. 281 and 282).

*Source of Infection.*—Discharges from nose and throat of infected persons or from articles freshly soiled with discharges from such individuals.

*Mode of Transmission.*—By direct contact with infected persons, by droplet infection, or by articles freshly soiled with discharges of nose and throat of infected individuals.

*Immunity.*—Susceptibility to disease is general, although some have natural immunity. Acquired immunity of only short duration follows recovery from the disease and is effective only against the particular type causing the infection.

*Prevention and Control.*—Isolation of infected persons during acute stage. Disinfection of discharges from nose and throat or articles freshly soiled by such discharges. Patients should be put to bed at the beginning of an attack. During epidemics, overcrowding should be avoided.

For additional information on influenza, see Beveridge, Burnet, and Williams (1944), Bodily, Corey, and Eaton (1943), Burnet (1943), Horsfall, Jr. (1941, 1942), Knight (1944*a*,*b*), Lauffer and Miller (1944), Miller (1944), Miller and Stanley (1944), Schaeffer (1942), Sharp, Taylor, McLean, Jr., Beard, Beard, Feller, and Dingle (1944), Stanley (1944), Taylor, Sharp, McLean, Jr., Beard, Beard, Dingle, and Feller (1944), Williams and Wyckoff (1945).

**Rabies (Hydrophobia, Canine Madness).**—Virus capable of passing through Berkefeld and the coarser Chamberland filters. It is reduced in virulence (attenuated) by drying over KOH, by exposure to 1 per cent phenol, and by heating to temperatures above 45°C. Virus may be preserved in 50 per cent glycerin for some time if kept at 7°C.

*Disease Produced.*—Rabies in an acute encephalitis due to a neurotropic virus acquired from the bite of a rabid animal, usually the dog. Disease characterized by depression, itching at site of primary infection, and fever. Patient becomes uneasy, swallowing difficult, salivation marked, followed by attacks of delirium. Paralysis of the face muscles, eyes, and tongue appears, gradually spreading to the trunk and limbs.

*Diagnosis.*—Cause of death may be determined by demonstrating the presence of Negri bodies in the nerve cells of brain or cord, or by emulsifying a small portion of the hippocampus in sterile saline and inoculating subdurally into guinea pigs or rabbits. Death occurs in about 16 days and Negri bodies can be demonstrated in the brain tissue.

*Source of Infection.*—Infected animals, usually dogs.

*Mode of Transmission.*—The virus is present in the saliva and is usually transmitted to man by the bite of a rabid animal. Infections have occurred from the licking of normal skin by rabid dogs.

Incubation period usually from 2 to 6 weeks, sometimes as long as 6 months, depending upon site of wound in relation to richness of nerve supply and distance of nerve path to brain.

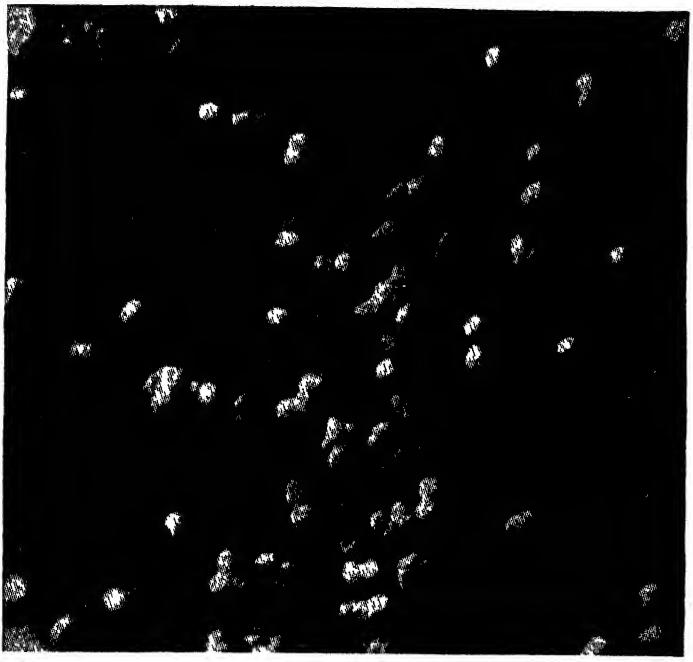

FIG. 281.—Electron micrograph of a suspension of purified PR–8 (Type A) influenza virus after it had been shadowed by the oblique deposition upon it of a thin layer of chromium. (*After Williams and Wyckoff.*)

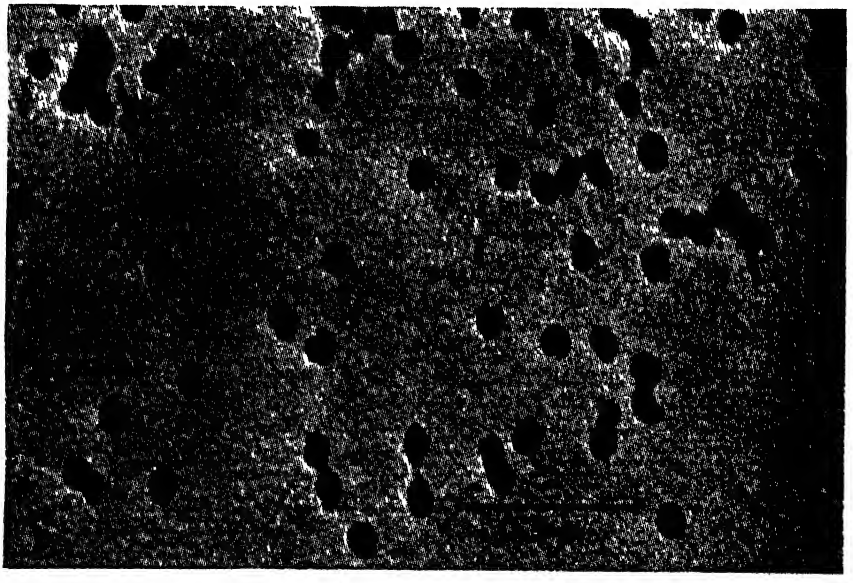

FIG. 282.—Electron micrograph of influenza virus B (Lee strain). (*After Sharp et al.*)

*Immunity.*—Susceptibility to disease is general. Active artificial immunity can be leveloped by use of a vaccine. The best known vaccine is that developed by Pasteur.

Vaccine is prepared by inoculating rabbits with street virus subdurally in series until the virulence is so stepped up that death occurs in 6 days. Since further increase in virulence is not possible, the virus is referred to as "fixed virus." As soon as the animal is dead, it is washed in a disinfectant solution and autopsied. The spinal cord is removed aseptically and suspended by a sterile thread within a large bottle the bottom of which is covered with pieces of potassium hydroxide as a drying agent. The bottle is kept in a dark place at a temperature of about 25°C. The virus is gradually attenuated by drying, the degree of attenuation varying directly with time.

For the first injection, it is customary to use cord that has been dried for 14 days (14-day cord); for the second injection, 13-day cord; for the third injection, 12-day cord; etc., until 3-day cord is used. From then on, the same preparation is used until a total of 21 consecutive injections has been given. For each injection about 1 cm. of cord is emulsified in 3 cc. salt solution and 2½ cc. inoculated subcutaneously. This treatment is generally sufficient to produce an active artificial immunity.

*Prevention and Control.*—A dog that has bitten a person should be isolated and observed for a proper period of time. If rabies is suspected, the animal should be killed and examined for the presence of Negri bodies in the brain. If examination is positive, a person bitten should be given antirabic vaccination immediately before symptoms appear.

The use of the vaccine has been instrumental in greatly reducing the mortality rate in rabies. In persons so treated, the death rate has dropped to 1 per cent.

**Psittacosis (Parrot Fever).**—Virus particles relatively large, approaching the size of the smallest bacteria. Particles visible under a light microscope. Virus filterable through membranes that retain bacteria. Virus stains Gram-negatively, produces lesions that resemble bacterial infections more than virus diseases, and is susceptible to bacteriostatic agents.

*Disease Produced.*—Psittacosis is a contagious disease of parrots resembling influenza and is transmissible to man. In man, the disease is characterized by high fever, headache, backache, thirst, changes in the tongue and pharynx, stupor or depression, fast pulse, diarrhea or constipation, swelling of the spleen, symptoms of an atypical pneumonia or of a typhoidal state, with *râles* and cardiac dullness, and the usual external signs of catarrh and pneumonia may be absent. Sputum light yellow in color and of extreme viscosity. White blood count is normal or slightly increased early, with leucopenia later.

*Diagnosis.*—Diagnosis made by inoculating sputum into white mice. The animals die usually within 5 to 14 days and impression preparations from the spleen show the presence of LCL (Leventhal-Coles-Lillie) elementary bodies. Serum contains complement-fixing antibodies.

Williams (1944) cultivated psittacosis virus on the chorioallantoic membrane of the developing chick and reported that the method was as sensitive as mouse intranasal inoculation for the detection of minimal amounts of virus. Large quantities of virus could be obtained by the method.

*Source of Infection.*—Canaries, pigeons, parrots, parakeets, love birds, and other birds. Birds that appear to be well occasionally transmit the infection.

*Mode of Transmission.*—Virus present in blood, saliva, and feces of infected birds. Disease transmitted by contact with such birds or their recent surroundings.

*Immunity.*—All ages susceptible. Disease more severe in higher age groups. Recovery from disease confers immunity.

*Prevention and Control.*—Disease may be diagnosed by presence of virus in saliva and blood during the first week of the infection. Serum contains complement-fixing antibodies. Infected persons should be isolated during the febrile and acute clinical stage. Masks should be worn when handling patients with coughs. Disinfection of discharges and articles soiled with such discharges. Infected birds should be disposed of by burning. Buildings in which infected birds were housed should be thoroughly cleaned and disinfected.

Strict regulation of traffic in birds of the parrot family. Education of public in dangers of birds of the parrot family particularly of those freshly imported.

