Microbes and You

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NEW YORK · CHICAGO
DALLAS · ATLANTA · SAN FRANCISCO

THE MACMILLAN COMPANY
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TORONTO

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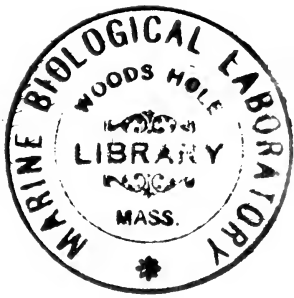
NEW YORK THE MACMILLAN COMPANY

Printed in the United States of America

First Printing



TO

Carol, Karen, and Robin

Preface

Microbes and You has been written as a text for an introductory, terminal, survey course in microbiology for students with little or no background in science. The book should fill a need in schools—both large universities and small liberal arts colleges—where students are required to complete at least one course in a biological science as part of their general education.

This text is not intended primarily for those planning to pursue microbiology as their major field of interest, nor for those individuals preparing for a career in medicine.

Because *Microbes and You* is designed for use in a cultural course in science, it is written in a style which should help to hold the interest of the reader. Practical everyday applications of microbiology are incorporated into the text, and the instructor can supplement the reading material with lectures and laboratory exercises slanted toward his particular course objectives. This text is more concise than other similar presentations, and students are likely to read it more thoroughly than longer textbooks.

Stanley E. Wedberg

Storrs, Connecticut

Acknowledgments

It is through the courtesy of a number of individuals, corporations, and publishers that many of the illustrations are included in this book. Specific acknowledgment is found in the legends accompanying these figures. The editors of the *Journal of Bacteriology*, the *Journal of Botany*, the *Journal of Experimental Medicine*, and the *Journal of Infectious Diseases* have been very cooperative in allowing material to be reproduced from original articles in their publications. Some of the prints are from the collection of the Committee for Visual Education in Microbiology of the Society of American Bacteriologists.

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I am indebted to my colleague, Dr. Robert C. Cleverdon, for his critical reading of the manuscript and for his invaluable suggestions for improvement of the text. My thanks are extended to Mrs. Gordon R. Hanks who typed and retyped the manuscript.

And in conclusion, I acknowledge sincere gratitude to Dr. Walter L. Kulp, Dr. Milton J. Foter, Mr. Vinton E. White, Miss Christie J. Mason, Dr. Leo F. Rettger, Dr. George Valley, Dr. George H. Smith, and Dr. Philip B. Cowles—my microbiology teachers—for the foundation they have given me in this science.

Contents

CHAPTER 1

Introduction

We are privileged to live in an age when, as the result of the hard labors and the genius of men, a new world is opened up for our study—a fascinating, vibrant, sub-visible world hidden from the eyes of human beings since the beginning of time. Since man first viewed these interesting microorganisms, or germs, only about two hundred years ago, much has been sacrificed, including the very lives of some of the microbe hunters who have attempted to ferret out the mysteries of these minute forms of life.

It might be well at this point to distinguish between *bacteria* and *microorganisms*. The former word includes only those single-celled, non-chlorophyll-containing plants which multiply by binary fission, or equal division. It may be a revelation to you to discover that bacteria are not "bugs," but plants. The word microorganism implies a much broader field and includes many different microscopic forms of both plant and animal life, including molds, yeasts, bacteria, protozoa, and even rickettsiae and viruses. Each of these organisms will be considered separately in later chapters. This book will deal principally with the bacteria, but because the technics for studying the other microorganisms are similar to those employed with bacteria, and because these other microscopic forms are oftentimes found intimately associated with bacteria, we shall not overlook them by any means.

These organisms face many of the same problems confronting

you and me—obtaining food, digestion, excretion, respiration, reproduction, etc. But in contrast to man, who possesses specialized organs for these important functions, the lowly bacteria must carry out all of these activities within the confines of a single cell, a cell so minute that 25,000 of them standing side by side would hardly occupy an inch!

Microbes cannot walk, so they are unable to crawl up over our shoes to get at us, but some do have the power of locomotion in liquids. These have been endowed with special hair-like projections, all part of the single cell, called FLAGELLA. By a whipping motion these flagella are able to propel the bacteria through liquids. The typhoid bacterium has been clocked at nearly 65 millimeters (about 2.5 inches) an hour! The swimming speed of a given organism is influenced by such factors as temperature, available food, nature of the suspending medium, age of the organism, and undoubtedly by other motivating forces. Some bacteria lack flagella, yet they make progress through liquids by a twisting, corkscrew-like motion. It should be made clear that many bacteria lack the power of independent motion; they just sit around and wait to be pushed.

There is little that is static in a science as young as microbiology. It is a dynamic phase of biology which has many practical, everyday applications. As a science, bacteriology is hardly more than one hundred years old. Some persons date this branch of science to 1857 when Pasteur first demonstrated that microorganisms could sour milk. Sir William Osler (1849–1919) said that this publication of Pasteur's, together with his demonstration that the transformation of sugar into alcohol and carbon dioxide was a phenomenon of life, set the date for a new era in medicine. This killed the notions that magic and air caused disease in some mysterious way, and it dragged the enemy into the open. All modern medical and surgical technics employed to prevent and to combat disease are based upon this germ concept. Too often, however, the word *germ* or *bacteria* conjures up in the mind of the layman but one thought—disease. Disease may be defined as an abnormal condition of any part or organ of the body or of the

mind. It is possible to name a number of diseases not caused by bacteria or other microbes, and with a little further probing one could undoubtedly think of many uses to which mankind has pressed microbes for useful ends. Yet if a psychologist uttered the word "bacteria" in an association test where the test subject is requested to give the first word that comes to his mind, the response would undoubtedly be "disease," "sickness," or some similar connotation more times than not.

Just how did this young branch of science originate? To state with finality that a given day of a given year launched microbiology is an impossibility. It is probably easier to pinpoint the beginning of the atomic age to the first successful application of the cyclotron or to that early morning hour on July 16, 1945, when the first man-made atomic bomb was set off in the desert near Alamogordo, New Mexico. Nevertheless, it must be admitted that a great deal of both pure and applied research preceded the first atomic blast. The same statement can be made with respect to microbiology. A number of modern inventions are the product of the accumulation of vast storehouses of smaller, minor discoveries which, when tied together, provided background material for the development of the finished product. The fortunate accidents which have been capitalized upon by clever observers should not be ignored.

At the same time that mention is made of the harmful effects produced by these organisms, the tremendous good that they accomplish for mankind should not be overlooked. The statement has been made that were it not for microbes, you and I would not be here. That is a rather strong assertion, yet there is plenty of evidence to add weight to such a contention. Without the chemical activity of organisms in their never-ending quest for food, no trees, plants, or animals would be consumed after their death unless they were burned or destroyed by some means other than biological activity. The vast accumulation of ancestors, plant and animal, would soon leave little room for the living. The chemical elements borrowed for a while by living things in the past would not be available for present generations, and in time life could very well grind to a creaking halt. Bacteria and other microorganisms

depend upon other living things for their food and for their very existence. Man's diet has been broadened as the result of microbial activity in the manufacture of such things as cheeses, sauerkraut, pickles, beer, and leavened bread, to mention but a few.

Once it had been established that bacteria were not "animalcules," as Antony van Leeuwenhoek (1632–1723) designated them in the seventeenth century, this new branch of plant science appropriately found its way into the field of botany, where it still remains as an ugly-duckling in some institutions. Botanists do not employ the same technics as those used by microbiologists, so it was only natural that the new science should eventually break away from botany and stand on its own two feet.

It seems unfortunate that so many bacteria in the distant past became unhappy with their saprophytic existence and decided to become pathogens, invading the tissues of plants and of animals. But this characteristic of microorganisms has presented one of the great challenges to microbiologists in the search for new ways to lengthen the span of man's life to the Biblical three score years and ten. We have come astonishingly close to this goal in recent years as the following statements will indicate.

Actuarial figures released by a large life insurance company demonstrate in a clear fashion the influence of scientific progress on the life span. The expectation of life at birth is five years greater today than it was a decade ago, and double that existing between 1879–1889, the earliest period for which experience tables are available. In round numbers, since 1911 we have gained something over twenty-one years, and since 1879 the average human life has been prolonged by thirty-five years! This is primarily a victory for preventive medicine, with a heavy assist in recent years from antibiotics. It is also a victory for nutritionists, who in the past two decades have told us more about what is good for us to eat than we ever knew before. An average human life two thousand years ago was about twenty-five years. In 1900 this figure had climbed to forty-nine and today we can look forward to better than sixty-eight years of life, females, on the average, living longer than males.

These changes in life expectancy will have, and already are having, a tremendous impact on problems connected with care of the aged. The social security program must carefully weigh these factors. We now have two chances out of three that an eighteen-year old will live to the retirement age of sixty-five. A forty-five-year-old man has seventy chances in one hundred of reaching sixty-five. Projecting our tables to 1980, about one person in ten will be sixty-five years of age or older, as compared with one person in twenty-five back in 1900. Some insurance companies have already adjusted premiums downward on straight life insurance policies as a reflection of this trend toward long life. We have not reached the end of possible means for increasing the span of human life, and before too many decades slip by, it seems fair to assume that the three score years and ten figure will be readily surpassed.

CHAPTER 2

Highlights in the History of Microbiology

THE DEVELOPMENT OF THE MICROSCOPE
THEORIES CONCERNING SPONTANEOUS GENERATION
THEORIES CONCERNING FERMENTATION
THEORIES CONCERNING DISEASE

THE DEVELOPMENT OF THE MICROSCOPE

Studying the history of some subjects represents a necessary evil to an undue number of individuals, but without some historical foundation, modern students of science would miss a great deal of the tingle that accompanied great and small discoveries. In microbiology it is possible to look back with considerable pride at the fascinating way in which small pieces of an intricate puzzle have fallen into place to bring the picture to its present stature.

Do you enjoy reading stimulating tales of adventure, such as those conjured up by Robert Louis Stevenson and others? The events described in these best sellers sound like commonplace happenings when they are compared with the adventures of the pioneers in microbiology. These scientists did not discover a mere island or a simple continent, they opened up an entirely new world! The discovery of microbes, some of which kill and maim, but others

of which work for the good of mankind, is a bright chapter in the history of biology. Long before the advent of television, the radio, the motor car, the refrigerator, and many other conveniences that enrich our lives to the point where we consider them as necessities, microbiological history was slowly being written. In 100 B.C. we find records of a Roman named Marcus Varro (116–27 B.C.) who speculated: "Certain minute invisible animals develop which, carried by the air, may enter the body through mouth or nose and cause serious ailments." How do you suppose Varro would react were he to peer through some of our modern instruments and see these microscopic living forms?

The eyes of what we term a normal individual cannot see objects smaller than about 30 microns (about $\frac{1}{1000}$ of an inch) in diameter, but by grinding lenses in certain ways we have created greater near-sightedness, if you will, which allows us to view objects much smaller than 30 microns. The origin of the first ground lenses is lost in history, although reference to magnifying glasses can be found in the writings of the ancient Greeks and Romans. As early as 1267 a Franciscan monk, Roger Bacon (1214–1294), clarified some of the principles of optics, and he is usually credited with being the founder of the science of optics. Bacon suggested, probably for the first time, that these lenses could be fashioned into spectacles for persons with poor eyesight. A report from Florence dated 1299 states: "I find myself so pressed by age that I can neither read nor write without those glasses they call spectacles, lately invented to the great advantage of poor old men when their eyesight grows weak." While reading the latest Book of the Month offerings did not take much of a person's time back in the thirteenth or fourteenth century, what a joy it must have been for the aged in the twilight of their lives to again see the world about them. We have good reason to assume that the crudeness of these early spectacles, while affording temporary help to failing eyes, might have done a great deal of harm over a period of time to persons suffering from certain eye disorders. Carefully compounded prescriptions for glasses were not available for centuries after these first spectacles were marketed.

By accident, or by logic, it was discovered that if a single ground lens could make things look larger, an object could be magnified still further by using two or more lenses set up in a definite relationship to each other. This compounding of lenses is usually credited to a spectacle maker in Holland in the year 1590. Considerable disagreement exists as to the exact name of this individual and as to the spelling of his name. Many books call him Janssen; other histories say his name was Zacharius (miscalled Jansen), the son of John, the spectacle maker. Other books refer to these persons as Hans Jansen and his son Zacharius, with the latter person being the discoverer of the principle of the telescope—two lenses in a tube. The earliest compound microscope was provided with a concave ocular and convex objective. Practical uses of compound lenses were not put to serious use in biological science until the middle of the seventeenth century by Robert Hooke (1635–1723), Antony van Leeuwenhoek (1635–1703), Marcello Malpighi (1628–1694), and others of whom more shall be written later.