**Acute Anterior Poliomyelitis (Infantile Paralysis).**—The virus is one of the smallest known, measuring 12 *mu* in diameter. It resists desiccation, freezing, 50 per cent glycerin, and exposure to 5 per cent phenol. It is thermolabile, being destroyed at a temperature of 45°C. or higher.

*Disease Produced.*—Poliomyelitis is an acute, systemic, infectious disease that involves the central nervous system. The disease is characterized by fever, headache, vomiting and constipation, drowsiness alternating with irritability, stiffness of neck and spine, tremor, and exaggeration of muscular reflexes. Later, paralysis may set in and may cause death in a few hours. There is a marked tendency for paralysis to improve after it has reached its height.

*Source of Infection.*—Nose and throat discharges of infected person; also from those not suffering from clinically recognized poliomyelitis. Virus present in bowel discharges.

*Mode of Transmission.*—It is believed that the virus enters by way of nose and mouth either from a carrier or from a person with a subclinical infection. There is some evidence that disease may be spread by water supplies and by swimming pools.

*Immunity.*—Children are generally more susceptible than adults. Active immunity produced after recovery from the disease. Second attacks have occurred only rarely.

*Prevention and Control.*—Infected individuals should be isolated for 2 weeks after symptoms appear. Disinfection of nose, throat, and bowel discharges, and articles soiled with such discharges.

All children with fever should be isolated in bed pending outcome of diagnosis. Protection of children from contact with others during an epidemic. Avoidance of nose and throat operations on children during an epidemic. During period when disease is prevalent, crowds should be avoided as far as possible.

For more information, see Carlson, Ridenour, and McKhann (1943) and Carlson and McKhann (1943).

**Varicella (Chickenpox).**—This is an acute, extremely contagious virus disease characterized by fever, mild constitutional symptoms, a cutaneous eruption involving the superficial layers of the skin lasting 3 to 4 days, leaving a granular scab. Vesicles tend to be more abundant on the covered parts of the body. Lesions also appear on scalp and mucous membranes of upper respiratory tract.

*Source of Infection.*—Virus present in lesions of skin and upper respiratory tract. Disease may be communicable before the eruption is in evidence.

*Mode of Transmission.*—From person to person by direct contact; by articles freshly soiled with discharges from infected persons.

*Immunity.*—Susceptibility to disease universal. About 70 per cent of persons have had the disease by the time they are fifteen years old. Recovery from disease usually confers permanent active immunity.

*Prevention and Control.*—Isolation of infected persons. Disinfection of discharges from nose and throat and articles soiled by such discharges.

**Infectious Parotitis (Mumps).**—Habel (1945) cultivated the virus in the yolk sac, the amniotic sac, and the allantoic sac of the developing chick embryo, and found the particles to be greater than 340 *mu* in diameter. If true, this is one of the largest viruses known. The virus was quite susceptible to the inactivating effects of heat, formaldehyde, ether, and ultraviolet light.

*Disease Produced.*—Mumps is an acute, specific, contagious disease characterized by fever and inflammation of the salivary glands. The parotid, submaxillary, and sublingual glands may be infected, although the parotid is most frequently involved. Sometimes the ovaries and testes may be attacked.

*Source of Infection.*—Secretions of the mouth and possibly the nose.

*Mode of Transmission.*—By direct contact with infected persons or by articles freshly soiled with discharges from mouth and nose of such individuals.

*Immunity.*—Susceptibility to disease is general. Recovery from infection usually confers permanent active immunity. Complement-fixing antibodies regularly appear, or increase in concentration, in the sera of persons during an attack of mumps or during convalescence.

For additional reading on immunity in mumps, see Enders, Kane, Cohen, and Levens (1945), Enders, Cohen, and Kane (1945), and Kane and Enders (1945).

**Common Cold.**—This is an acute, highly communicable catarrhal infection of the nose, throat, larynx, sinuses, trachea, and larger bronchi, and lasting usually from 2 to 7 days. The etiologic agent is one or more viruses, although at one time numerous bacteria were believed to be the cause of the infection.

*Source of Infection.*—Discharges from nose and throat of infected persons or from articles freshly soiled with discharges from such persons.

*Mode of Transmission.*—Usually directly by droplets of infected saliva sprayed into the air during coughing, sneezing, and talking, or indirectly from articles freshly soiled with such discharges.

*Immunity.*—Susceptibility to disease is general. Temporary active immunity of approximately 1 month follows recovery from the disease.

*Prevention and Control.*—Infected persons should avoid contact with others. Rest in bed during the acute stage is advisable. Nasal and mouth discharges should be kept away from others and disposed of, preferably by burning.

**Rubeola (Measles).**—This is a specific, highly contagious disease, characterized by fever, catarrhal symptoms of the eyes, nose, and throat, an early eruption of the mouth, a cutaneous rash, and a desquamation during convalescence.

*Source of Infection.*—Secretions of nose and throat of infected persons.

*Mode of Transmission.*—Directly from person to person; by droplets of infected saliva sprayed into the air during coughing, sneezing, and talking; by articles freshly soiled with discharges of an infected individual.

Measles is one of the most easily transmitted of the communicable diseases.

*Immunity.*—Susceptibility to disease is general. Disease occurs most commonly in children between five and fourteen years of age. Permanent acquired immunity usually follows recovery from disease.

*Prevention and Control.*—Isolation of infected persons during period of communicability. Disinfection of articles soiled with fresh discharges from nose and throat of infected persons.

Passive immunity may be transferred to healthy individuals before symptoms of measles appear, by the injection of convalescent serum or serum from a person who has recovered from the disease. Such passive immunity may persist for about 4 weeks. During an epidemic, convalescent serum may either prevent the disease or modify the severity of the attack. In the latter instance, a mild case of measles is usually sufficient to produce a lasting immunity.

**Rubella (German Measles).**—This is a specific, mild, virus infection characterized by fever, a cutaneous eruption sometimes resembling that of measles, which usually appears without other symptoms but is almost always accompanied by enlargement of the postauricular, suboccipital, and cervical lymph nodes.

*Source of Infection.*—Secretions of mouth and nose.

*Mode of Transmission.*—Directly from person to person; by droplets of infected saliva sprayed into the air during coughing, sneezing, and talking; by articles freshly soiled with discharges of an infected individual.

*Immunity.*—Susceptibility to disease is general. Permanent acquired immunity usually follows recovery from disease. Disease occurs most commonly in children. Disease more prevalent in adults than measles.

**Encephalitis lethargica (Infectious Encephalitis).**—A disease of the central nervous system characterized by mild fever, inflammation, lethargy, paralysis of the cranial nerves, and in some cases spinal and peripheral nerve involvement. Fever occurs during acute stage or on each exacerbation.

Four types of the disease have occurred in this country: (1) the Vienna type or type A, (2) the St. Louis type, (3) the eastern equine type, and (4) the western equine type. The Vienna type is the most chronic and variable in course.

*Source of Infection.*—Unknown. Birds are believed to be a reservoir of the equine types.

*Mode of Transmission.*—Mosquitoes are believed to be the most important natural vectors.

*Immunity.*—Permanent active immunity is believed to follow recovery from the disease.

*Prevention and Control.*—Isolation of patients and protection from mosquitoes during febrile stage. Control of species of mosquito responsible for disease transmission.

**Yellow Fever.**—This is an acute, specific virus disease characterized by sudden onset, fever, chills, prostration, headache, muscular pain, some destruction of red blood cells, congestion of the mucous membranes, black vomit, a mild albuminuria, and jaundice. Leucopenia is the rule. The disease is of short duration.

*Source of Infection.*—Blood of infected persons, monkeys, and probably other wild animals.

*Mode of Transmission.*—By the bite of *Aëdes aegypti*, and other species of mosquitoes of which *Hemagogus* appears to be the most important in South America.

*Immunity.*—Susceptibility to disease is general. Permanent acquired immunity follows recovery from the disease. Active immunity of about 4 years' duration may be developed within 10 days by inoculation of virus attenuated by growth in living tissue in vitro (tissue culture).

*Prevention and Control.*—Isolation of infected persons during the first 4 days of fever, and protection from mosquitoes. Control of breeding places of the mosquito *A. aegypti*. Immunization of exposed population by the use of attenuated virus is the only feasible method for the control of yellow fever.

For additional information on viruses, see American Public Health Association (1945), Bawden (1945), Burnet (1945), Harvard School of Public Health Symposium (1940), Horsfall, Jr. (1943), Mudd (1944), Mudd and Anderson (1944), Rous (1943), Schaeffer (1942), and Shope (1943).

## RICKETTSIAL DISEASES OF MAN

The rickettsial diseases of man may be defined as specific infections transmitted by the bite of arthropods and characterized by continued

fever, accompanied by a rash. The pathological lesions occur chiefly in the blood vessels, being due to the presence of the organisms and not to their toxins.

**Organisms.**—The rickettsial organisms are so named in honor of Howard Taylor Ricketts who was the first to give a description of the organisms in connection with his studies on Rocky Mountain spotted fever and later on typhus fever. He succeeded in isolating from the blood of typhus fever patients very short bacillus-like rods measuring about 0.3 $\mu$ in diameter and 2 $\mu$ or less in length. The organisms were stained readily by Giemsa's stain and possessed a faintly stained bar through the middle, giving each organism the appearance of a diplobacillus. The organisms were nonmotile. Rickett's observations were later confirmed by da Rocha-Lima (1916)

Following the discovery of Ricketts, Hegler and Prowazek (1913) and others reported the presence of similar organisms in the blood of typhus fever patients and in lice that had fed on infected persons.

The rickettsias are Gram-negative, coccoid or rod-shaped organisms found typically in arthropods. The species pathogenic for man occur intracellularly in the tissues of their animal and arthropod hosts. The organisms have not been grown on the usual laboratory media. However, they can be cultivated in the various tissue culture media. From the standpoint of size, the rickettsias occupy a position intermediate between the bacteria and the viruses. With the exception of one pathogenic species, they do not pass through filters that retain bacteria.