One of the earliest reports on the existence of microorganisms can be found in the writings of an Austrian Jesuit priest, Athanasius Kircher (1601–1680), who reported on the cause of plague as seen in blood of infected individuals. Since his microscopes were extremely crude affairs and his lenses had magnifying limits of only about 32 diameters, it is quite doubtful that Kircher actually saw the organisms we attribute today as the etiology of this scourge. Kircher was trained in physics, medicine, mathematics, and music, and in 1658 he published a treatise on medical microscopy.

The name of Galilei Galileo (1564–1642) should not be passed over without at least a mention of his work on lenses. Some historians go so far as to credit this man with the discovery of the compound microscope, but because Galileo failed to leave complete records of his work and his findings, other individuals who had left such reports were credited with many discoveries which might originally have stemmed from the brain of Galileo. It is of interest to note that the word "microscope" was coined in 1625 by Giovanni Faber. Hooke is credited with the discovery of what we know

today as cells, and his name is included as a milestone in cytology. He published his "Micrographia" in 1665 in which his compound microscope was described and pictured. Another pioneer microscopist was Malpighi, who viewed, probably for the first time, circulation of blood in capillaries. Among his interests are included studies of animal and vegetable materials, and his re-

Fig. 1. Antony van Leeuwenhoek (1632–1723)—The "Father of Microbiology." (*Courtesy of the Lambert Pharmacal Company, Division of the Lambert Company, St. Louis, Missouri.*)

searches are recorded in papers submitted to the Royal Society of London.

One of the most interesting of the microbe hunters was Antony van Leeuwenhoek, who was born in Delft, Holland, the son of well-to-do tradespeople. This person's early years are not well documented, but when his father died, Antony's mother wanted him to become a government official, a member of a respected profession. When he was sixteen years old he left school and

became an apprentice in a dry goods store in Amsterdam until he reached the age of twenty-one. For the next twenty years Leeuwenhoek ran his private dry goods establishment, but little is recorded of this period in his life. It is believed, however, that he was twice

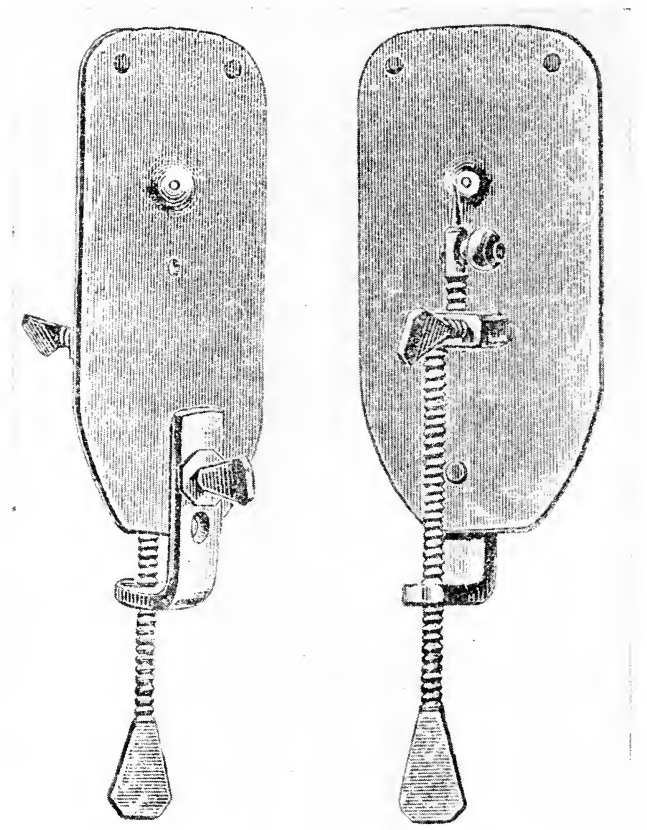


Fig. 2. One of Leeuwenhoek's microscopes. (*Courtesy of the American Optical Company, Instrument Division, Buffalo, New York.*)

married and had children, but most of the children died. Somewhere during this period he was appointed janitor of the City Hall of Delft, and sandwiched in between his official duties he began his passionate lens-grinding activities which must have

drained off most, if not all, of his spare time. He is reported to have constructed 247 complete microscopes with magnifying powers of from 40 to 270 diameters. These might more accurately be described as simple lenses rather than microscopes. Some 419 individual lenses are credited to his patient grinding activities during his lifetime.

Leeuwenhoek's neighbors did not look kindly on the strange things to which Antony devoted his energies. But Leeuwenhoek, the only man in Holland who knew how to grind pieces of clear rock crystal in such a way as to magnify objects too small to be seen with the naked eye, actually felt sorry for his neighbors and once made the statement: "We must forgive them, seeing that they know no better." Science has not always been a respectable profession, and much laboratory work was conducted behind closed doors. After all, had not Galileo been imprisoned because he dared to suggest that the earth moved around the sun? Burning at the stake was the price paid by some who ventured to cut up a human body in an attempt to discover what made it function.

Learned men of this interesting era spoke Latin, but poor Leeuwenhoek could only read the Dutch Bible. He was a religious man and referred to God as the Maker of the Great All. Because of his relative ignorance, Antony was not fettered with a great deal of nonsense subscribed to by the so-called learned professions. He built up his storehouse of knowledge by employing the scientific method, unbiased by the printed word which was so often based upon fallacy and not upon fact. A half-dozen observations of a given reaction were not sufficient for Leeuwenhoek before he put his findings in writing. Each experiment had to be repeated hundreds of times to eliminate any chance misconception of what had taken place. However, once he felt that he could record his observations, strong-willed Leeuwenhoek could be swayed by no one. Many writings describe the man as being strongly opinionated; perhaps he had every justification for being so sure of himself. Who among these Latin speaking scholars had soiled his hands by working tediously in poorly lighted laboratories to squeeze out one scientific fact from the secrets to be

unfolded to those who pursue the truth? They could read the works of others written in fancy Latin, but they were not scientists in any sense of the word, according to Leeuwenhoek. Just as it is true today, this devoted scholar had little or no time for his family.

From all appearances Leeuwenhoek richly deserves the title often given to him—"Father of Microbiology," although many persons today reserve that honor for Louis Pasteur who lived nearly two centuries later. If a father is one who gives origin, then we must admit that Pasteur, as great as he was, must be relegated to the rank of stepfather since he came after Leeuwenhoek. The majority of persons today give the honor to Leeuwenhoek, and apparently the Society of American Bacteriologists has leanings in that same direction since this group prints a small picture of Leeuwenhoek on the cover of its monthly publication, the *Journal of Bacteriology,* and has done so since the Society was founded in 1899.

The careful records compiled by this man and sent in great volume to the Royal Society of London are sufficient evidence that this microbe hunter was much more patient and much more conservative than many scientists are willing to be before publishing results of their experimentations. We recognize from his drawings many organisms which we associate with certain parts of our bodies, such as scrapings from the teeth. The Royal Society to which Antony sent his observations had a most humble origin. A band of individuals, curious about the surrounding world and strong-willed enough to overcome opposition, risked public ridicule and even death to eke out the truths of science. They joined a sort of secret fraternity and did not come out in the open until the reign of Charles II when they emerged as the Royal Society of London and gained respectability. The membership boasted such names as the founder of the science of chemistry, Robert Boyle, Samuel Pepys, Isaac Newton, Christopher Wren, and others of equal stature in the scientific world.

While most of Leeuwenhoek's countrymen scoffed at his ex-

periments and his boasts of the "beasties" he saw under his lenses, one man, Regnier de Graaf (1641–1673), became truly curious and eventually was accorded the rare privilege of peeping through some of these lenses. To say that the observer was agog would phrase the reaction in mild terms. Having been appointed a corresponding member of the Royal Society for his interesting observations on the subject of the human ovary, de Graaf implored the Society to request a written account from Leeuwenhoek of his unbelievable discoveries. As suspicious and jealous as Antony was, he finally consented to the Society's invitation, and in his humble, unpolished way he wrote a letter entitled "A Specimen of Some Observations Made by the Microscope Contrived by Mr. Leeuwenhoek Concerning Mould upon the Skin, Flesh, etc.; the Sting of a Bee, etc." Quite a title. It is perfectly true that Leeuwenhoek had not mastered the fine art of writing, but what a contribution this man made to biological science! When the Royal Society, in its reserved manner, asked that this original letter be followed by others, they did not have long to wait. Records show that during the next fifty years hundreds of such communications reached the Society from Leeuwenhoek's laboratory in Holland. While he tended to ramble in his writings and loved to discuss topics not always pertinent to the subject at hand, each letter he wrote did contain some gem, or gems, of a scientific nature. Bacteria were first described by him in a letter written on October 9, 1676, to Henry Oldenburg, secretary of the Royal Society. And in a letter written in 1783 he sketched the three principal shapes of bacteria we accept today: the rods (tube-like), the spheres (circles), and the spirals (snake-like).

Insatiable curiosity led this man to examine a wide variety of objects under his lenses, including stagnant water, rain water, scrapings of his teeth and the teeth of perfect strangers when he thought their brown stains might reveal something his own white teeth might not harbor, bodies of a wide variety of insects, the intestinal contents of frogs, horses, and humans, spermatozoa of man and lower animals, human skin, whale fibers, hairs of sheep,

beaver, and elk, wood from many types of trees, etc. He was amazed to discover that the sperms of an ox and of a mouse were much alike in size.

It was not until he examined drops of water, however, that Leeuwenhoek began to see amazing creatures—a thousand times smaller than the limits for the human eye. Since the beginning of time these sub-visible creatures had wreaked their havoc, had killed the innocent child as well as the adult rascal; had played important roles in the essential process of decay and putrefaction, in soil fertility, and in fermentations resulting in the production of wine and other beverages. "They stop, they stand still as 'twere upon a point, and then turn themselves round with that swiftness, as we see a top turn round, the circumference they make being no bigger than that of a fine grain of sand," he wrote. It is always a pleasant experience to observe the reaction of students peering through a microscope and viewing for the first time the nervous activity of the strange, new world in a drop of stagnant water. Students are informed that there are such things in existence, but how would a person react were he living back in the seventeenth century looking at this same drop of stagnant water and seeing sub-visible families that no other person had ever seen before? Leeuwenhoek was jubilant!

As we examine these early records of his observations we find them startlingly accurate. He once wrote that one of his "beasties" was one-thousand times smaller than the eye of a large louse. We have since learned that the eyes of all adult lice are no smaller nor larger than the eyes of sister and brother lice.

His findings in stagnant water drove him mercilessly on in his observations, and he naturally wondered whether these "animalcules" arrived on earth in rain water. Samples of rain water were carefully collected in clean containers, and examination of drops of this fluid revealed no organisms. However, after dust and lint had fallen into his container and sufficient time had elapsed, he was able to show that life abounded in his stored liquid.

While trying to find out what made pepper bite his tongue, he cut the condiment into pieces for easier microscopic examination.

but when these pieces were still too large to be conveniently placed under his lenses, he decided to soak the pepper in water to facilitate cutting into smaller units. Lo and behold, four days later the pepper water was teeming with life. This might be considered as the first bacteriological medium devised for growing organisms in the laboratory. With his calculating mind he reported to the Royal Society that a single drop of his pepper water contained more than 2,700,000 of these little animals—more than the total population of his native country! This was, after all, a rather startling revelation, and scoffers were in the majority. A few individuals, however, remembered how accurate his previous observations had been, and they tried to get Leeuwenhoek to reveal his technic of manufacturing microscopes. He was a jealous man and refused the request, but he finally did condescend to submit his calculations to show how he had arrived at his conclusions relative to the pepper water populations.

People might look at some of his instruments, but touch them—never. In his famed letter of October 9, 1676, to the Royal Society he wrote: "My method for seeing the very smallest animalcules and minute eels, I do not impart to others; nor how to see very many animalcules at one time. That I keep for myself alone."

Barnett Cohen in 1937 offered the following explanation of Leeuwenhoek's success which others at the time could not seem to duplicate. "One can augment the effectiveness of a simple lens by suitably utilizing the inherent optical properties of the spherical drop of fluid containing the objects under observation. The advantages of a water-immersion objective are too well known to require comment, but the added advantage of what amounts to the super-position of a relatively thick meniscus lens (of water) may be worthy of mention. There is apparently no way to prove that Leeuwenhoek did actually employ either of the simple devices set forth above; but certainly, their production was well within the facilities and competence of that clever manipulator."