The most important species-producing rickettsial diseases of man are (1) *Rickettsia prowazeki*, the cause of typhus fever; (2) *Dermacentroxenus rickettsi*, the cause of Rocky Mountain spotted fever; (3) *R. tsutsugamushi*, the etiological agent of tsutsugamushi disease; (4) *R. wolhynica*, the cause of trench fever; and (5) *R. burneti*, the causative organism of Q-fever.

**Typhus Fever.**—This disease is generally differentiated into the European or epidemic type (louse-borne), and the murine or endemic type (flea-borne).

*Epidemic or Louse-borne Typhus.*—The etiological agent of this type is *R. prowazeki*. The onset of the disease is often sudden and characterized by headache, chills, fever, and general pains, a macular eruption on the 5th or 6th day, and toxemia. The fever falls usually by rapid lysis after about 14 days. The organisms may be found in the blood vessels of the skin, kidneys, muscles, brain, and testes. Case mortality varies from 10 to 40 per cent, depending upon epidemics and age.

*Source of Infection.*—Infected persons.

*Mode of Transmission.*—Organism is transmitted from man to man by the louse *Pediculus humanus* which has fed on an infected person. Organisms present in feces of louse. Cells inoculated by scratching louse feces into the wound produced by the bite, or into other superficial breaks in the skin. Dirty clothing contaminated with louse feces may disseminate the organism into the air from where it may reach the respiratory tract. The incubation period is from 6 to 15 days, most often 12 days. Disease is prevalent among people living under crowded and unhygienic conditions.

*Immunity.*—Susceptibility to disease is general. Acquired immunity follows recovery from disease, but it is not always permanent.

Serum from epidemic typhus patients agglutinates certain strains of *Proteus* designated *Proteus* $OX_{19}$ and $OX_2$. This test is referred to as the Weil-Felix reaction. The former strain is more commonly agglutinated and is the strain customarily employed. The reaction usually becomes positive during the second week of the disease, reaches its height about the time of convalescence, then disappears rather rapidly.

*Prevention and Control.*—Delousing of infected persons and isolation from others. Use of insecticides on clothing and bedding of patient and special treatment of hair for louse eggs. Use of vaccine for immunization. Vaccine prepared by growing rickettsias in the yolk sac of the developing chick embryo. The suspension after purification is inactivated by formalin. The vaccine is generally given in three injections and confers considerable protection. Reimmunization should be practiced every few months where danger of the disease is present. Vaccination reduces risk of infection, modifies the course of the disease, and lowers mortality rate.

Incidence may be greatly reduced by improving living conditions, by more frequent bathing, and by reduction in louse infestation.

*Endemic or Flea-borne Typhus.*—The etiological agent of this type is *R. prowazeki* var. *mooseri*. The clinical features of this type are identical with those of the epidemic or louse-borne type except that the symptoms are generally much less severe. The rash in endemic typhus usually does not appear before the 5th day and may comprise only a few macules, which tend to disappear in a day or so. The death rate for all ages is about 2 per cent.

*Source of Infection.*—Infected rats and other rodents, especially *Rattus norvegicus*.

*Mode of Transmission.*—Organism transmitted from rodent to man by the bite and infected feces of fleas, commonly *Xenopsylla cheopis*. The incubation period is from 6 to 14 days, most often 12 days.

*Immunity.*—Susceptibility to disease is general. Acquired immunity follows recovery from disease, but it is not always permanent.

Serum from endemic typhus patients also agglutinates certain strains of *Proteus* designated $OX_{19}$ and $OX_2$. The former strain is more commonly agglutinated and is the strain customarily employed.

*Prevention and Control.*—Control of rat population by trapping, poisoning, and ratproofing. Trapping and poisoning must be continuous to be of any practical value. Ratproofing is the only method that may be considered of permanent value.

Vaccine prepared against endemic typhus in the same manner as for the epidemic type has produced good protection in animals but has not been tried in humans.

For more information on typhus fever, see Donovick and Wyckoff (1945), Lebrón and Otero (1943), and Weiss (1943).

**Rocky Mountain Spotted Fever (Tick-borne).**—The etiological agent is *Rickettsia rickettsi*. Rocky Mountain spotted fever is a specific infectious disease characterized by sudden onset, fever, headache, pains in muscles and joints, chills, a macular eruption, usually first on the extremities and rapidly spreading over most of the body, irritability and hyperesthesia of the skin, an enlarged spleen, and catarrh of the respiratory tract. Patients show a history of either a tick bite or exposure to ticks. The death rate varies with age and locality; in this country, for all ages, it is about 20 per cent.

*Source of Infection.*—Rickettsias found in infected ticks. In the Eastern and Southern United States, the common vector is the dog tick *Dermacentor variabilis;* in the Northwestern United States it is the wood tick *D. andersoni;* in the Southwestern United States, it may occasionally be the lone-star tick *Amblyomma americanum*. In Brazil, the common vector is *A. cajennense*. The infection is passed from generation to generation in ticks. It is probably maintained in ticks by larvae feeding upon susceptible wild rodents.

*Mode of Transmission.*—Disease transmitted by bite of infected tick or contact with tick material, such as blood or feces on the unbroken skin. The organisms invade all the tissues of the tick. The incubation period is from 3 to 10 days.

*Immunity.*—Susceptibility to disease is general. Acquired immunity follows recovery from disease, but it is not always permanent.

White blood cell count usually shows a slight increase over the normal. Serum from spotted fever patients agglutinates *Proteus* $OX_{19}$ and $OX_2$. Since typhus fever serum also agglutinates the same organisms, it is not possible to distinguish between the two diseases by this reaction. As is true in typhus fever infections, agglutination reaction usually becomes positive toward the end of the second week, reaches its peak during convalescence, then disappears rather rapidly.

*Prevention and Control.*—All ticks on patients should be removed and destroyed. Avoidance of areas known to be infested with ticks. Removal of ticks from body as promptly as possible without crushing. Destruction of ticks in infested areas by clearing and burning vegetation; by destruction of small animals known to be infested with ticks.

Two types of vaccines are employed. One is made from infected ticks and the other from rickettsias grown in the yolk sac of the developing chick embryo. The former is prepared by emulsifying infected ticks in a 0.5 per cent solution of phenol. The latter is prepared by the same procedure as used in the preparation of typhus fever vaccine (see page 687). The two vaccines are of comparable value in prevention. Vaccination lessens the chances of infection and lowers the mortality rate. Protection is good for 1 to 2 years. Vaccine is of no value after infection is once acquired.

**Tsutsugamushi Disease.**—The etiological agent is *Rickettsia tsutsugamushi* (*R. nipponica, R. orientalis*). This is an acute, specific infection of man prevalent in Japan and characterized by sudden onset, fever, malaise, chills, and headache. Primary sores develop at the site of the insect bite, caused by the secretions of the insect and accompanied by adenitis of the regional lymph nodes. A skin eruption appears on the trunk, may spread to the arms and legs, and ordinarily disappears after several days. The spleen and liver are congested, and there may be cloudy swelling in the parenchymatous organs, and cellular necrosis. Convalescence usually occurs in 2 to 3 weeks.

*Source of Infection.*—Infected larval mites of *Trombicula akamushi* and other related species. The infection is passed from generation to generation in mites and maintained by feeding upon diseased wild rodents, especially mice and rats.

*Mode of Transmission.*—Disease transmitted to man from a reservoir probably in field mice and other rodents by the larval form of the mite *T. akamushi*. The larvae contract the infection by feeding on infected rodents.

*Immunity.*—Susceptibility to disease is general. Acquired immunity follows recovery from disease, but it is not always permanent.

Serum from patient usually agglutinates *Proteus OXK* but not the $OX_{19}$ or $OX_2$ strains. The agglutination reaction usually becomes positive toward the end of the second week, reaches its peak during convalescence, then disappears rather rapidly.

*Prevention and Control.*—Wearing of miteproof clothing has been recommended in areas infested with mites. Grass and scrub on localities for camp sites should be cut level with the ground and burned. The camp area should also be burned preferably with a flame thrower. Cots should be used to keep bedding from contact with the ground. Sleeping on ground should be avoided. Body should be kept clean by thorough soaping and scrubbing of the skin.

No satisfactory vaccine has been developed against tsutsugamushi disease.

For additional information on the rickettsial diseases, see American Public Health Association (1945), Cox (1938), Dyer (1944), Harvard School of Public Health Symposium (1940), Parker and Steinhaus (1943), Pinkerton (1942), and Plotz, Wertman, and Bennett (1946).

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# CHAPTER XXVII

## HISTORY OF BACTERIOLOGY

The history of disease dates back to biblical times, but it has been comparatively recently that bacteria were first seen and recognized as the causative agents of infections.

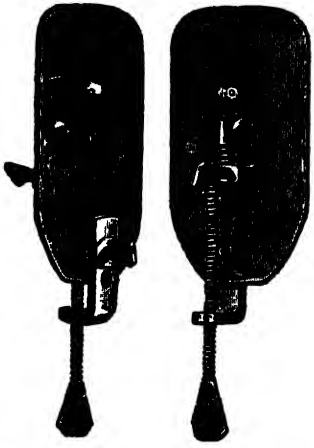

FIG. 283.—One of van Leeuwenhoek's microscopes (ante 1673). (*From "Origin and Development of the Microscope," courtesy of Royal Microscopic Society, London.*)

Benedetti (1493) believed that disease was contracted by touch and that the causative agent was taken up by various articles used by the patient. From these observations, he emphasized the necessity of purifying the clothes.