It is recorded that the Royal Society instructed two of its members—Robert Hooke and Nehemiah Grew—to build the best,

most modern instrument they could devise. It was a memorable day for science on November 15, 1677, when this microscope was presented to the Society and all could see for themselves that the poor Dutch lens grinder from Delft was not fabricating his findings. This instrument did not, however, measure up to the ones that Leeuwenhoek had perfected. An invitation for Leeuwenhoek to become a Fellow of the Society was soon on its way, and he accepted the high honor and promised to serve faithfully during the rest of his life. He never went back on his word. But he never sent them a single microscope. In fact, he possessed one instrument that no one was allowed to even look at—not even members of his immediate family.

In the tail of a small fish he saw capillary blood vessels through which the blood passes from the arteries to the veins. This completed Harvey's discovery of the theory of blood circulation. As Antony watched the blood cells passing through capillaries, practically in single file, a bright idea occurred to him which eventually resulted in probably one of the earliest cures for a hangover. He wrote that after a night of drinking he awoke in the morning feeling sluggish, because his blood thickened, he postulated. Several cups of black coffee in the morning, taken as hot as possible until sweat broke out on his face, made him feel better. If this treatment did not cure his sluggishness by opening up the capillaries or by the thinning of his blood, he felt sure that no prescription by an apothecary could cure the condition either. He chanced to examine some of his teeth scrapings after such a hot coffee episode, and he found that the small organisms scraped from his front teeth no longer exhibited their frisky movements. His back teeth, however, where the coffee had not come in contact at such a high temperature, still showed active organisms when scrapings were examined under his lenses. Selective heating in flasks revealed the truth of his suspicions that the organisms could be killed by heat.

Of all the microbiologists none was so accurate, none so completely honest, and none had such common sense as Leeuwenhoek,

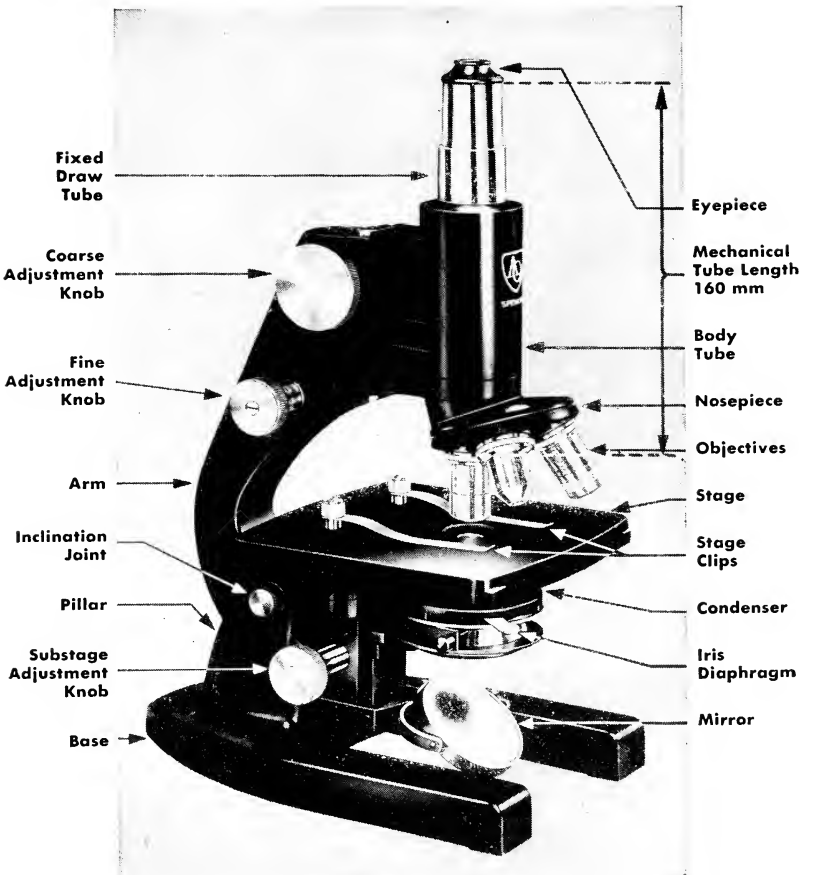


Fig. 3. Parts of the compound microscope. (*Courtesy of the American Optical Company, Instrument Division, Buffalo, New York.*)

but when he died in 1723 at the age of ninety-one, this field of science went into a dormant stage for almost 150 years. Competition stirs activity. Leeuwenhoek was not in competition with anyone except himself. Had he cooperated with others, microbiology might have used his work as a springboard, instead of waiting until Louis Pasteur (1822–1895), Robert Koch (1843–1910), and others gave it the necessary impetus during the Golden

Age of Microbiology, commonly designated as the period between 1850 and 1900.

As we increase the magnification of our lenses with a light-type instrument, we must have a greater concentration of light if we are to see our objects clearly. An important contribution in this direction was the introduction of the immersion lens which provides a homogeneous refraction system for the light as it passes from

Fig. 4. Ferdinand Cohn (1828–1898). (*By permission from* Introduction to the Bacteria, *by C. E. Clifton. Copyright, 1950. McGraw-Hill Book Company, Inc.*)

below up through the lenses. In other words, by placing a drop of oil on top of the preparation on the slide to be examined, if the oil has the same index of refraction as the lenses of the microscope, the light coming from below will not be lost after hitting the object, but will continue through the oil and will be reflected through the objective lens of the instrument. Along with this improvement, the perfection of the substage condenser by Ernst Abbe (1840–1905) about 1870 made possible a more brilliant illumination of the microscopic field.

With the use of improved microscopes observers were better

able to study in more detail the finer characteristics of bacterial morphology which spurred schemes for attempting to classify these newly discovered living forms. A German botanist, Ferdinand Cohn (1828–1898), worked out between 1872–1876 the first scheme for classifying bacteria as plants rather than as animals. Can we

Fig. 5. Table model electron microscope. (*Courtesy of Radio Corporation of America, RCA Victor Division, Camden, New Jersey.*)

say that modern bacteriology began with this piece of work? At this point the useful magnification of instruments was quite similar to those employed today. A clear view of an object is not possible when the object is smaller than half the wavelength of the light being used to illuminate the field. This sets the limitations of

light-type microscopes and explains why we can't just keep setting up stronger and greater numbers of lenses to raise magnifying powers of the instruments. When we employ light of shorter wavelength, such as ultra-violet light, as our source of illumina-

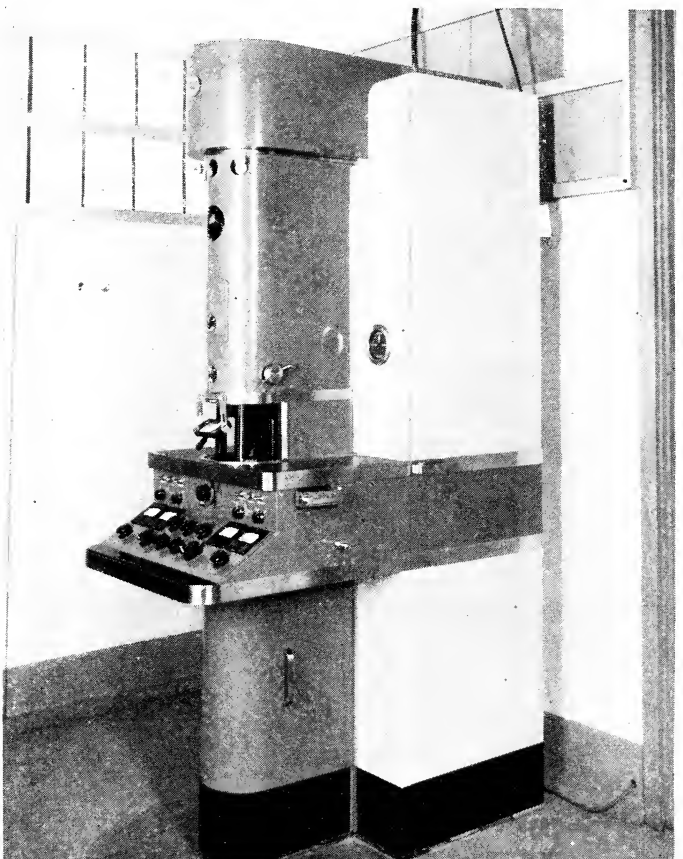


Fig. 6. Universal type electron microscope. (*Courtesy of Radio Corporation of America, RCA Victor Division, Camden, New Jersey.*)

tion, we can increase the magnifying power about two or three times that of ordinary light instruments. However, even with ultra-violet light we are unable to photograph these particles which we now call viruses.

Research continued in an effort to devise some means of increasing useful magnifications without sacrificing resolving power (the ability to detect small objects that are close together), and this resulted in the development of the electron microscope in which electrons replace light waves. The wavelength of an electric beam is about 1/100,000 that of light, and the Radio Corporation of America developed an electron scope with magnification as high as 100,000 diameters in one of the earliest attempts to employ this new principle. Recent improvements have pushed these limits up to 200,000 diameters, whereas the practical limits of optical scopes is 2500–3000 diameters. Certain magnetic and electric fields act on an electric beam in the same manner that a lens acts on a light beam. High velocity electrons and electromagnetic or electrostatic "lenses" serve in place of the condenser, objective, and ocular of optical instruments. The image produced is viewed on fluorescent screens or is registered on a photographic plate.

THEORIES CONCERNING SPONTANEOUS GENERATION

Having developed the tools necessary to see these microscopic plants we know as bacteria, a second major problem confronting biologists was the question of the origin of life. Could living things arise spontaneously from dead matter (ABIOGENESIS), or do all living things have to have parents (BIOGENESIS)? While attempting to thrash out the solution, many discoveries were made which were incidental to the main objective, but which contributed greatly to the over-all growth of the science of microbiology.

It is just as true today as it was centuries ago that certain persons greatly influence the thinking and the beliefs of their time. Almost all so-called scientists from the time of Aristotle (384–322 B.C.) to the middle of the nineteenth century, believed that animals could be generated from non-living matter. The ancient teachings of Aristotle were accepted, unfortunately, and because of the man's stature, progress along certain lines was materially retarded. According to this Greek naturalist, living things were the result of passive *matter* and active *form*, the latter

representing the soul. Only after the soul enters the matter, does life originate. He said in 354 B.C., "Animals sometimes arise in soil, in plants or in other animals."

Facts are established by repeated, confirmed observations. But as stated in the introduction of this book, scientific facts remain only until something proves them to be otherwise. The develop-

Fig. 7. Francesco Redi (1626–1697). (*From* Elementary Bacteriology, *J. E. Greaves and E. O. Greaves, 5th. ed. Copyright 1946, W. B. Saunders Company, Philadelphia.*)

ment of newer knowledge may completely nullify previously accepted facts.

Some unusual recipes for creating living forms are inserted here for your edification. They were based upon repeated observations and were accepted at the time. Publius Vergil (70–19 B.C.) in the *Georgics* suggested this technic for producing swarms of insects: "First, a space of ground of small dimensions is chosen; this they cover with the tiling of a narrow roof with confining walls, and add four openings with a slanting light turned toward the four points of the compass. Then a bullock, just arching his horns upon his forehead of two years old, is sought out; whilst he struggles fiercely, they close up both nostrils and his mouth; and

when they have beaten him to death, his battered carcass is macerated within the hide which remains unbroken. Then they leave him in the pent-up chamber, and lay under his sides fragments of boughs, thyme, and fresh cassia. This is done when first the zephyrs stir the waves, before the meadows blush new colors, before the twittering swallow suspends her nest upon the rafters. Meanwhile, the animal juices, warmed in the softened bones, ferment; and living things of wonderful aspect, first devoid of feet, and in a little while buzzing with wings, swarm together, and more and more take the thin air, till they burst away like a shower poured down from the summer clouds; or like an arrow from the impelling string, when the swift Parthians first began to fight." This formula sounds like the hallucinations of an alcoholic, but the "facts" were undoubtedly believed to be true, at least by Vergil.

An interesting comment by Homer indicates that he knew the origin of flies. He put these words into the mouth of Achilles: "But I greatly dread that flies may enter into the mighty son of Menoities through the wounds made by the bronze weapons, and beget worms in him and defile his corpse." The proof of Homer's words was not forthcoming until 1668 when Francesco Redi (1626–1697), poet and physician of Arezzo, showed that if meat was properly covered with gauze, no maggots would develop on the meat, and only when the egg-laying flies gained access to the meat, were maggots able to arise.

Theophrastus Paracelsus (1493–1541), a Swiss medical philosopher, offered his formula for the creation of human beings (homunculi). "Place certain substances in a bottle, stopper it, and bury it in a dung heap. Every day certain incantations must be uttered over the submerged bottle. In time, a small being will appear in the bottle." However, he did admit that he was never successful in keeping the homunculus alive after taking it from the bottle. His instructions, I'm sure you will agree, were rather vague, and he never was able to demonstrate publicly his spontaneous generation.

Should you desire to produce mice, Jean Baptiste van Helmont (1577–1644), a physician and alchemist, offered this: "Place a

dirty shirt in a vessel containing wheat, and after twenty-one days' storage in a dark place, to allow fermentation to be completed, the vapors of the seeds and the germinating principle in human sweat contained in the dirty shirt will generate live mice." An English naturalist says of the views of a doubter of abiogenesis: "So may we doubt whether, in cheese and timber, worms are generated, or if beetles and wasps in cow dung, or if butterflies, locusts, shell-

Fig. 8. Theophrastus Paracelsus (1493–1541). (*From* Elementary Bacteriology, *J. E. Greaves and E. O. Greaves, 5th. ed. Copyright 1946, W. B. Saunders Company, Philadelphia.*)

fish, snails, eels, and such life procreated of putrefied matter which is to receive the forms of that creature to which it is by formative power disposed. To question this is to question reason, sense and experience. If he doubts this, let him go to Egypt, and there he will find the fields swarming with mice begot of the mud of Nylus, to the great calamity of the inhabitants."

These examples are enough to give you an idea of how fantastic concepts can become when a limited bit of knowledge is available. In the light of our present information we can partially explain how many of these recipes for creating life resulted in the evolution

of animals under the conditions set forth by these early workers. Other formulae are probably pure fabrications.

After Leeuwenhoek and others enlightened the world with the discovery of bacteria, theories concerning the origin of these small (microscopic) forms of life were soon forthcoming, just as there had been explanations for the creation of large (macroscopic) visible forms of life. John T. Needham (1713–1781), a Roman Catholic priest, firmly believed that a "productive" or a "vegetative" force was responsible for the creation of living things. This was in opposition to Georges Buffon (1708–1788), the naturalist, who felt that all life possessed certain chemical constituents in common. After death, he postulated, these constituents were released and remained very active until they could locate and combine with other similar particles and form a new microscopic organism. Needham was one of the first research workers to conduct scientific laboratory experiments in support of abiogenesis, and the Royal Society was convinced by his proof of the theory, which resulted in his election as a Fellow of the Society. Not to be outdone, the Academy of Science in Paris made him an Associate Member of their organization.

These honors irked Lazzaro Spallanzani (1729–1799) who repeated the work of Needham and arrived at the opposite conclusion—life does not arise spontaneously. Needham had boiled meat juice or vegetable infusions in corked flasks, and upon standing he found that life had been generated in these containers. Spallanzani (the maker of the doll in Offenbach's opera, *Tales of Hoffman*) boiled his infusions for a longer period and then sealed the openings of his flasks in a flame. None of these revealed spoilage. To quote Spallanzani: "I used hermetically sealed vessels. I kept them for one hour in boiling water, and after opening and examining their contents, after a reasonable interval I found not the slightest trace of animalcules, though I had examined the infusion from nineteen different vessels." This might be considered to be the first laboratory proof that abiogenesis was not founded upon fact. He criticized Needham for using such porous material as cork which allowed the entrance of microorganisms into his boiled

infusions, especially during the cooling stage when negative pressure within the flasks tended to suck contaminated air into the vessels.

Spallanzani, exhibiting a truly scientific approach to the problem, tried to beat his own theory disproving spontaneous generation. His critics claimed that in boiling his infusions for such a long time, he had devitalized the substrate and organisms could not grow. Spallanzani took some seeds which he had found to be

Fig. 9. Lazzaro Spallanzani (1729–1799). (*From* Fundamentals of Bacteriology, *M. Frobisher, 4th. ed. Copyright 1949, W. B. Saunders Company, Philadelphia.*)

good food for microbes, and he roasted these seeds until they were black—certainly devitalizing them, if such was the case. When he added water to this charred medium, he found that access to air soon provided the necessary germs which readily multiplied in his seed infusion. This proof was finally accepted by most persons, and he was proclaimed all over Europe, but a few persisted in their denunciation of the man because they felt that sealing the flasks had removed a vital force in air necessary for microbial growth.

To combat this criticism Franz Schultze (1836) passed air through his infusions after forcing the air through caustic potash

and sulfuric acid to filter out any suspended organisms. He aspirated his flasks daily for three months, and at the end of that period no flasks exhibited the slightest suggestion of microbial growth. Theodore Schwann (1837) passed the air through heated tubes before it was allowed to come in contact with his boiled infusions, and he showed that growth was absent. Some biological historians credit Schwann as founder of the science of disinfection. It was only natural that opponents should accuse these two workers of chemically devitalizing the air by the chemical treatment and by the heating technics they employed. One of the greatest contributions to microbiology resulted from this argument when Schroeder and von Dusch (1853–1854) suggested the use of wool stoppers in flasks to allow ready access of air without devitalizing it in any way. These wool plugs are capable of mechanically screening out the tiny microbes, and the bacteria-free atmosphere can provide the necessary vital factors for growth of any organisms present in the infusions. As long as these plugs are kept dry, they are effective, but wet stoppers allow migration of organisms through an otherwise effective filter. Today laboratories throughout the world employ non-absorbent cotton as a standard procedure for stoppering test tubes and flasks to be used in the cultivation of microorganisms.

To add weight to Spallanzani's contention that microbes must come from other microbes, he took a flask of broth containing actively growing bacteria, and he diluted this culture until only a few bacteria were present in each drop of infusion. By placing these drops under his microscope, he was able to observe that simple method of reproduction which we now call BINARY FISSION—equal splitting. These cells became longer, pinched in the center, and finally separated into two organisms. He was probably the first person to observe this process, but his keen observations were for the most part lost in the many controversies relative to spontaneous generation. The pressure being exerted on science for more and more proof of commonly accepted theories spurred microbiological progress, just as the pressure of war results in rapid expansion of practically all scientific knowledge.

Useful applications of Spallanzani's discovery were made by Nicholas Appert (1750–1841), a French confectioner, in 1810 when the French government offered 20,000 francs to the first person who could perfect a method for preserving food. He founded the modern canning industry when he demonstrated that food placed in sealed containers and boiled for suitable time periods could be stored indefinitely without spoilage. Appert did not try to explain how this technic worked, but Joseph Gay-Lussac (1778–1850) stated that air was necessary before fermentation and spoilage could occur. Since Appert's containers were sealed when hot, a vacuum was created, and both Joseph Priestley (1733–1804) and Antoine Lavoissier (1743–1794) had previously contended that oxygen, a constituent of air, was essential for life.

In spite of mounting evidence that microbes must have parents, Felix-Archimede Pouchet (1800–1872), a famous naturalist and member of the French Academy of Sciences, lead concerted attacks in support of the theory of abiogenesis. The Academy had made an offer in 1860 to anyone who could, by scientific methods, provide indisputable proof one way or the other on the question of spontaneous generation. When Pouchet gave a paper before the Academy presenting his views, many influential scientists rallied to his support and Louis Pasteur was compelled to make public his opposite point of view. Pasteur insisted that air contained the necessary spark that reproduced cells. Microbes ride through the air on dust particles, and he insisted that a dust-free atmosphere harbored no microbes. We know today, however, that droplets expelled from the nose and throat of man and lower animals can also contain bacteria, even in the absence of dust particles.

An English physicist, John Tyndall (1820–1893), added support to Pasteur's claims when he demonstrated that an open vessel containing a fermentable infusion would remain sterile when placed in a dust-free, or optically empty atmosphere within a chamber. By passing a beam of light through his chamber, Tyndall was able to see whether motes were dancing in the light beam. If no motes were evident to the eye, he found that there was no growth of organisms in his infusions.

Pasteur felt that since bacteria cannot walk, they must have vehicles to carry them. Dust particles provide this necessary transportation. Pouchet's rebuttal is contained in this quotation: "How could germs contained in the air be numerous enough to develop in every organic infusion? Such a crowd of them would produce a thick mist as dense as iron."

Undoubtedly the first air analysis experiments were those performed by Pasteur as he added new evidence in support of his dust-borne theory of microbial dissemination. He noted that when he broke the tip from his flasks sealed with their infusion contents while hot, air rushing into the vessels confirmed the existence of a vacuum. This observation gave Pasteur an idea which he was to employ later in analyzing different air samples. If dust carried microbes, then the streets of Paris on a windy day were certainly a good source of organisms. So he prepared a number of such sealed flasks and opened them in the streets of the French Capitol. Every flask revealed microbial activity upon incubation. Similar flasks opened in the relatively calm, dustless atmosphere of his cellar showed some infusions positive for growth, while still others remained sterile. Even fewer flasks were positive when the vessels were opened on the Jura Mountains at an altitude of 2500 feet, and only one flask in 20 showed growth when the vacuum of the flasks was broken in the clear air on the Mer de Glace at a height of 6000 feet. Pouchet performed similar experiments, but every one of his containers opened at the 9000 foot level of the Pyrenees showed growth of microorganisms. Pouchet became so incensed when Pasteur continued to report results supporting the dust theory, that Pouchet challenged Pasteur to a duel, which never materialized. Wouldn't present day science leap ahead if every time research workers disagreed the issue had to be settled with drawn swords? It would be a boon to the sword manufacturers, but science would undoubtedly lose many brilliant scholars whose adeptness with the sword was limited!

How can these opposite results obtained by these two workers be explained? The one difference in their research was the medium employed for growing the organisms. Pasteur used sugar,

yeasts and water, a relatively easy medium to sterilize. Pouchet, unfortunately, had chosen a hay infusion as his substrate, and we know that such material abounds with SPORES, those resistant bodies so difficult to kill by mere boiling for the usual time periods. Pouchet's experiments did help eventually to prove that spores are more resistant to heating than are vegetative cells, but he did not have spontaneous generation. These tough endospores help to explain the many irregularities obtained by the pioneers as they fumbled and groped with ideas and technics in their search for proof of one theory or another. The great resistance of these spores to heat was not conclusively demonstrated until 1877 when Ferdinand Cohn described them in the so-called hay bacillus, *Bacillus subtilis*. Cohn also showed that spores could be made to germinate into vegetative cells which were not as resistant to boiling and to treatment with chemicals as were the spores. In fact, our present methods for sterilizing materials in bacteriological laboratories and in hospitals are based upon the time, and the temperature, necessary to inactivate these resistant stages in the growth of some, but not of all, bacteria.

Although Pasteur was essentially a chemist, he had a flair for microscopy, and his ability to see through the clouds of misconceptions to the clear air of reality led him to some of the greatest discoveries in biology up to that time. To overcome all criticism about devitalizing the air by heating it, by passing it through strong chemicals, or by screening it through such an innocuous substance as wool or cotton plugs, Pasteur placed a fermentable substrate in a flask and boiled this medium for a sufficiently long time to insure destruction of all living things in this infusion. He then heated the neck of the flask in a flame and drew the glass out into an S-shaped curved capillary tube which he left open to the outside air. Since microbes cannot ambulate, he postulated that it would be impossible for contamination of his infusion to occur unless he tilted the flasks and allowed some of the sterile liquid to come in contact with the tip of the capillary tube containing dust particles. Others had amply demonstrated that boiling infusions did not devitalize them, and since ready access to vital

substance in the atmosphere was not impaired, microbes inclined to generate spontaneously had every opportunity to do so in his open flasks. We know that no growth took place until Pasteur either broke off the neck of his flasks or until he tilted the contents

Fig. 10. Louis Pasteur (1822–1895). (*Courtesy of the Central Scientific Company, Chicago, Illinois.*)

into the dust-laden tip. This was conclusive proof that spontaneous generation was a myth, and the arguments of his opponents had been nullified once and for all. Some of these flasks are still on display at the Pasteur Institute in Paris, France, and they remain bacteria-free to this day. Pasteur was hailed as a hero and

the French Academy awarded him in 1862 the prize they had offered in 1860 to the first person who could prove or disprove abiogenesis.

One of the highlights of Pasteur's life occurred on April 7, 1864, when he delivered his now-famous address at the Sorbonne in Paris in defense of his disproof of spontaneous generation: "There is no condition known today in which you can affirm that microscopic beings come into the world without germs, without parents like themselves. They who allege it have been the sport of illusions, of ill-made experiments, vitiated by errors which they have not been able to perceive, and have not known how to avoid." In another passage he described how he watched his flasks, pleading for them to give him a sign of life, and could not . . . "for I have kept from them, and am still keeping from them, that one thing which is above the power of man to make; I have kept from them the germs which float in the air; I have kept from them life." He postulated his germ theory when he stated so concisely that life is the germ and the germ is life.

THEORIES CONCERNING FERMENTATION

Another overwhelming problem needing clarification and sound proof before microbiology could become a firmly established science was the riddle of fermentation. What initiates this change in fruit juices and other sugar-containing substances, and what maintains the reaction? Is fermentation the same as decay? Once bacteria had been discovered many persons pondered over the question revolving around whether bacteria were the cause or the direct result of fermentation.