Fracastoro (1483–1553) postulated the existence of seeds of disease, which he named *seminaria prima.* These seeds were capable of being disseminated from person to person. He believed that the seeds were transmissible (1) by direct contact, (2) by fomites, and (3) at a distance. The term "fomes" (plural, fomites) may be defined as any substance such as clothing, capable of absorbing, retaining, and transporting infectious agents. The seeds of infection were believed by him to invade and multiply until the entire mass was involved. Fracastoro accurately described the great epidemics of typhus fever, plague, rabies, syphilis, and foot-and-mouth disease which occurred in Italy from 1505 to 1528. It is not known if he considered the seeds of infection as living or dead agents.

### LIVING ORGANISMS AS CAUSE OF DISEASE

Varro (116–26 B.C.), Columella (about 60 B.C.) and others, suggested that disease might be caused by living microscopic organisms. The theoretical deductions of Fracastoro on the existence of seeds of disease led to renewed speculation on the nature of the infectious agents.

Kircher (1602–1680) believed that infections were produced by living organisms of microscopic dimensions. Lange (1619–1662) supported the views of Kircher. He firmly believed in the doctrine of *Pathologia animata,* that disease was produced by the entry and growth of microscopic living agents into the body.

During the same period, Marten (1720) expressed his views concerning the etiological agent of tuberculosis, which coincided exactly with the ideas held by present-day investigators. His work may be said to have been a couple of centuries ahead of its time. Since he could not offer any support for his statements, many doubted his views. Plenciz (1762) was perhaps the first to suggest that the seeds of contagion were air-borne and lie dormant for a time before giving rise to innumerable organisms.

FIG. 284.—van Leeuwenhoek examining a specimen with one of his microscopes. Note the size of the microscope as compared to present-day instruments. (*Courtesy Lambert Pharmacal Co.*)

**Antony van Leeuwenhoek.**—The discovery of living microscopic organisms is generally accredited to the Dutch microscopist Antony van Leeuwenhoek (about 1672). He was not a trained scientist but a skilled craftsman in glass blowing, metalworking, lens grinding, etc. Leeuwenhoek amused himself by grinding lenses and making his own microscopes, which were nothing more than simple magnifiying glasses. The magnifying powers of some 400 microscopes that he made ranged from 40 to 300 diameters. A sketch of one of his microscopes is shown in Fig. 283. Leeuwenhoek examined rain water, well water, infusions of peppercorns, tartar scraped from teeth, throat washings, etc. His lenses are not to be compared to the modern high-power objectives, but the pictures that he

sketched in his letters to the Royal Society of London leave no doubt that he actually saw living microscopic organisms (Fig. 285).

**Compound Microscopes.**—Although van Leeuwenhoek is generally referred to as the first bacteriologist and protozoologist, he was not the first individual to make or use a microscope. Compound microscopes were in use many years before his epoch-making observations. Pieter Harting (1866) believed that the compound microscope was first invented by Zacharias Janssen (about 1590).

## FERMENTATION

Industrial fermentations such as occur in the preparation of wines, bread, vinegar, beer, etc., were widely practiced by the ancients, but they

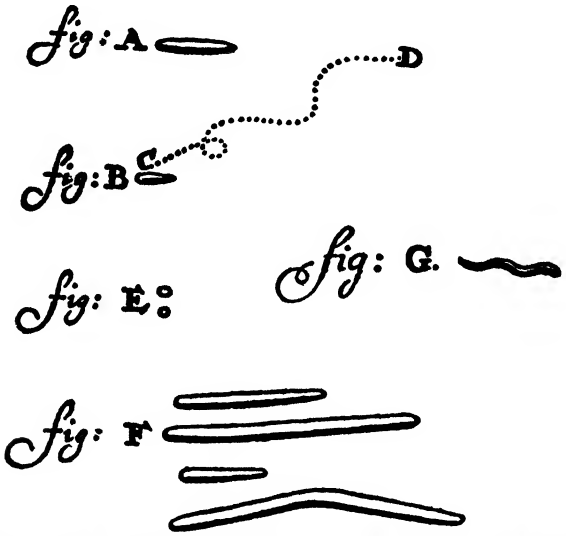


FIG. 285.—Leeuwenhoek's drawings of bacteria. These are the oldest known figures of bacteria.

were little understood. It was not until the nineteenth century that their mechanisms became known. The knowledge gained played a very important role in the founding of the science of bacteriology.

Gay-Lussac (1810) introduced some grapes into a bell jar standing over mercury. He filled the jar several times with hydrogen to remove any oxygen present. Then he ruptured the grapes by means of an iron rod and noted the effect. For a period of 25 days, fermentation failed to occur. However, when oxygen was readmitted into the jar, fermentation soon occurred. He showed that the oxygen soon disappeared and was replaced by carbon dioxide. Gay-Lussac concluded that oxygen was necessary to start fermentation but, once started, the gas was no longer required.

During the early part of the nineteenth century, the yeast plant was discovered and offered a new method of approach to the phenomenon of fermentation. Cagniard-Latour (1836) was probably the first to express the belief that yeast was a living microscopic plant. The following year, he stated that fermentation was probably the result of the growth and multiplication of yeast cells in sugar solutions with the production of alcohol and carbon dioxide. He even noted that yeast cells multiplied by a process of budding.

FIG. 286.—Zacharias Janssen, inventor of the compound microscope.

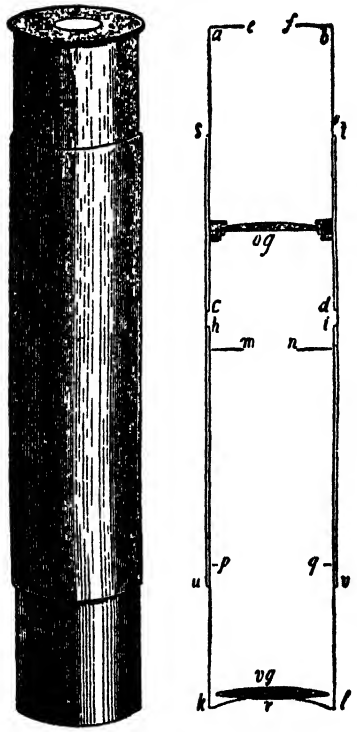


FIG. 287.—The Middelburg microscope, believed to be the invention of Zacharias Janssen (1590). (*From "Origin and Development of the Microscope," courtesy of the Royal Microscopic Society, London.*)

Schwann (1837), working independently of Cagniard-Latour, also discovered the yeast cell and expressed the belief that it was a living organism belonging to the vegetable kingdom. He stated that, in addition to sugar, a nitrogenous compound was necessary for the growth of the organisms. The plants utilized the sugar and the nitrogen compound and eliminated alcohol and carbon dioxide as waste products. His reasons for believing that yeasts caused fermentation were (1) They were always found to be present during the fermentation process, (2) the destruction of the cells resulted in a cessation of the fermentation process, and (3) the substance

that caused fermentation (enzymes) was regenerated and increased in amount during the fermentation. This latter phenomenon is characteristic only of living cells. Schwann was the real founder of the germ theory of fermentation.

Unfortunately, the biological theory of fermentation made slow progress, owing to the fact that the majority of scientists of that period were chemists and physicists. Berzelius (1779–1848) and his pupils Wöhler (1800–1882) and Liebig (1803–1873) were chemists imbued with the belief that all vital phenomena could be explained on purely chemical grounds. These three chemists vigorously opposed the biological theory by ridiculing the teachings of Schwann and Cagniard-Latour in every possible manner. Liebig believed that fermentation and putrefaction were the result of the chemical instability of certain substances that were capable of transferring their instability to other substances. He called these unstable bodies "ferments." The ferments were believed to arise from some constituent of the solution when exposed to air but, after fermentation had started, oxygen was no longer required.

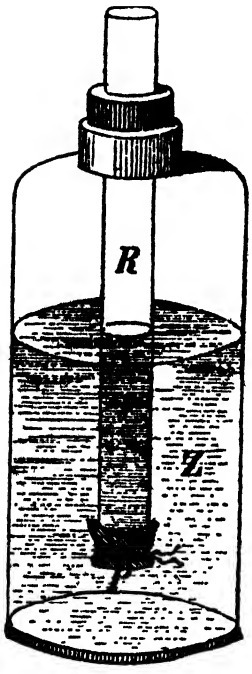

FIG. 288.—Mitscherlich's experiment.

Mitscherlich (1841) showed that yeast cells were not able to pass through the pores of filter paper (Fig. 288). He wrapped a piece of filter paper around one end of a short length of glass tubing and suspended it in a jar of sugar solution. The inside of the tube was filled with the same solution to which had been added a few yeast cells. Fermentation occurred inside of the tube but not in the surrounding solution. Although the experiment was convincing for the biological theory of fermentation, Mitscherlich explained the result by stating that yeast did not act as a vital agent, but merely by its presence. He adhered to the chemical teachings of Berzelius, Wöhler, and Liebig.

**Louis Pasteur.**—The early discoveries in the field of fermentation were made by Pasteur. About 1854, Pasteur interested himself in this field and shortly thereafter published his first report on the fermentation of sugar to lactic acid. He noted a grayish deposit and scum in liquids undergoing the lactic fermentation. When some of the scum and sediment were examined under the microscope, they were found to be composed of masses of minute round bodies. Transfer of some of these bodies to a fresh solution of sugar resulted in a lactic fermentation. This process could be repeated indefinitely. Pasteur stated that the spherical cells were living organisms

and that they were responsible for the fermentation. When sterilized solutions of sugar were exposed to the air, the same type of fermentation was again produced. Examination of the scum and sediment revealed the same globoid bodies. He argued that the yeast cells came from the air because no fermentation occurred or yeast cells appeared in the solution when sterilized solutions were protected from the air.

During the next 20 years, Pasteur devoted most of his time to a study of all types of fermentations. He showed that each type was produced by a specific organism and that in the absence of living cells no fermentation occurred. He described various "diseases of wines" which were caused by the presence of foreign organisms, resulting in abnormal fermentation.