We can find Biblical references relative to the transmissibility of ferments, including the "little leaven that leaveneth the whole loaf." Since earliest times man has employed the process of fermentation for making bread rise, for souring milk, and for making alcoholic beverages without knowing how it all came about. Successes and failures could never be explained. However, until the nineteenth century was well along, we had little, if any, concrete evidence on the matter. Cagniard-Latour in 1836, Theodore

Schwann (1810–1882) in 1837, and Friedrich Kützing (1807–1893) in 1837 independently reported that yeasts play a role in the process of fermentation. Witnessing budding in yeast water, Charles Cagniard-Latour (1777–1859) referred to these objects as living substances. Ferments, as far as he was concerned, were composed of cells susceptible to reproduction by a sort of budding process, and these living objects were capable of acting upon sugars through "some effect of their vegetation." Schwann described yeasts as vegetative germs. These adherents of a biological theory to explain fermentation were scorned by most scientists of their day because the biological explanation was in direct contradiction to the physico-chemical theory of that renowned German organic chemist, Justus von Liebig (1803–1873). Von Liebig held sway from 1840–1860, and few individuals dared to question his decisions. When he announced that microbes were not the cause of fermentation, his faithful followers went along with the idea and helped to perpetuate the untruth. Molecules are in a constant state of motion—are chemically unstable—according to von Liebig, and when small amounts of decomposing stuff are mixed with fresh fermentable material, a chain-like reaction is initiated and continues until the fermentation is complete. Fermentation was believed to be a natural physical decomposition of large molecules with bacteria and other organisms capitalizing on this more readily available food supply for their metabolism. This supported the concepts of Georg Stahl (1660–1734) expressed in 1697 when he suggested that the process of fermentation was the result of the shattering of molecules by forces either from within or from without. Just what set off the reaction was never made quite clear by any of the proponents of the mechanistic theory. In 1869 when Berzelius, the renowned Swedish chemist of Upsala, supported the mechanistic approach with his explanation that it was due to contact of catalytic forces, things looked dark for the opponents.

The genius of Louis Pasteur arrived on the scene in time to set up one of the more famous controversies in microbiology: Pasteur's biological vs. von Liebig's mechanistic theory of fermentation. Believing that the proof of such knotty problems lay in the experi-

mental method, Pasteur went to work in his laboratory. He did not sit down and try to explain reactions until he had enough careful experimentation to back up his contentions. He was a Professor at the University of Lille, France, right in the heart of the wine and beer industries, and just at this time France was experiencing serious wine spoilage which no one seemed able to control. Pasteur was commissioned to undertake a study in the hope that a "cure" might be found for the "disease" of the wine. In his investigations he found that each type of microbe will produce a predictable ferment. Pasteur's first paper on fermentation appeared in 1857. He described a grayish color in sugars undergoing fermentation, and his microscopic observations revealed the presence of small globules or short rods which, when transferred to fresh sugar solutions, perpetuated the process. Heating solutions so inoculated resulted in no fermentation. He stressed that spoilage of wine could be directly attributed to the action of certain microbes which produced undesirable end products and "diseased" the wines. By selectively heating the fresh juice after it was bottled, such diseases could be prevented. This prescribed heating has since been given the designation of PASTEURIZATION.

In further studies on fermentation, Pasteur reported that among other end products in the reaction was amyl alcohol. If von Liebig's theory were true, then amyl alcohol should be a constituent of sugar merely waiting release when the larger molecules shattered. This discovery made such a theory untenable since amyl alcohol is too different from sugar in its structure. Lavoisier and Gay-Lussac had reported that the weight of carbon dioxide gas and alcohol formed in sugar fermentation was practically equal to the weight of the sugar. By a series of clever experiments, Pasteur was able to give uncontestable proof that fermentation was a biological process, initiated and perpetuated by living substances.

Moritz Traube (1826–1894) in 1858 was apparently the first person to suggest the existence of ENZYMES, those remarkable digestive juices so essential to all life. The work of Edward Buchner (1860–1917) in 1897 confirmed the enzyme theory when

he was able to demonstrate the presence of zymase, the ferment which attacks glucose. A temporary wave of excitement arose when Buchner demonstrated that a yeast-free extract could still cause fermentation—apparent evidence in favor of von Liebig's original non-biological concept. But it had to be admitted by even the most ardent supporters of the mechanistic theory that the enzymes had originally come from a living cell; not any old juice could start the reaction on its way.

During the course of his fermentation studies, Pasteur discovered ANAEROBES, those interesting microorganisms which grow in the absence of free atmospheric oxygen, in contrast to the aerobes which can develop only when free atmospheric oxygen is available. Fermentation is life without air, according to Pasteur. The first step involves growth of the organisms, and air is the source of assimilated oxygen. Alcohol production in this stage of fermentation is insignificant. During the second stage the yeast is compelled to act upon the sugar in the absence of atmospheric oxygen, and the essential gas is abstracted from the sugar. Pasteur's final conclusions were that (1) ferments are living organisms, (2) each ferment is produced by a special organism, and (3) ferments are not formed spontaneously. Liebig and his proponents believed fermentation to be a function of death, but Pasteur proved it to be a function of life.

If wine could be diseased, the next logical step was to assume that human beings and other living things could also be afflicted as the result of specific microbial activity. It is of interest to note that Robert Boyle (1627–1691) in 1663 had stated that until the nature of fermentation was clearly understood, we could hardly expect a logical explanation of disease. As a direct result of the Pasteur–von Liebig controversy, the solution of many other problems, including the disposal of wastes, the purification of water, etc., were given a decided impetus, and progress was recorded. Lord Joseph Lister (1827–1912), a surgeon of Glasgow, Scotland, was so impressed with Pasteur's conclusions with respect to fermentation that he decided to apply these same biological principles

to the fermentation of wounds which too often were the aftermath of surgery. Could he kill the fermenting agents before they could destroy flesh?

An Institute was erected in Paris in honor of Pasteur, and the building was dedicated in 1888. Pasteur was its first director, and he remained there until his death on September 28, 1895. He was succeeded by Emile Duclaux (1840–1904) who made the Institute

Fig. 11. Joseph Lister (1827–1912). (*Courtesy of Kelly and Hite,* Microbiology, *Appleton-Century-Crofts, Inc., and Ethicon Suture Laboratories, Inc., New Brunswick, New Jersey.*)

a great research center for scholars from all over the world. A scientific journal was begun by Duclaux in 1887, before the Institute actually was dedicated, and this famous journal is still devoted to the publication of articles relative to microbiology and related fields.

THEORIES CONCERNING DISEASE

Another vital concept that had to be crystallized before microbiology could emerge on a firm foundation was the *etiology* (cause) of disease. Without reviewing the minute history of this phase of our science, and eliminating the evil spirits as the cause of man's

miseries, we can note that the ancient Greeks suggested worms, too small to be seen, as the explanation of disease. Marcus Varro, in about 100 B.C., expressed the idea that invisible animals are carried through the air and enter the body by way of the nose and mouth. The Italian physician, Hieronymus Fracastorius (1483–1553), in 1546 postulated the theory of contagion, but since he had not actually seen the inciting agents, his thoughts represented pure

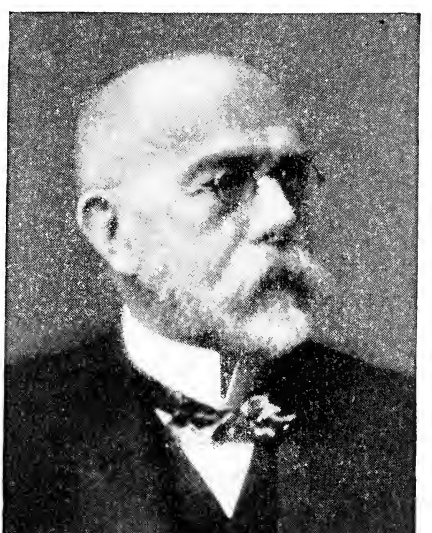


Fig. 12. Robert Koch (1843–1910). (*By permission from* Introduction to the Bacteria, *by C. E. Clifton. Copyright, 1950. McGraw-Hill Book Company, Inc.*)

speculation. Some two hundred years later (1762) an Austrian physician, Marcus von Plenciz (1705–1786), put forth a new concept that not only were diseases caused by microscopic organisms, but each disease was caused by a specific germ capable of being transmitted to other individuals via the air.

Glossing over these early suggestions, we come to the year 1840 when Jacob Henle (1809–1885), a German pathologist, laid down the principles for our present germ theory of disease which led directly to the fundamental work of Robert Koch (1843–1910).

Koch emphasized the importance of proving concepts by employing the scientific approach of actual laboratory experimentation. Before an organism could be said to be the cause of a specific disease, the agent had to fulfill the following postulates which Koch formulated in 1882:

1. The suspected organism must be found in every case of the disease.
2. The organism must be isolated in pure culture from every case of the disease.
3. These isolated pure cultures, when introduced into susceptible animals, must be capable of reproducing the original disease in its typical clinical form.
4. The same organism must be re-isolated from the injected test animal.

If the postulates were fulfilled, Koch was willing to admit that the cause of that particular disease had been proven. We should point out here, however, that Koch's postulates cannot be applied in some cases. When you try to select a *susceptible* animal in postulate three, you find that human beings are the only animals you can use in some diseases and that is not always practical. It can be said, however, that accidental laboratory infections have permitted proof of the etiology of some diseases, such as typhoid fever, which man alone seems to contract. The strictly parasitic nature of such microorganisms as the leprosy organisms does not allow their isolation on ordinary laboratory media, and that sets up a block in the necessary cycle of proof. There are some diseases in which the clinical symptoms are the result of multiple infections, and trying to prove that a single organism is the cause of the disease may lead to confusing results. The common cold, for example, probably is initiated by a virus, or by viruses, but the misery of a cold seems to be associated with the activity of secondary invaders, usually bacteria, which are opportunists.

In 1863 Casimer Joseph Davaine (1812–1882) reported that the blood of animals infected with anthrax contained rod-shaped organisms which could be transferred by blood from infected

animals to the blood of healthy animals and could cause the same disease. Pasteur reported similar findings with respect to silk worm disease caused by protozoan parasites. Robert Koch confirmed the findings of Davaine and expanded materially on the subject as we shall see later in the chapter.

Because Koch is considered high on the list of famous microbiologists, second probably only to Pasteur, his accomplishments deserve more detailed evaluation than many other workers mentioned in this chapter on the highlights of microbiology. He was a rural practitioner in Wollstein, Germany, and at the time that news of Pasteur's work reached him, he was employed at an insane asylum in Hamburg. The potential possibilities in this new biological field intrigued him, and his tendency to let his medical practice fall by the wayside became more pronounced with the passage of time. His first microscope was a present from his wife on his twenty-eighth birthday.

In this dynamic period during the latter half of the nineteenth century, Koch became increasingly alarmed at the tremendous economic loss being incurred as the result of anthrax infection in domestic animals. Those who could least afford to lose their animals—in some cases the family's sole support—seemed to be taking the brunt of this unconquerable disease. Perfectly healthy-looking sheep would die during the relatively short space of a single night, and postmortem examination would reveal the telltale black blood so characteristic of anthrax. The farmer, or members of his family, might contract horrible-looking boils, and in some instances they would contract the pneumonic type of the disease from breathing in the infectious agent, with painful death culminating their losing battle with respiration.

Koch laboriously examined untold numbers of blood specimens drawn from healthy sheep and he compared these samples with the blackened blood of the stiff carcasses of the infected animals. Without exception he found that the blackened blood contained rod-like sticks among the remaining undissolved blood-cells, but these rods were never found in blood from healthy sheep. Since those rods exhibited no locomotion, Koch was hesitant about calling

them microbes. We know today that typical anthrax bacteria are non-motile, but movement was accepted as an important criterion of microbes by many workers in those days.

Being a man of modest means and unable to equip his laboratory with large numbers of animals for experimentation, Koch was

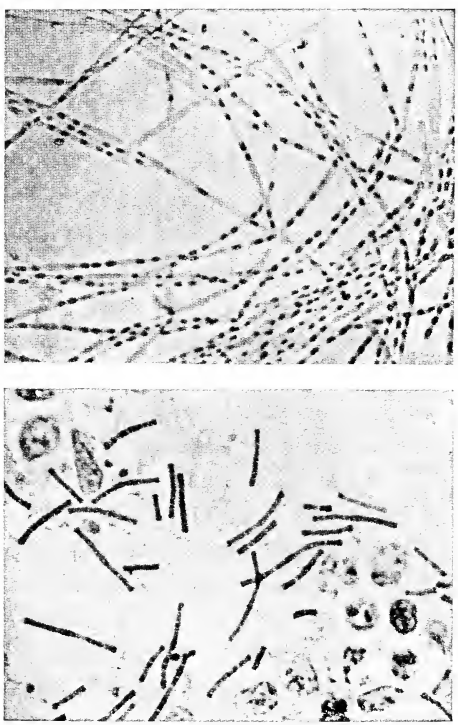


Fig. 13. Koch's photographs of anthrax bacilli. (*From* Fundamentals of Bacteriology, *M. Frobisher, 2nd. ed. Copyright 1942, W. B. Saunders Company, Philadelphia.*)

obliged to conduct much of his early research under field conditions. This added complications but it also added weight to his final conclusions. Mice were available and were relatively cheap to maintain, but he wasn't even sure that they were susceptible to this disease. He proposed to inject some of the infected blood containing these rod-shaped "things" into mice, but with no con-

venient syringe handy for the injection, he was obliged to improvise, an important necessity for those individuals who do extensive research. If these rods were really microbes, he hoped that the mice would contract the disease and show active multiplication of the organisms within the body. His improvised syringe turned out to be splinters of wood, sharpened to a point, washed thoroughly, and heated in a drying oven to destroy any other microbes the wood might harbor. He made a small slit with a knife at the base of the mouse's tail and inserted the sharpened splinter which had been dipped into the blood of a recently expired sheep that had been suffering from anthrax.

After a night's sleep, which we can presume was probably not too restful as he pondered over his problems, he returned to his laboratory and examined the injected mice. They were all dead, and at autopsy he found the internal organs and the blood to be harboring these rod forms. Whereas he had injected only a few microbes, there were millions of them to be seen in the dead mice, an indication that active multiplication had occurred. Most persons would have accepted this as conclusive proof that the rods were the etiological agent in the disease. Koch, being a cautious research worker, repeated his experiment over and over again, transferring the blood of successive dead mice to new healthy mice using his splinter technic. The results always came out the same.

At this juncture Koch hit upon an idea which was to develop into a simple yet vital technic in microbiology. He wanted to cultivate his suspected organisms in such a manner that he could watch actual multiplication occurring before his very eyes. If he could see this step in their development, he felt certain that he was on safe ground if he announced that anthrax was caused by a microbe which grew and multiplied within the susceptible animals. For some reason known only to Koch, he decided to place some liver from an infected mouse into some fluid drained from the eye of a recently slaughtered ox. We know today that such body fluids are normally free of bacteria, and are an excellent culture medium for growing organisms away from living hosts.

For hours he watched intently to see whether these rods under his microscope changed their size or their shape, but nothing happened. Then suddenly he was able to observe that the rods were becoming longer, and these elongated forms eventually broke in half and formed two short rods. These short rods in turn lengthened and subdivided by splitting in the very center, until within a few more hours he had many times the number of organisms that he started out with in his ox-eye fluid medium. This was truly an astounding discovery. Here was an organism probably one-billionth the size of a sheep. By gaining access to the internal organs of a sheep, the microbe could set up housekeeping, settle down, and have a family within a relatively few minutes, and before a single day had passed, untold numbers of offspring could point with pride to their parent who came over on a blade of grass. The multiplication of rabbits is put to shame when compared with the ability of bacteria to reproduce several times during a single hour! When these overwhelming hordes of microorganisms had parasitized enough cells of the sheep, the afflicted animal could no longer overcome their deadly effect, and the sheep succumbed.

After growing these bacteria away from the originally infected sheep for generation after generation, would the far-removed offspring be able to establish themselves once more in the species of animal from whence they originated in his experiments? This was most important for Koch to know, and he promptly set about to find the answer. He still had some of the mice he had killed with blood-soaked splinters dipped in the juices of previously sacrificed mice. It had been weeks since these offspring of the original anthrax bacteria had been near sheep. If he could inject some infected mouse blood back into sheep and have the larger animal contract the disease, he felt that he could say with assurance that these microbes were the cause of anthrax. As he hoped, the sheep died with typical clinical symptoms of the disease. Not wanting to shout from the housetops that he had solved the problem, Koch very cautiously sent out feelers by casually telling some of his intimate friends and colleagues what he had done. They naturally raised questions which he could not always answer to their satis-

faction. One of these embarrassingly perplexing problems was that of the "curse" of some fields in which grazing sheep died like flies long after any infected sheep had succumbed on the premises. If bacteria were spread from one person to another or from one animal to another, how did Koch propose to explain the infectivity of these grazing lands after the broiling sun had baked the area for months and the freezing winds of winter had subjected the land to such rigorous treatment? At the moment Koch had no answer, but he proposed to find the explanation if one was to be had.

It was a puzzling situation indeed, for had he not seen these same organisms shrivel up and disintegrate on his glass slides when they were allowed to dry? Certainly that would indicate that the bacteria were dead and no longer able to generate their own kind. Fortunate accidents have resulted in many great discoveries, and fate decided to step in at this juncture and give the answer to a man who was clever enough to capitalize on an accident. One of his ox-eye fluid cultures happened to be left out on his laboratory table for a twenty-four-hour period. Normally such preparations would have been discarded in some chemical fluid, but Koch decided to take just one more look at this dried specimen under his microscope before discarding the slide. Lo and behold, his thread-like germs had been transformed into a string of glistening bead-like structures. Upon closer examination he discovered that these beads were within faintly outlined rod-like objects, slightly suggestive of his original organisms. At the time he felt that some contaminating bacteria had found their way into his carefully prepared slides, so he didn't think too much about it. Nevertheless, he did, again quite by chance rather than by design, keep his slide for several more months before he came across it once more and decided to confirm his original observation of the beads. Things had not changed in the interval, the beads were there glistening as before. He added some fresh ox-eye fluid to the dried slide and watched under his lenses to see what these supposedly contaminating forms would turn out to be. Within a few hours he really was jolted when before his eyes he watched the individual beads dis-

appear and turn into the original rod-shaped microbes so characteristic of the ones in the anthrax-infected animals. Like a flash, the truth of the situation occurred to him. Here was the answer to the "cursed" pasture land; these beads were spores capable of withstanding drying for extended periods of time, and the rigors of winter could not kill them. Those pasture lands were contaminated with resistant forms of these bacteria, and when sheep grazed on these fields, they ingested the spores and became infected. Now he was ready in 1876 to announce to the world the cause of anthrax.

With self-assurance for the first time in many long, arduous months, Koch determined to journey to Breslau to visit an old friend who had encouraged him during his early ventures in research. This friend was Professor Ferdinand Cohn, the first person to work out a scheme for classifying bacteria in the plant kingdom. He carried along some of his deadly anthrax organisms and some of his mice on this particular trip, since he proposed to show Professor Cohn the completeness of his findings with respect to the deadly disease. Cohn invited anybody who was anybody in the scientific world to attend a lecture by Koch in which he was to present his research findings. Few had ever heard of this man who had been working quietly without the aid of fancy laboratory equipment, but many came to the presentation more out of curiosity than faith in what Koch might have to say. Because Koch was not an outstanding orator, he used the demonstration technic of teaching in place of the lecture method, and he put on a show that amazed the most learned scientists in the audience. He gave his theories and then proved his beliefs with actual animal experimentation. The renowned Professor Julius Cohnheim (1839–1884), who first demonstrated that pus was composed largely of white blood cells, and who was without doubt the leading authority on disease in Europe, was most impressed with the clarity and conclusiveness of Koch's presentation. Koch had converted a disciple! Because of Cohnheim's influence, Koch was compelled to turn away scholars who came in droves to study under his tutelage. When he announced to members of his class, including

Paul Ehrlich (1854–1915), what he had heard and seen relative to the etiology of anthrax, Koch found himself with still other followers who all wanted to join in these investigations of microbes.

This was the year 1876, and Louis Pasteur had made a rather sweeping statement just seven years previously to the effect that man held the power to wipe parasitic maladies from the face of the earth. To say that this pronouncement was scoffed at is to put it mildly. Koch was now leading the fight in that direction, and while we must agree that we still have a long way to go before Pasteur's statement can be fulfilled, the strides we have made would astonish Pasteur, were he to rise today from his tomb in the basement of the Pasteur Institute in Paris.

Koch's reputation continued to grow, and even though the years immediately following his announcement of his anthrax findings were not prosperous ones for this great discoverer, he emerged in 1880 with an appointment by the German government to the position of Extra-ordinary Associate of the Imperial Health Office, with a fine, well-equipped laboratory and with assistants to help him in his research activities. People clamored to be allowed to study under him in Germany, and the list of his pupils is a "Who's Who in Microbiology." Laboratory procedures were in a very chaotic state in 1860, and Koch felt that order had to emerge from this chaos if microbiology ever expected to amount to very much.

If each disease is caused by a single species of microbes, Koch realized that he would have to devise some technic for separating organisms from other organisms. There are few places in nature where a pure culture of an organism can be found. Bacteria are usually mixed with all forms of microscopic and macroscopic life in the keen battles for survival. Fate once again came to Koch's rescue and he was smart enough to capitalize on the chance observation. It seems that a sliced, cooked potato had been left on one of the tables in his laboratory. He happened to observe several colored spots on this potato and his curiousity got the better of him. What are those colored spots? Streaking a little of the pigmented material on a glass slide in a drop of water, Koch was thrilled to find that each spot was composed of a pure culture of

microbes. Nature had provided the answer to the problem of culturing microbes as separate species. If a single microorganism carried through dust in the air, as Pasteur had shown, landed on a cooked potato, it was capable of multiplying into a visible growth called a COLONY. All members of a colony represent the progeny of the original organism and hence we have a pure culture. If he could just work out a few of the details, he had the answer to what might have become a perplexing problem. Finally, with the assistance of two military doctors, Friedrich Loeffler (1852–1915) and Georg Gaffky (1850–1918), he announced that by streaking mixtures of microbes over the surface of a fresh boiled potato free of other organisms, colonies of the bacteria contained in the mixtures could be made to develop on the potato. When these colonies were well-enough isolated, they could be picked from the potato with the assurance that all microbes from such a colony were alike. This was revolutionary!

Before too many organisms had been cultivated, however, Koch began to appreciate that the food requirements of bacteria varied tremendously, some being a great deal more exacting about the diet set before them. Joseph Schroeter (1835–1895) first separated chromogens from each other in 1872 by growing them on such solid substances as potatoes, coagulated egg white, starch paste, and meat, but he ran into difficulty when he tried to cultivate nonpigmented organisms. Koch came along and knew what he wanted—a solid, transparent, sterile medium. Gelatin, as a solidifying agent, seemed to best fulfill these requirements, and since mycologists had been using this material for thirty years, Koch adopted it in 1881, thus revolutionizing bacteriological technic. As is so often true with new ideas, time modifies their original seemingly wonderful characteristics. Gelatin has two major limitations. Some bacteria can utilize the substance as a source of food, thus turning it into a liquid. If one is seeking lytic ferments, gelatin serves a useful purpose, but the enzyme also makes the medium useless from the standpoint of trying to isolate pure cultures on a solid medium. A second serious defect of gelatin is that it is a

liquid at body temperature, that vital warmth needed to cultivate many pathogenic organisms.

At this point let us introduce a man who had worked in Koch's laboratory—Dr. Walther Hesse (1846–1911)—and his wife, Fanny Eilshemius (1850–1934), his faithful technician. He had labored

Fig. 14. Frau Fanny Eilshemius and Dr. Walther Hesse. (*Courtesy of Morris C. Leikind. From the* Journal of Bacteriology, *1939, 37, 487.*)

on the bacteriology of the atmosphere using gelatin as a solidifying agent for his many bouillon concoctions necessary to cultivate the organisms in the air. His maddening failures when the gelatin was attacked by the organisms drove Hesse to seek new solidifying agents. Mrs. Hesse made an epic suggestion for which Koch is often given more credit than is probably due him. She had been using agar-agar (derived from the Malayan *agal-agal,* which

means *very gelatinous*) as a solidifying agent in her own jelly and jam recipes at home. She picked up this technic from her mother, who in turn received it from some Dutch friends who had lived in Java. In the East Indies agar-agar had been employed for generations as a thickening agent for both soups and jellies. Why not try agar-agar in place of gelatin, she suggested to her husband. An historic moment in microbiology was reached by this simple, yet necessary, substitution. An occasional contribution of an unknown individual can make discoveries of lasting value. Oliver Wendell Holmes (1809–1894) stated that medicine learned "from a Jesuit how to cure agues, from a friar how to cut for the stone, from a soldier how to treat gout, from a sailor how to keep off scurvy, from a postmaster how to sound the eustachian tube, from a dairymaid how to prevent smallpox, and from an old market woman how to catch the itch-insect." To this imposing list we can add a housewife who helped her husband solve the perplexing problem of pure culture isolation technics. Without delay this new substance derived from Japanese seaweed (*Gelidium corneum*) was reported to Koch, probably in the latter half of 1881. Koch adopted it and in his famous publication of 1882 in which he announced his preliminary investigation on the tubercle organism (*Mycobacterium tuberculosis*), he made reference to agar-agar in one brief sentence. Fanny Hesse, who had been born in 1850 in New Jersey in the present locality of Jersey City, died in 1934, with few bacteriologists realizing that the credit often ascribed to Koch originated with her suggestion to her research-minded husband.