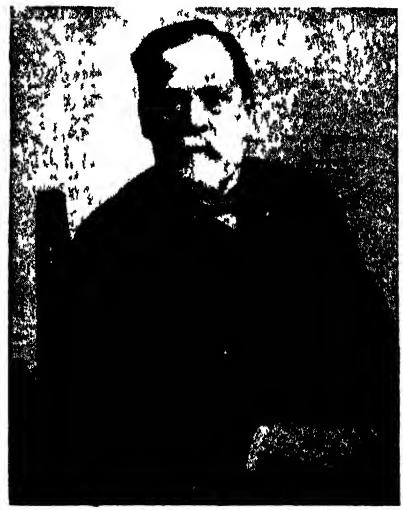

FIG. 289.—Louis Pasteur.

Pasteur devoted the period from 1865 to 1871 to a study of the silkworm disease that was ravaging France at that time. This memorable work was completed after 6 years of intensive research. In 1871, Pasteur again returned to his studies on the various types of fermentations. He expressed the view that all fermentations were anaerobic reactions and that they occurred in the absence of free atmospheric oxygen.

Buchner (1896) subjected yeast cells to great pressure and collected the cell-free juice. He found that this liquid, when added to a solution of sugar, was capable of inducing alcoholic fermentation. This observation brought to an end the controversies of Pasteur vs. Berzelius, Wöhler, and Liebig. It showed that both views were correct in that fermentation took place in the absence of living cells by means of enzymes, but that a living organism was necessary to elaborate the enzymes (ferments).

## SPONTANEOUS GENERATION

The ancients believed that living organisms were created through the combined action of heat, earth, air, and putrefaction. Several of the more ardent supporters of the theory even went so far as to offer recipes for spontaneous generation. The most famous of these was the one advocated by van Helmont (1652). He stated that, if a piece of dirty linen cloth were stuffed into the neck of a jar containing grains of corn, a fermentation ensued and after a lapse of 21 days mice appeared. What surprised van Hel-

mont was that the mice that appeared were always of adult size and fully formed.

For the next 200 years, scientists and philosophers were concerned with the problems of spontaneous generation, some supporting the theory and others vigorously opposing it. Redi (1668) expressed the view that maggots found in putrid meat were the offspring of flies. He covered pieces of meat and fish with fine mesh gauze and placed them under gauze-covered supports. Flies were allowed to come in contact with all parts of the gauze, but no maggots developed. He believed that he actually saw flies deposit their eggs on the gauze. Redi also showed that when maggots were kept in a closed vessel flies developed. Convincing as these experiments were, the idea of spontaneous generation still persisted.

Leeuwenhoek, the discoverer of bacteria, believed that the animalcules that appeared in infusions came from the air, where they were present as seeds and developed into active organisms after they gained entrance into infusions. Joblot (1718) boiled hay infusion and placed equal quantities into two jars. One was covered with parchment while still hot; the other was exposed to the air. The open jar was soon teeming with bacteria; the covered jar remained sterile. When the closed jar was exposed to the air, it soon became heavily contaminated. These observations undoubtedly refuted the doctrine of spontaneous generation, but there were still some who did not regard them as conclusive.

During the next few years, a strong controversy developed between Needham, a proponent of the theory of spontaneous generation, and Spallanzani. Needham (1749) believed that in every particle of organic matter there existed a vegetative force that caused the organic matter of the medium to be converted into living forms. Spallanzani (1765) carried out hundreds of experiments to refute the teachings of Needham. He heated infusions in various kinds of flasks, after which they were stoppered with cork, wood, or paper. He was not certain that he had excluded all air that was believed to act as a carrier of the seeds. Some of his infusions were boiled after all air had been removed, then distributed into sterile flasks aseptically and hermetically sealed. He concluded not only that the infusion must be sterile but that the air must be free from living organisms. If both are sterile and the containers properly sealed, organisms will not develop in the medium. Needham criticized the experiments by stating that the prolonged heating had destroyed the vegetative force of the liquid. Spallanzani continued to answer every objection put forth by Needham, but still he was not able to convince the proponents of the doctrine of spontaneous generation.

Schulze (1836) devised an experiment (Fig. 290) in which an infusion contained in a flask was sterilized by heat and the overlying air was freed from living organisms by filtration. The flask containing the medium

was boiled over a sand bath and, while steam was streaming from the vessel, an absorption bulb was attached to each end. One bulb contained sulfuric acid and the other a solution of potassium hydroxide. Schulze applied his mouth to the open end of the potash bulb and aspirated fresh air into the vessel containing the medium. The air, in passing through the sulfuric acid, was freed from bacteria. The medium remained sterile for

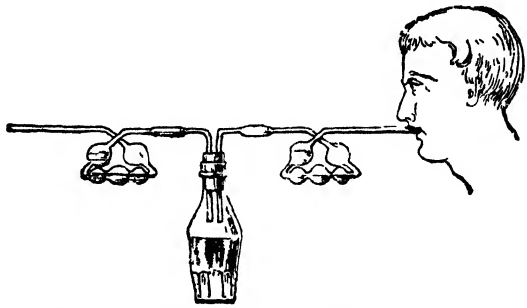

FIG. 290.—Schulze's experiment (1836). (*After Lafar.*)

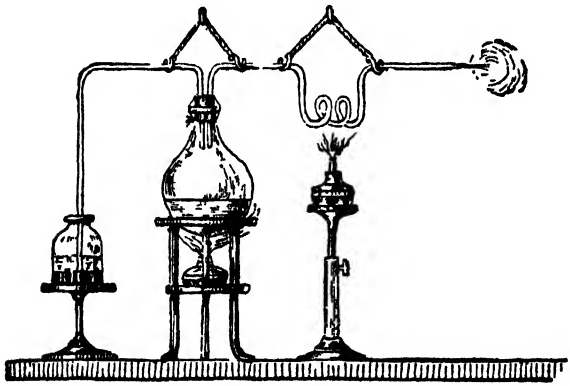

FIG. 291.—Schwann's experiment. (*After Lafar.*)

more than 2 months. A control flask showed growth in 2 days. The experiment demonstrated that the air in the flask was not the cause of growth in the liquid.

Schwann (1837) performed a similar experiment in which he did away with the absorption bulbs (Fig. 291). One tube from the flask entered a vessel containing mercury; the other opening was connected to a piece of glass tubing having several spiral twists. The infusion was brought to a boil and, while the steam was streaming from the open end, heat was also applied to the spiral. When the contents of the flask had cooled, the open spiral end was sealed in a flame. The contents of the flask remained sterile for 6 weeks. When the flask was disconnected, putrefaction rapidly took

place. Helmholtz (1843) in a similar type of experiment confirmed the findings of Schwann.

Schröder and von Dusch (1854) filtered air through cotton instead of heating or drawing it through sulfuric acid. Later, Schröder (1859) stoppered flasks with cotton. Solutions boiled in flasks plugged with cotton remained sterile, demonstrating that the cotton prevented bacterial organisms from entering the flasks. This was probably the origin of the use of cotton stoppers for keeping laboratory glassware and culture media sterile.

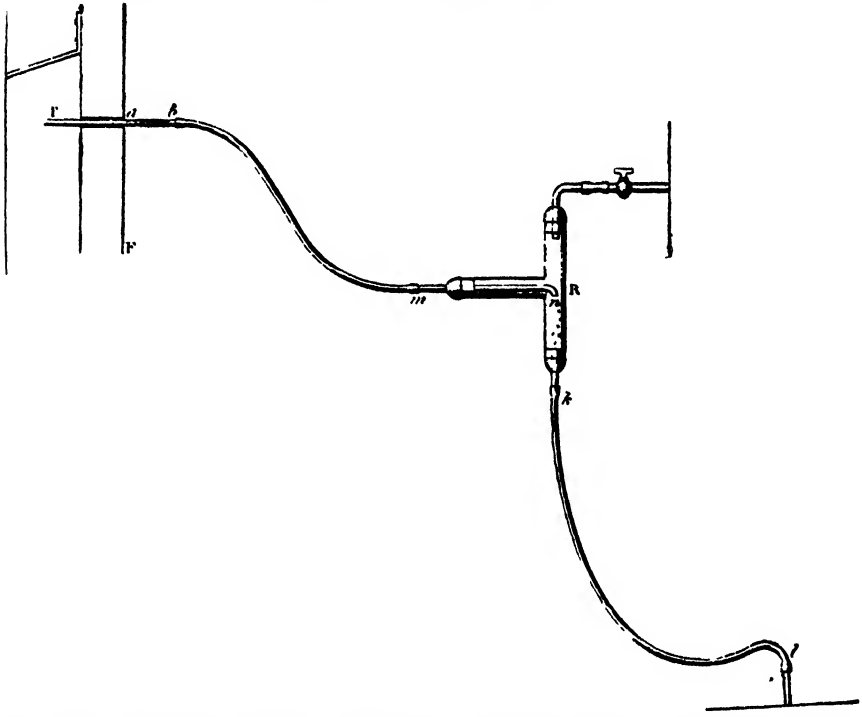


FIG. 292.—Experiment of Pasteur. Aspirated air filtered through a pledget of guncotton (*a–b*).

Convincing as these experiments were, they still did not convert the proponents of the doctrine of spontaneous generation.

About this time, Pouchet (1858) came into prominence by bitterly opposing the teachings of Pasteur and Spallanzani. He performed many experiments which convinced him that living organisms were able to spring from dead organic matter. The experiments of Schwann and Schulze were repeated by him with opposite results. Pasteur devoted the next several years to devising experiments to refute the teachings of Pouchet. The apparatus used in one of these experiments is shown in Fig. 292. The aspirated air was filtered through a pledget of guncotton (*a–b*).

At the end of the experiment, the guncotton was removed from the tube and dissolved in a mixture of ether and alcohol. The sediment that settled out at the end of 24 hr. was examined under the microscope and found to

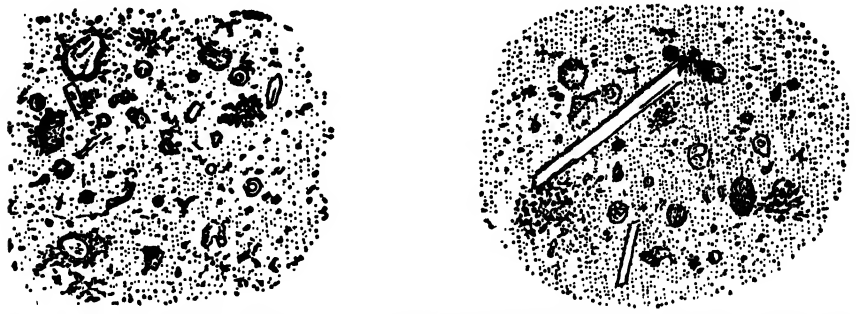

FIG. 293.—Air particles collected on the piece of guncotton (*a–b*) in Pasteur's experiment (Fig. 292).

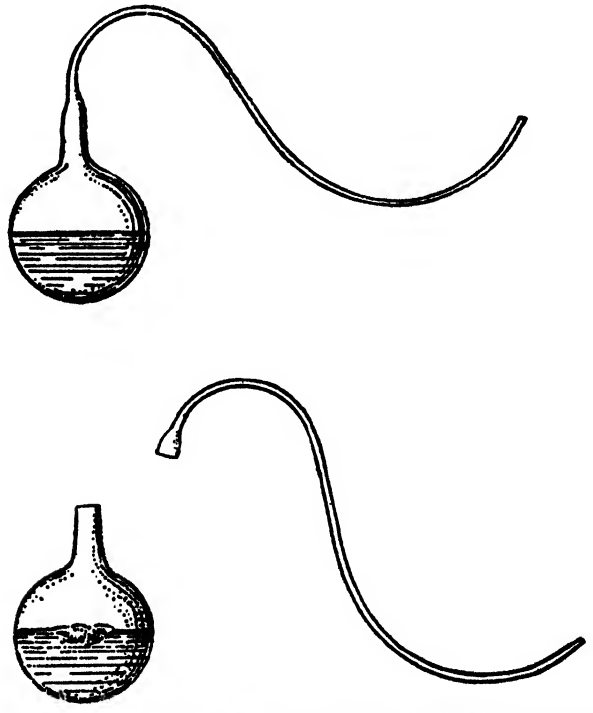

FIG. 294.—Long-neck flasks used by Pasteur to prevent entrance of dust to the interior.

consist of many organisms (Fig. 293). He also filled flasks with infusion and bent the necks downward in such a manner that organisms from the air could not ascend the tube and enter the flask (Fig. 294). The infusion was then boiled to destroy any living organisms present. The flasks re-

mained sterile for months. When the necks were cut off, putrefaction rapidly set in. Pasteur showed that the number of organisms present in air varied with the locality. He exposed 20 flasks at an altitude of 2550 ft. and 20 at 6276 ft. At the end of the experiment, the former group showed 12 flasks sterile and 8 contaminated; the latter group showed 19 sterile and only 1 contaminated. These experiments again demonstrated that bacteria were present in air and also that the numbers present decreased with altitude. Still Pouchet refused to be shaken by Pasteur's latest experiments.

Pouchet exposed flasks at an elevation of 9000 ft., and much to his satisfaction all of them became turbid. He obtained air from the summits

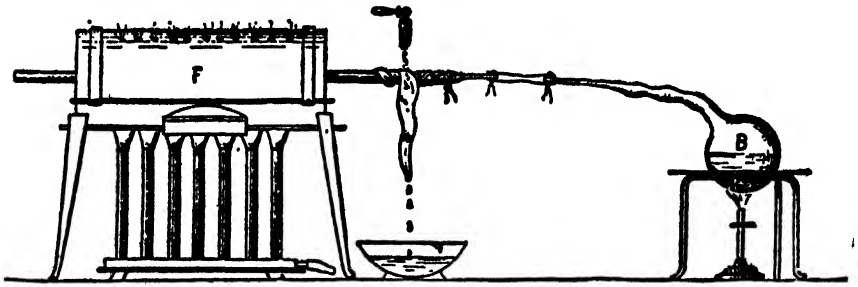


FIG. 295.—One of Pasteur's experiments to refute the doctrine of spontaneous generation.

of Mont Blanc and Monte Rosa and found that organisms were present. His experiments gave results just the opposite from those obtained by Pasteur. It was finally arranged to have Pasteur and Pouchet appear at Chevreul's laboratory in the Museum of Natural History in Paris. Pasteur performed a number of convincing experiments before a commission appointed to decide the controversy, but Pouchet refused to carry out any experiments. This was sufficient to convince the leading scientists of the day that the conclusions of Pasteur were correct. Pouchet finally gave up the problem and dropped from public view.

Bastian (1872) continued to propound the beliefs of Pouchet for nearly 40 years, during which time he published many books and papers on the subject. In the end, he appeared to be alone in his fight for the establishment of the doctrine of heterogenesis.

Tyndall (1876) noticed that, when a beam of light was passed through air laden with dust particles, there was a scattering of the light rays into its spectrum. If the dust particles were absent, no scattering effect was noted. After this observation, he employed a beam of light to reveal the presence of dust particles. Tyndall devised a special chamber to study the development of organisms in culture media. Two small holes in the sides

and the entire front were covered with glass. A hole in the top was covered with a piece of sheet rubber, in the center of which was inserted a long thistle tube. Two bent tubes were inserted into openings in the top for passage of air. The tubes were bent several times to prevent the entrance of any particles into the chamber. The floor was perforated with holes to accommodate 12 large test tubes. The interior surfaces of the chamber were moistened with glycerin to gather the floating dust particles coming into contact with the liquid. The chamber was allowed to stand undisturbed for several days until a beam of light showed the interior to be free from floating dust particles. Then the tubes were filled with culture media through the thistle tube. Since this tube was held in the flexible sheet of rubber, the stem could be easily directed into each test tube. The tubes were finally immersed in a boiling brine bath and held at that temperature for 5 min. The protected tubes of medium in the chamber remained sterile for months, and the control tubes exposed to the air showed growth after a few hours. He concluded that the contamination of the medium and the scattering of the light rays by the dust particles ran parallel.

Tyndall noted that sometimes 5 min. heating of the tubes in a boiling brine bath did not always sterilize the medium. He believed that some organisms were relatively thermolabile and destroyed in 5 min. at 100°C. and that others were thermoresistant to a high degree. Cohn (1876) arrived at the same conclusion independently of Tyndall by different methods. Tyndall showed that, if the medium was boiled for 1 min. on five successive days, it remained sterile. This appeared to be the first reference to use of the discontinuous method (Arnold) for the sterilization of culture media.

Cohn showed that the thermoresistance of some organisms was due to the presence of certain highly refractile bodies known as spores. Old cultures of hay infusion revealed many spores, whereas fresh cultures showed a much smaller number. He stated that, when spores were transferred to a fresh medium, germination into vegetative cells occurred. Cohn is generally given credit for being the first observer to see and describe spores of bacteria.

## PUTREFACTION

For centuries, putrefaction was considered an important factor as the cause of disease. Complications known as septicemia, pyemia, putrid intoxication, and putrid infection were believed to be the result of the absorption of putrid substances into the system. This belief was the outcome of observations on the injection of putrid materials into animals, which resulted in the development of symptoms resembling those noted in septic infections. Gaspard (1822) conducted many experiments on animals

and birds by injecting them with putrid substances, and described in great detail the symptoms and lesions that followed. Gaspard's work was repeated by others and aroused great interest at that time.

Magendie (1823) was the first to show that a dose of putrid material was lethal if injected intravenously, but inert if given by mouth. He placed animals on a grill above a putrid mass of material and found that no ill effect resulted from the inhalation of the foul gases given off. This observation refuted the ancient belief that disease was caused by the penetration of foul odors into the respiratory tract.

Schwann (1837) suggested and actually demonstrated that putrefaction was a biological process. This observation was later confirmed by Pasteur (1863), who stated that putrefaction was due to "ferments" of the genus *Vibrio*. Following the report of Pasteur, some workers attempted to show that putrefaction was due to chemical poisons while others believed it was due to the growth of living organisms. Later, Brieger (1881) isolated a large number of compounds known as ptomaines and believed to be the cause of disease. At present, it is definitely established that ptomaines are not capable of producing disease.

## PYEMIA AND SEPTICEMIA

Piorry (1837) believed that certain infections, leading to the formation of abscesses in various parts of the body, resulted in an escape of pus into the blood stream. He called this condition "pyemia," which may be defined as a form of blood poisoning produced by the absorption of pyogenic microorganisms into the blood, usually from a wound or local inflammation. It is characterized by multiple abscesses throughout the body. Septicemia may be defined as a poisoned condition of the blood due to the presence of pathogenic bacteria. It is sometimes referred to as blood poisoning. Donné (1836), Bonnet (1837), and others determined the composition of pus by microscopic examination.

Coze and Feltz (1866–1869) injected putrid materials into dogs and rabbits and showed that blood from these animals, when injected into normal animals, produced similar symptoms. Their work showed that blood increased in virulence by animal passage. They succeeded in demonstrating the presence of bacteria in the blood of diseased animals and in persons suffering from typhoid fever, scarlet fever, puerperal fever, etc. Davaine (1872) confirmed the findings of Coze and Feltz.