When our source of agar was cut off during World War II, the United States was obliged to seek a new supply. After some intensive searching, beds of the species *Gelideum cartilagineum* were discovered off the coast of California, and much of our wartime agar came from this source.

By perfecting new technics and by making use of this newfound agar base for his culture media, Koch began a long series of fruitful discoveries, complementing much of the work of his French colleague in microbiology, Louis Pasteur. Koch proved

the etiology of cholera epidemics after his studies in Calcutta, India, where the disease was endemic. The Emperor of Germany bestowed the Order of the Crown, with star, on Robert Koch for his brilliant discoveries. His laboratories in Berlin became the focal point for training laboratory technicians. The germ theory of disease was now firmly established, and Koch, together with his ever-widening circle of trained personnel, began a chain reaction of discoveries. Once we knew the cause of disease, isolated the germ, and found ways to destroy it, we were able to cut out a link in the chain of the progress of disease, giving us the greatest control in the spread of diseases of man in recorded history. What has been accomplished has already been reviewed in the introductory chapter under the discussion of life expectancy since the turn of the twentieth century.

Pasteur had not been idle while Koch and his disciples were busy perfecting new technics, and Pasteur's announcements also began to stir the imagination of men. Lord Lister carried out his first aseptic surgery with the help of a fine mist of carbolic acid playing on the field of operation, after soaking his instruments in the same solution to destroy these germs that Pasteur and Tyndall had so conclusively demonstrated were present in the air. This is the foundation of modern surgical procedures, based today upon a combination of asepsis and disinfection.

We can divide this history of microbiology into three periods. People do not always agree with the limiting dates of these periods, but in general we can say that until the year 1850 most microbiology was purely speculative in nature. From 1850 until about 1900 the important fundamental discoveries were made, preparing for the so-called modern era from 1900 to the present. Too often students of this science feel that everything has been discovered; nothing remains to be done. This may be the tendency of some individuals in the twilight of their lives, but any real student of bacteriology can hardly read a single printed page in a research journal, without realizing that much remains to be done. If one thinks in terms of bacterial diseases, we will have to agree that many of the former scourges of mankind have been fairly-well

checked. But the field of Virology is a young, very young, outgrowth of our science of microbiology, and it hides many secrets.

This chapter was purposely titled "Highlights in the History of Microbiology." Included in the list of persons mentioned were individuals whom Hitchens and Leikind so aptly described as the "bead stringers" in contrast to the "bead collectors." Each bead represents a scientific fact. But isolated facts, to be really useful, must be strung together by those rare individuals who have the insight to string these beads into a useful necklace. Bead collectors are by far more numerous, but this does not detract from their usefulness in science. Other famous bead stringers have played vital roles in the science of microbiology, but we shall weave their contributions into subsequent chapters as we discuss specific phases and applications of their discoveries.

CHAPTER 3

Bacteria Are Classified as Plants

DIFFERENCE BETWEEN PLANTS AND ANIMALS
CLASSIFICATION OF BACTERIA

DIFFERENCES BETWEEN PLANTS AND ANIMALS

Before the perfection of ground lenses opened up a new vista for scientists to explore, it was a relatively simple matter for an individual to distinguish a plant from an animal. After all, an animal was an animal, and it was a mighty peculiar person who could not tell a plant from an animal! The microscope, however, made the distinction more complex. Since bacteria have some characteristics of each kingdom, the question of where to place them in a systematic scheme was a good topic for brisk debate during the infancy of this new branch of science. As evidence continued to accumulate, it became increasingly clear that more plant traits were being exhibited by bacteria than were the animal characteristics. Biologists today generally agree that we should consider these microorganisms as plants. However, the decision is not a unanimous one; a few die-hards are still a bit hesitant in making the concession.

It is only a natural reaction for beginning students in bacteriology to evidence surprise that bacteria are plants, especially after they view motility of microorganisms under the microscope for the first time. Who ever saw a plant that could swim under

its own power? We can answer that inquiry by stating that locomotion, in itself, is a poor criterion for judging whether a living thing should be classified as a plant or as an animal. There are other more important differences which have been accepted by persons who devote their lives to this field of study we call *Taxonomy*—the science of classification.

While bacteria do not exhibit all of the characteristics generally ascribed to plants, they do have more plant features than animal characteristics. The presence of a firm, thick, demonstrable cell wall, the ability to combine simple substances for their own use (CHEMOSYNTHESIS), and the capacity to utilize only relatively simple compounds taken in solution (HOLOPHYTIC NUTRITION), are strong arguments favoring the classification of bacteria as plants. The dividing line between the plant and the animal kingdoms might be said to pass through the bacteria.

CLASSIFICATION OF BACTERIA

It has been convenient for scientists to place living things into various groups, or classifications, in order to point out their relation to other living things, and to demonstrate how much more specialized some organisms are than others. The "big words" employed by scientists are often frightening to the novice, but don't think for one moment that these same words are not also disturbing to workers who have been engaged in the field for a good many years. Not all bacteriologists agree with the names given to microorganisms by taxonomists. The very fact that taxonomy is not static, that changes are continually being suggested and adopted by those individuals most concerned, indicates that even the leaders in the field cannot always agree. Compromises must be made if we are to have any kind of a workable system for classifying microbes. This agreement becomes important not only for American biologists, but for scientists engaged in laboratories throughout the world. An International Microbiological Congress meets every few years in an attempt to thrash out knotty biological problems, and the questions relative to taxonomy have a habit of finding their way near the top of the agenda. Without

such international understanding, science would soon turn into a series of closed cells, each country adhering to its own, oftentimes narrow, opinions. The concept of *One World* is also important in biology.

Classification supplies valuable information to a person trained in the field. It is often uninteresting to many students who are taking a terminal survey course in microbiology, and it is not the intention of this book to perpetuate this natural reluctance to learn new names. But a brief discussion of this topic will help to emphasize the relative position that bacteria occupy in the plant kingdom.

Since some plants closely resemble others, and are quite distinct from still other plants, we can group them together under appropriate headings. The following is the generally accepted simplified breakdown, or table of organization, for bacteria:

KINGDOM: *Plant*
PHYLUM: *Thallophyta* (exhibit no roots, no stems, and no leaves.)
CLASS: *Schizomycetes* (microscopic, unicellular, chlorophyll-free plants that reproduce asexually by fission, and exist either as rods, spheres, or spirals).
ORDER:
I. *Eubacteriales* (The true bacteria, including most of the organisms discussed in an elementary course of this type.)
II. *Actinomycetales* (Elongated cells with a definite tendency to branching.)
III. *Chlamydobacteriales* (Filamentous, colorless, alga-like bacteria which may or may not be ensheathed.)
IV. *Myxobacteriales* (Slime bacteria, exhibiting group movement as a unit; crawling, creeping motion away from the center of the colony.)
V. *Spirochaetales* (Slender, flexuous cell body in the form of a spiral with at least one complete turn —from 6 to 500 microns in length.)

Each order, in turn, is subdivided into families; the families contain genera, and the genera include various species. It should be made clear that sharp lines of demarcation do not always exist between these man-made groupings.

It seems important to review the thinking that preceded this final classification whereby bacteria are catalogued under the *Thallophyta.* You may recall that Antony van Leeuwenhoek, the first person to leave written descriptions of bacteria, referred to these organisms as "animalcules," since their active motility suggested small, darting animals. Carl von Linne (1707–1778), the Swedish botanist who is better known as Linnaeus, couldn't decide where to place bacteria in his *Systema Naturae,* in which he listed all plants and animals recognized up to that time. However, he finally did call bacteria animals in the class *Vermes* and the order *Chaos,* where they remained until more could be learned about them. The first organized attempt to bring order out of this chaos was in 1774 with the work of Otto F. Müller (1730–1784), a Dutch naturalist, who placed bacteria among the ciliated protozoa. He included a genus he called *Vibrio,* a term still in common usage. Felix Dujardin (1801–1860), not knowing whether bacteria were plants or animals, reached a happy solution by naming them Zoophytes, which means animal-plants. Christian Ehrenberg (1795–1879) published his grouping of organisms in 1839, and he includes four genera familiar to modern bacteriologists: *Bacterium, Spirillum, Spirochaeta,* and *Vibrio.*

As early as 1857 Karl Nageli (1817–1891) introduced the word *Schizomycetes* (fission fungi) and this is the class under which we find bacteria in modern classification schemes. This helped to set the pattern for workers interested in supporting the idea that bacteria belong to the plant kingdom. A great lift was given to this school of thought when Ferdinand Cohn in 1872 published the first systematic classification of bacteria. He pointed out that grouping these organisms into genera and into species was not only possible, but that it was logical. It was not, however, until Gualterio Migula at the turn of the twentieth century classified bacteria on the basis of morphology (size, shape, and structure)—especially motility and arrangement of the flagella—that wide acceptance of any scheme was encouraged. The technical difficulties involved in trying to stain flagella soon became only too apparent, and Orla-Jensen in 1909 expanded the base for criteria employed in taxonomy

by including biochemical characteristics along with morphology. C.-E. A. Winslow (1877–) had made the same suggestion in 1908 when he classified the *Coccaceae*. Other schemes were advocated from time to time, but the general acceptance of any classification was still lacking.

Through the initiative of a volunteer committee consisting of A. C. Abbott, Professor of Hygiene and Bacteriology at the University of Pennsylvania, H. W. Conn, Professor of Biology at Wesleyan University in Middletown, Connecticut, and E. O. Jordan, Assistant Professor of Bacteriology at the University of Chicago, a national organization called the Society of American Bacteriologists (abbreviated S.A.B.) was founded in 1899 at New Haven, Connecticut. This group naturally became interested in the problem of bacterial taxonomy, and at its annual meeting held at Urbana, Illinois, in 1915, a committee was appointed to review the problem of classification and to report back to a later annual gathering of the S.A.B.[1] A progress report was submitted in 1916 and the final recommendations were published in 1920. While this report was not adopted as official by the S.A.B., it did serve as a valuable framework for future deliberations. Other taxonomists pursued the problem further with the result that bacteria were finally classified into groups on the basis of all

[1] Some of you may be wondering how one goes about becoming a member of a national scientific society. The S.A.B. is not like an exclusive college fraternity. Members are not picked after a period of "rushing," criticism, and selection. To quote Article III, Section 2a of the S.A.B. Constitution: "Any person interested in the objects of the SOCIETY shall be eligible for election as a member." And to quote Article II: "The objects of the SOCIETY shall be to promote scientific knowledge of bacteriology and related subjects through discussions, reports and publications, to stimulate scientific investigations and their applications, to plan, organize and administer projects for the advancement of knowledge in this field, and to improve professional qualifications."

If you care to join nearly five thousand individuals who are presently listed on the membership rolls of the S.A.B., you merely have a member nominate you on a prescribed formal application blank, have another member second the nomination, send in your annual dues, and after the National Council approves the nomination (which is practically an automatic matter), you are a member of an outstanding national scientific society! Anyone who plans to work in the field of microbiology should be encouraged to join the S.A.B. and to help promote its important program.

considerations—morphology, cultural characteristics, habitat, biochemical reactions, etc. Through the leadership of Robert E. Buchanan and David H. Bergey, among others, the 1923 publication of the *Manual of Determinative Bacteriology* came into being. Modern terminology calls this *Bergey's Manual.* The sixth edition of this bacteriologists' bible was published in 1948, and this volume lists 1,630 species of bacteria with descriptions of each organism. *Bergey's Manual* is widely accepted today as the standard reference work in the field.

Just as it is customary for human beings, at least in large areas of the civilized world, to have a first and a last name, scientists employ a two-name system, called the *Binomial System of Nomenclature,* for plant and animal designations. Linnaeus introduced this system to science, but evidence tends to show that he was not its originator, contrary to popular opinion.

Latin, or Latinized, names are used in biology. Man, for example, is called *Homo sapiens. Homo* is the generic name, while the species name is *sapiens.* When writing such scientific names we underscore them, or in print we italicize the names. Sometimes we find organisms with three names, not to be outdone by many humans, such as *Thiobacillus thiooxidans* Beijerinck. The discoverer of the organism, in this instance Beijerinck, is occasionally honored in this way. Notice too that the generic name is always started with a capital letter, while the species name is written with a small letter. Names of individuals tacked on the end are always capitalized. These details may seem very exacting to beginners in science, but orderliness is important.