The germ theory of disease was now gaining momentum, largely because of the work of Pasteur on the biological origin of fermentation. Lister greatly reduced the number of deaths from surgical infections by employing aseptic precautions, which was additional evidence in favor of the germ theory of disease. Davaine (1872–1879) injected a rabbit with a few drops of putrid blood. This resulted in death after 40 hr. He found that

the transfer of blood from rabbit to rabbit for 25 transfers increased the virulence enormously. Because of the progressive increase in virulence, the quantity of blood required to reproduce the disease in each succeeding rabbit became less. Final settlement of the relationship of microorganisms to disease was made by Koch (1878). He demonstrated, without a doubt, that diseases were caused by microorganisms and that each disease was caused by a specific organism. Koch's success was due largely to the use of dyes for staining the bacteria and the condenser system invented by Abbé for increased illumination.

Ogston (1880) followed Koch's technique for the examination of over 100 acute and chronic abscesses for bacteria. Chronic abscesses gave negative results, but acute abscesses showed the presence of groups and chains of organisms. Pus heated or treated with phenol was noninfectious, but unheated or untreated pus gave positive results. He differentiated two kinds of cocci: streptococci and micrococci. Ogston's work was very complete and accurate, and exerted a tremendous influence on the ideas of that period.

## SPECIFIC CAUSES OF DISEASE

Bretonneau (1778) is generally credited as being the real founder of the doctrine of the specificity of disease as understood today. He stated that the diseases of typhoid, diphtheria, malaria, and dysentery were produced by specific elements and by no others. His conception of specificity was etiological. However, he failed to connect the specific elements responsible for disease with microscopic animalcules.

Henle (1840) believed that each disease was caused by a specific organism and that it could not be caused by any other organism. To show that an organism was the cause of a specific disease, he postulated that the organism must be isolated and found capable of again reproducing the disease in another animal. These postulates are generally referred to as Koch's postulates (1878) because they were restated by Koch; in reality, they are Henle's postulates.

The work of Henle exerted a tremendous influence on the scientists of that period. His work acted as a stimulus which led to the microscopic examination of a large number of disease products. Within the next few years, many organisms were isolated from diseased tissue and believed to be the causes of those diseases.

## EARLY ATTEMPTS TO CLASSIFY BACTERIA

The first attempt to classify microscopic plants and animals was apparently undertaken by Müller (1773), who described, arranged, and named with great accuracy a number of microscopic organisms. Later classifications were proposed by Ehrenberg (1838), Dujardin (1841), Perty (1852),

Cohn (1854), Zopf (1885), and Migula (1897). Ehrenberg made no distinction between bacteria and protozoa. He classed them all in a group known as the *Infusoria*, and he regarded them as animals from his belief that they have stomachs. Ehrenberg recognized 22 families of organisms.

Dujardin placed bacteria in the family *Vibrioniens* which he characterized as extremely slender filiform animals, without appreciable organization or visible organs of locomotion. He recognized three genera: *Bacterium*, *Vibrio*, and *Spirillum*.

Cohn believed that bacteria showed more of the characteristics of plants than of animals and suggested that they be classified in the plant kingdom. Nägeli (1857) appeared to be the first to suggest the use of the term *Schizomycetes* (fission fungi) to include all organisms classified as bacteria.

Cohn (1872) believed that it was a difficult task to classify bacteria into genera and species. He pointed out that so many species looked alike that a classification based on morphology alone was insufficient. Physiological as well as morphological differences were necessary to distinguish bacteria accurately. This appears to be the first reference to the use of physiological characters for the classification of bacteria.

Cohn suggested the following classification of bacteria:

Tribe I. *Sphaerobacteria.*
  Genus 1. *Micrococcus.*
Tribe II. *Microbacteria.*
  Genus 2. *Bacterium.*
Tribe III. *Desmobacteria.*
  Genus 3. *Bacillus.*
  Genus 4. *Vibrio.*
Tribe IV. *Spirobacteria.*
  Genus 5. *Spirillum.*
  Genus 6. *Spirochaete.*

Cohn believed in the morphological fixity of bacterial species; otherwise he could not have classified microorganisms. Nägeli was not in accord with Cohn's views. He brought forth evidence to show that every species of bacterial organism may appear in several types on the basis of morphological and physiological differences. Nägeli believed that one form could be converted into another. Some of the scientists who were in harmony with the findings of Nägeli were Lankester (1873), Billroth (1874), Warming (1875), Zopf (1879), and Buchner (1882).

Zopf believed that the change from one bacterial form into another was dependent chiefly or entirely upon environment. In spite of his views on the variability of species, he proposed the following classification of bacteria on the basis of morphology:

I. *Coccaceae*—spherical forms.

II. *Bacteriaceae*—spherical, rod, and filamentous forms, without distinction between base and apex.

III. *Leptothriceae*—spherical, rod, and filamentous forms, the latter with distinction between base and apex.

IV. *Cladothriceae*—spherical, rod, and spiral forms, and showing dichotomy.

## DEVELOPMENT OF BACTERIOLOGICAL METHODS

Pollender (1849) and Davaine (1850), working independently, first saw the anthrax organisms in the blood of cows affected with the disease. The rods were present in enormous numbers in the spleen. Brauell (1857) performed inoculation experiments and showed that the disease was transmissible from man to sheep and from horse to horse. Davaine, through a

FIG. 296.—Robert Koch.

long series of researches, definitely proved that the rods were the cause of anthrax. He found that the organisms were constantly present in the blood of animals suffering from the disease and that in the absence of the rods no illness was present. Since the germ theory of disease was not accepted by all scientists of that period, the work of Davaine did much to place it on a solid foundation.

Koch in 1876 took up the study of anthrax where Davaine had left off. He placed minute pieces of infected spleen tissue in drops of sterile blood serum and studied the cultures from hour to hour to see what took place. Koch saw the organisms grow in the form of long filaments inside of which appeared oval, granular bodies. He came to the conclusion that these bodies were spores. In order to rule out the possibility that the spores were contaminants, he was able to show that they grew into anthrax vegetative cells. Koch showed that the disease was transmissible from

mouse to mouse and that the lesions that appeared in the animals were identical.

Koch inoculated mice with organisms that were morphologically identical with the anthrax rods, but he was not able to produce the disease. He concluded that each disease was produced by a specific organism. Most of the early technical methods originated in his laboratory.

**Staining bacteria.**—Various dyes for staining bacteria now began to be used. The first dyes were obtained from natural sources, the best known representatives being hematoxylin from logwood and carmine from certain insects. Coal-tar dyes first made their appearance about 1856. Hoffmann (1869), probably the first to stain bacteria, used carmine and fuchsin. Weigert (1871) employed methyl violet for staining bacteria in histological sections. Salomonsen (1877) used fuchsin for staining bacteria.

The present-day methods for staining bacteria are essentially the same as those first advocated by Koch (1877). He prepared thin smears of bacteria on cover slips and dried them. The films were fixed in alcohol and then stained with various dye solutions, such as methyl violet 5*B*, fuchsin, and aniline brown. He appears to have been the first to stain bacterial flagella.

Rapid progress on new staining procedures now followed. Weigert (1878) introduced Bismarck brown and Ehrlich (1881) methylene blue. Koch employed methylene blue when he discovered the tubercle bacillus in 1882. Loeffler (1884) added a small amount of potassium hydroxide to a solution of methylene blue to neutralize the acidity of the preparation. This dye solution is known as Loeffler's methylene blue and is extensively employed at the present time. Since the methylene blue now prepared in this country is neutral in reaction, the addition of the alkali is no longer necessary. Potassium hydroxide may cause too rapid oxidation of the methylene blue, resulting in a solution having poor keeping qualities. In 1882, Ehrlich first announced the method for staining the tubercle bacillus. He added aniline oil to methyl violet and fuchsin as an intensifier. Smears stained with these solutions revealed the presence of long, slender, deeply stained, rod-shaped organisms. Ehrlich found that the organisms were not decolorized when treated with 30 per cent nitric acid so he called them "acid-fast." He used vesuvin as a counterstain for methyl violet, and methylene blue as a counterstain for fuchsin. Ziehl substituted phenol (carbolic acid) for the aniline oil, and Neelsen decolorized with sulfuric acid instead of nitric acid. This modification is known as the Ziehl-Neelsen staining solution. In 1884, Gram discovered the staining technique bearing his name. He was working on a new method for staining bacteria in tissues, but discovered a new differential stain for bacteria.

**Cultivation of Bacteria.**—The development of various staining procedures was followed by studies on the nutritive requirements of bacteria.

Pasteur (1861) employed a carbohydrate medium composed of 100 parts of water, 10 parts of candy sugar, 1 part of ammonium tartrate, and 1 part of yeast ash. Mayer (1869) improved Pasteur's medium by using chemically pure salts as found in yeast ash instead of the ash as such. Cohn (1872) recommended a medium composed of 0.5 gm. potassium phosphate, 0.5 gm. magnesium sulfate, 0.05 gm. calcium phosphate, 1.0 gm. ammonium tartrate, and 100 cc. distilled water. Miquel (1883) suggested the use of Liebig's beef extract or an infusion prepared from lean beef. Loeffler (1881) cultivated organisms on a medium composed of beef infusion to which was added 1 per cent peptone and 0.6 per cent sodium chloride. The reaction was made weakly alkaline with sodium phosphate buffer. This is essentially the composition of nutrient broth as used at the present time. Koch (1881) worked on the perfection of solid media, which were required for use in his isolation experiments. He recommended the addition of 2.5 to 5 per cent gelatin to nutritive fluid media. Two disadvantages to the use of gelatin as a solidifying agent are that it is fluid at body temperature (37°C.) and that it is liquefied by many bacteria. At the suggestion of Frau Hesse, the wife of one of Koch's assistants, agar was substituted for the gelatin. This overcame the objections to the use of gelatin. In 1882, Koch introduced coagulated blood serum as a solid nutritive medium for the cultivation of the tubercle bacillus. Petri (1887) recommended the use of covered dishes bearing his name. Petri dishes or plates have not been altered since the time of Koch and are standard equipment in bacteriological laboratories throughout the world.

**Isolation of Species.**—The separation of organisms into pure species was first attempted by Klebs (1873). He introduced a small amount of inoculum into a sterile medium. When visible signs of growth appeared, a small amount of the culture was transferred to fresh medium. This was repeated several times. He believed that one organism would eventually overgrow the others with the result that a pure culture would be obtained. Hoffmann (1865) employed solid media in the form of pieces of potato and bread. Schroeter (1872), in his work on the cultivation of the pigment-producing bacteria, employed potato, starch paste, flour paste, bread, egg albumin, and meat as solid substrates. He was able to obtain pure colonies of bacteria by such a technique. Koch (1881) also used potato medium but greatly improved its preparation. A potato was first soaked in a 1:1000 solution of mercuric chloride, then sterilized in steam, and finally cut in two with a sterile knife. The two halves were allowed to fall apart into a sterile dish. The cut surface was then inoculated with the bacterial culture.

Brefeld (1875), working on pure cultures of molds, laid down the rules required to separate them into species: (1) The inoculation of a sterile medium should be made from a single spore, (2) the medium should be

favorable to the growth of the species, (3) the culture should be protected from aerial contamination. The method was difficult to apply to the isolation of pure bacterial species because the cells are considerably smaller than mold spores. Lister (1878) made high dilutions of a culture in sterile medium in the hope that growth from a single organism would eventually be obtained. His method proved successful and was adopted by Hansen (1883) for the propagation of pure species of yeasts. Salomonsen (1877) aspirated contaminated blood into long capillary tubes, which he was able to observe under the microscope. The tubes were broken where isolated spots (colonies) appeared. He found that each spot often contained only one kind of organism.

Coze and Feltz (1866), Davaine (1872), and Koch (1876) employed the method of animal inoculation for the isolation of pathogenic organisms. Koch showed that the anthrax organism could be separated from other nonpathogenic species by injecting the mixed culture into mice. Only the anthrax organism invaded the tissues of the host, with the result that a pure culture of the organism could be recovered from the blood of the diseased animal.

Koch (1881) devoted considerable time to a solution of the problem that was to place him in the front rank as a bacteriologist. He was the first to obtain pure cultures of bacteria by the streak-plate method. Koch poured melted gelatin medium on glass plates and placed them under a bell jar to avoid aerial contamination. Then he inoculated a needle with a mixed culture of bacteria and made several cross lines over the surface of the solidified gelatin. After incubation, different colonies of bacteria appeared on the gelatin medium. These were transferred to tubes containing sterile nutrient gelatin slants. In 1883, Koch recommended the pour-plate procedure for the isolation of pure cultures of bacteria. Instead of streaking a culture of bacteria over the surface of solid nutrient gelatin, he now mixed the organisms with melted gelatin and poured the mixture on cold sterile glass plates kept under bell jars. The separation of colonies by this procedure was much better and soon became the method of choice. The methods advocated by Koch for the purification of bacteria are the same as those used today, except that agar is now universally employed in preference to gelatin for reasons already given.

**Sterilization by Filtration.**—Tiegel (1871) appears to be the first to use filtration as a method for the removal of bacteria from liquids. He passed anthrax cultures through porous cells composed of unburned clay. Pasteur and Joubert (1877) substituted plaster of Paris for the clay. Miquel and Benoist (1881) used a mixture of asbestos and plaster of Paris. Chamberland (1884) employed cylinders composed of unglazed porcelain. This type of filter is still widely used under the name "filtre Chamberland, système Pasteur." In 1891, Nordtmeyer employed a filter composed of

infusorial earth or kieselguhr. These are called "Berkefeld" filters after the name of the owner of the mine from which the material was first obtained.

**Sterilization by Heat.**—The value of hot air for the sterilization of materials was determined by Koch and Wolffhügel in 1881. Koch, Gaffky, and Loeffler (1881) employed streaming steam at 100°C. and pointed out its limitations. The discontinuous method of steam sterilization was recommended by Tyndall (1876). Pasteur noted the increased sterilizing effect of superheated steam for the destruction of microorganisms. Modern laboratory models known as autoclaves were made available about 1884.

FIG. 297.—Joseph, Lord Lister. (*From Bulloch, "History of Bacteriology," Oxford University Press, London.*)

**Sterilization by Chemicals.**—Lister (1868) revolutionized surgery by his antiseptic treatment of wounds. He atomized a solution of phenol or carbolic acid into the air in the immediate vicinity of the patient during an operation. Koch (1881) compared the action of a large number of antiseptics and germicides on certain bacteria. Small pieces of thread were impregnated with bacteria, then dried. The dried threads were immersed in an antiseptic solution and removed after definite intervals of time. The threads were washed free from antiseptic and then dropped into tubes of medium to test for growth. After testing some 70 antiseptics, Koch came to the conclusion that mercuric chloride was the most potent germ-killing chemical. Geppert (1889) confirmed the above findings but pointed out that Koch failed to distinguish between bacteriostatic and bactericidal. On adding ammonium sulfide to the transfer medium to precipitate the mercury from the bacteria at the end of the experiment, he found that the organisms were not dead as Koch had reported, but still living. Krönig and Paul (1897) laid the foundations of our present methods for the evaluation of germicides. They pointed out that accurate comparisons of germicides were possible only if certain factors are controlled.

The pour-plate and the streak-plate methods, advocated by Koch for the purification of bacterial species, now resulted in the isolation of a considerable number of disease-producing organisms. Up to 1881, two organisms had been definitely proved to be the cause of disease. *Bacillus*

*anthracis* was shown to be the cause of anthrax and *Spirochaete obermeieri* of relapsing fever. In 1882, Koch isolated the organism of tuberculosis, and Loeffler and Schütz discovered the organism of glanders; Koch (1884) isolated the organism of Asiatic cholera and Loeffler the organism of diphtheria; Gaffky (1884) discovered the bacillus of typhoid fever, and Rosenbach isolated pure cultures of *Staphylococcus* and *Streptococcus;* Bumm (1885) proved that pure cultures of the gonococcus are the cause of gonorrhoea, and Escherich isolated *Escherichia coli;* Fränkel (1886) cultivated the pneumococcus in pure culture; Bruce (1887) isolated *Brucella melitensis*, the causative agent of Malta fever, and Weichselbaum cultivated the meningococcus; Kitasato (1889) isolated the tetanus bacillus; Kitasato and Yersin (1894) discovered the plague bacillus; van Ermengem (1897) cultivated the organism of botulism; and Shiga (1898) isolated the cause of bacillary dysentery.

**Anaerobic Methods.**—Leeuwenhoek, as early as 1680, showed that "animalcules" can exist in the absence of air. Spallanzani (1776) observed that organisms developed in a high vacuum and could live at least for 16 days. These observations were forgotten until 1861, when Pasteur again drew attention to the existence of organisms that are capable of living in the absence of free oxygen. Pasteur showed that yeasts and other organisms could live and multiply in the absence of air. He introduced the terms "aerobe" and "anaerobe." Many methods were devised for the production of anaerobic conditions. From the time of Pasteur to the present day, hundreds of different types of apparatus have been described and recommended for the cultivation of anaerobic bacteria.

## ATTENUATION OF BACTERIA

The pioneer discoveries in this field were made by Pasteur. In 1880, he isolated the organism of chicken cholera and cultivated it in pure culture. Injection of the organisms into normal chickens usually resulted in a fatal infection. Occasionally, chickens recovered from the disease and became refractory to a second inoculation. The first dose conferred sufficient immunity to protect the chickens against a subsequent infection. Pasteur noted that the virulence of a culture depended to a large extent upon its age. As the time between transfers was increased, the ability of the organisms to produce an infection decreased. When he used very old cultures, the organisms failed to produce cholera in chickens but still retained the ability to immunize the fowls against the disease. He used the term "attenuated" to describe such cultures. This was probably the first experiment performed to demonstrate the value of an attenuated culture for the elaboration of immune bodies in the host without the appearance of symptoms of the disease. This same principle was applied to the preparation of vaccines of the organisms of anthrax, swine erysipelas, and rabies.

Pasteur devoted a number of years to a study of anthrax. He found that the organism did not grow at a temperature above 45°C. but did grow at 42 to 43°C. A virulent anthrax culture kept at 42 to 43°C. failed to produce disease in laboratory animals but was still capable of eliciting an antibody response. This attenuated culture of the anthrax organism was used as a prophylactic to protect animals from a subsequent dose of virulent bacilli.

His next problem was concerned with the disease known as swine erysipelas. A well-defined bacillus was discovered in the blood of pigs suffering from the disease. Attenuation was accomplished by passage of the organisms through rabbits. Inoculation of pigs with this attenuated vaccine did not produce the disease but did confer protection against a subsequent dose of virulent organisms.

Perhaps the crowning achievement of Pasteur's work was his investigations on rabies. It was shown that the virus of the disease attacked the central nervous system. The incubation period of the disease showed considerable variation, depending upon the distance from the site of inoculation to the central nervous system. Pasteur inoculated the virus into dogs directly under the dura mater and showed that the incubation period before symptoms appeared was greatly shortened. He noted that dogs that had recovered from the disease became immune to a second inoculation. Pasteur prepared his vaccine by removing the spinal cords of rabbits dead of the disease and by drying them for varying periods of time. As the drying period was increased, the virulence of the virus decreased. In this manner, he prepared virus of varying degrees of virulence. The virus employed for the first injection was dried for 14 days. Pieces of the cord were emulsified and injected into a patient bitten by a rabid dog. For 12 more daily injections, virus of gradually increasing virulence was employed. Finally, cord taken from an animal dead of virulent virus was emulsified and injected. In this manner, immunity was conferred upon the patient and symptoms of the disease did not develop. If treatment was not begun early, the immunity conferred by the vaccine was not always sufficient to destroy the virus before it reached the central nervous system, with the result that symptoms of the disease appeared. This treatment, with slight modifications, is now used in all parts of the world and has reduced the incidence of rabies to a very small figure.

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