Most of the bacteria studied in an elementary course in microbiology fall into the order *Eubacteriales*—the so-called true bacteria. But when disease-producing (PATHOGENIC) organisms are considered, interesting members of some of the other orders under the class *Schizomycetes* will be considered. They will include the *Actinomycetales* under which is found the tuberculosis organism (*Mycobacterium tuberculosis*), and the order *Spirochaetales* includes the syphilis spirochaete, *Treponema pallidum,* to mention just two.

CHAPTER 4

Microbes Must Eat

PREREQUISITES FOR A GOOD MICROBIOLOGICAL MEDIUM

- Proper moisture content
- Readily available food materials
- Correct *p*H
- Sterility
- Desired physical properties

GELATIN VS. AGAR

CLASSIFICATION OF MEDIA

PREPARATION OF STANDARD NUTRIENT AGAR

It may be difficult for you to visualize a living entity the size of a microbe sitting down to an abundant repast, but like every other living thing the microscopic organism must ingest food to provide energy and to allow metabolic processes to take place. Being single cells, bacteria have not been endowed with specialized organs, such as a mouth, a stomach, or intestines. But an ingenious process has been evolved whereby a single-celled organism, whether it be a plant or an animal, can absorb dissolved nutrients directly through the cell wall membrane—HOLOPHYTIC feeding. The outer shell is fastidious in that it selects what shall go into the cell and what shall be secreted or excreted. Such food intake is called DIFFUSION and the exacting nature of the cell wall depends

upon the permeability of its membrane. The passage of fluids across a membrane is termed OSMOSIS.

We all know individuals who are particular about their diets; they refuse to eat this or that because they just don't like it. Microbes also exhibit this type of rejection, in some cases to a marked degree. While differences in the permeability of the cell walls partially explains this selectivity, we find some foods that can be absorbed only to be rejected by a cell that doesn't thrive on them. Some organisms have had their own way for such a long time that they are no longer able to metabolize certain foods. Since a number of organisms are unable to swim around in their quest for food, the nutrients must be provided nearby at the right time, and in an acceptable concentration, otherwise the cells may starve to death. Such extreme dependence upon a narrowly defined diet sometimes works to the disadvantage of pathogenic bacteria.

When more than a bare minimum of food is available the organism gets larger, just as man puts on weight, but the microbe goes us one step better. When it reaches a predetermined size, it splits in two by a process called BINARY FISSION (equal division). Under optimum conditions the dividing process occurs once in about twenty to thirty minutes, with notable exceptions including *Mycobacterium tuberculosis* whose generation time is much greater. But this splitting cannot go on indefinitely, because natural checks and balances come into play to keep other forms of life from being forced from the face of the earth by growth of overwhelming numbers of microbes.

Some persons keep cows, others keep pigs or sheep, and the breed of scientists we call a microbiologist, tends microbes. In each case the nutritional needs of the living things being cared for must receive careful attention by the keepers. While minute amounts of food are required by a single bacterial cell, the masses of organisms capable of being generated in only a few hours put increasing demands upon the food supply. In general, we are not interested in prolonging the life span of microbes the way we are striving to increase man's life expectancy. However, in the process of

training microbiologists or in research investigations, increased numbers and increased longevity of bacteria may be important. We cannot do too effective a job by studying only a few of these microscopic forms, but masses of actively growing cultures can provide valuable information relative to the physiology and the ultimate identity of a given species.

In addition to preparing a diet which is most favorable for as many species of bacteria as possible, we can also blend chemicals with our nutrient materials in such a manner that selectivity of growth can be accomplished. Often, particularly in clinical laboratories when we are attempting to isolate the etiological agent in a given disease, it is highly desirable to inhibit the growth of abundant organisms which we know are normally not pathogenic, and to encourage the cultivation of our suspected pathogens. Such a medium employs the principle of BACTERIOSTASIS for its selectivity. No culture medium has yet been devised which will allow all known bacteria to grow under one set of conditions. To meet this problem, microbiologists have been forced to concoct hundreds, even thousands, of combinations of substances to fulfill growth requirements. There are a few basic media to provide general uniformity in running such standardized tests as milk and water analyses, otherwise no two laboratories would be able to compare a given sample with reproducible results. A great boon to uniformity has been the manufacture by biological supply houses of dehydrated media, and standardized procedures play an important part in modern laboratory technics.

PREREQUISITES FOR A GOOD MICROBIOLOGICAL MEDIUM

Any material employed to grow bacteria is termed a CULTURE MEDIUM, and the resultant growth is termed a CULTURE. A satisfactory microbiological medium should fulfill the following prerequisites. It must:

1. Have the proper moisture content.
2. Contain readily available food materials.
3. Have the correct acid-base balance, called *p*H.

4. Be sterile—in a bacteriological sense.
5. Provide desired physical properties (clarity, liquid, solid, etc.).

Since modern microbiology is founded upon our ability to isolate pure strains of specific species of organisms through the use of culture media, it seems wise at this point to elaborate on these five prerequisites of a good microbiological medium.

PREREQUISITE I: PROPER MOISTURE CONTENT

When we analyze bacterial cells, we find that their moisture content approaches 80%, with a range approximately 15% either side of this figure. The technic generally used in the determination of water content of cells is to dry them at 100–110° C. in the air, or in a vacuum oven at a lower temperature, and to observe their weight loss. Slimy capsular layers surrounding some organisms tend to raise their relative moisture level. In compounding media we try to provide a high water content, usually 75 to 95%, to fulfill this growth requirement of microorganisms.

Bacteria are more closely related to aquatic plants than to terrestrial plants; they thrive best when the organisms are surrounded by moisture containing readily available food substances. An abundance of water is just as important for bacterial growth as is the presence of available food, since moisture serves as a vehicle for the food and provides transportation for the egress of waste products built up within the cell during metabolism. Water is the most universal solvent. Its specific heat aids in the absorption of heat liberated during metabolism of the cells, and equally important is the conductivity of water which facilitates the dissipation of heat generated by living organisms.

The concentration of food in solution directly affects the speed of flow and the direction of flow of water with respect to the suspended cells. Too high a concentration of food draws water from the organism and tends to shrink the cell, while too low a concentration of nutrients induces the entrance of excessive water through the cell wall with resulting swelling and eventual harm to the organism.

PREREQUISITE II: READILY AVAILABLE FOOD MATERIALS

Organisms vary tremendously in their nutritional requirements, varying from simple inorganic salts up to and including living tissues of species of plants or animals. But in general most common bacteria are not too exacting, with the result that many types of food substances can be utilized for food. Minute amounts of accessory growth substances, or vitamins, are required by some organisms, and to that end some species manufacture their own vitamins. This brings in an interesting sidelight in nutritional studies. If an organism requires a given vitamin and this vitamin is not provided by the organism's own chemosynthetic activity, the vitamin content of foods can be determined by attempting to grow these vitamin-dependent microbes on this food. This is called a VITAMIN ASSAY determination, employing biological rather than strictly chemical test tube technics in the analysis. Experience has demonstrated the value and the accuracy of this biological method.

Organisms can be classified on the basis of their nutritional needs into two major groupings, the AUTOTROPHS and the HETEROTROPHS. The former bacteria use simple elements or simple combinations of inorganic material (iron, manganese, etc.) as their principal source of food, and carbon dioxide is utilized as a carbon source. Heterotrophs, in general, are unable to assimilate carbon from carbon dioxide and their energy is primarily derived through the chemical breakdown (analysis) of more complex food materials (nitrites, nitrates, etc.), especially organic compounds. An organic compound is a carbon-containing compound derived from plants or from animals. Heterotrophs can be further subdivided into SAPROPHYTES which thrive on dead organic matter, and into PARASITES which depend upon living cells for their survival. There are degrees of parasitism. The term FACULTATIVE PARASITES is used to designate those organisms capable of thriving on either living or on inanimate materials, such as common laboratory media. Strict parasitism is not nearly as common as is facultative parasitism. It is generally conceded that heterotrophs probably evolved

from the autotrophs, although this conjecture provides the subject for lively discussions in seminar sessions. It should be emphasized that while it is convenient to speak of various nutritional groupings of organisms, clear-cut differences do not always exist. There are plenty of "in between" forms to complicate these neat cataloguing schemes.

If bacteria were compelled to depend upon nature to supply the exact chemical substances required for their existence, survival of these microscopic forms might probably be even more difficult than is actually true under existing conditions. Whenever the food particles (molecules) are too large for heterotrophs to absorb directly, the cells must secrete digestive juices, called ENZYMES, to attack these large molecules as the initial step in making the food parcels small enough to pass through the membrane of the organism via the fluid menstrum. Enzymes may be defined in simple language as organic catalysts[1] produced by living cells. A catalyst may be considered to be an agent which accelerates a chemical reaction without itself being consumed in the reaction. A little bit of catalyst goes a long way, and it has something which "sparks" a reaction.

The orderliness of science demands categorizing and classification. Enzymes are no exceptions. We can divide these digestive ferments into two major groupings, the EXTRACELLULAR enzymes and the INTRACELLULAR enzymes. When an organism is obliged in its search for food to secrete enzymes to attack large molecules *surrounding the cell,* these juices are referred to as extracellular enzymes. While it is true that they are formed within the living cell, such exo-enzymes are able to leave the confines of the cell and

[1] If you would like to demonstrate a catalytic reaction as a parlor trick, take a piece of lump sugar and try to make it burn with the flame from a match. It will not ignite. Now dip the sugar into some cigarette ash and the sugar will burn with a faint blue flame when a lighted match is touched to the catalyst in the cigarette ash on the lump of sugar. No doubt a good chemist using micro-technics could analyze the end products of such combustion, and the catalyst in the original ash would be found to be intact. To prevent damage during a demonstration, it is recommended that the experimenter carry out the ignition over a plate or vessel to catch any hot dripping sugar.

to carry out their assigned functions extracellularly. Once the food has been broken down to a size permitting passage through the semi-permeable covering of the organism, then the absorbed food is further attacked within the cell by the endo-enzymes, which convert the food into an available form. A rebuilding process may also be employed by intracellular enzymes to convert the food into chemical configurations required in the metabolism of the specific microbe.

Enzymology is a complex science, and a book of this nature is not intended to do much more than introduce the beginning student to general concepts needed to familiarize him with the underlying technics employed by single-celled, mouthless, microscopic organisms when they are faced with the problem of obtaining food "too tough to chew" without some preliminary breakdown.

The action of enzymes may be influenced by many of the same forces affecting other chemical reactions. Temperature, moisture, acid balance (*p*H), presence of chemical poisons, and other factors have their effect on enzymes. Enzymes are highly specific for given substrates. Enzyme "A" only works on substrate "A," and substrate "B" can only be attacked by enzyme "B." Since physiological reactions determine to a great degree where organisms are placed in classification schemes, it becomes apparent that there is a close relationship between enzymes which initiate physiological reactions and taxonomy. Higher plants have the advantage over bacteria in that the former possess *chlorophyll,* that green pigment so essential to higher plant life. Chlorophyll is another example of a catalyst, where the cholorphyll is activated by sunlight to allow water and carbon dioxide gas to combine into plant substance in a process called photosynthesis (putting together in the presence of light).

Much of the food taken in by bacteria must be drawn upon for respiration. When free gaseous atmospheric oxygen is involved in respiration, we speak of this as AEROBIC RESPIRATION, in contrast to ANAEROBIC RESPIRATION which involves the breakdown or re-arrangement of molecules in a food substrate as a means of

obtaining oxygen. All living cells must have oxygen, but the origin of this oxygen varies with the type of respiration employed by the cell. Some organisms have adjusted their lives in such a way that they can respire either aerobically or anaerobically. Such bacteria are termed FACULTATIVE. Some persons go so far as to subdivide the facultative microbes into the FACULTATIVE AEROBES, which grow either aerobically or anaerobically but prefer aerobic respiration, and FACULTATIVE ANAEROBES which prefer anaerobic to aerobic respiration.

When bacterial cells are subjected to chemical analysis, a number of elements can be detected. Sometimes an element may be fortuitous, just entering the cell wall for the ride at the same time that essential elements are being absorbed. Still other elements, even in minute traces, are vital to survival and multiplication of the organism. The five pillar elements—carbon, hydrogen, oxygen, phosphorous, and nitrogen—must be supplied in an available form to all living cells. In addition to these five, however, trace amounts of other elements must be included in good bacteriological media. The names of the essential elements for cellular growth are conveniently remembered in the expression: C HOPKINS CAFE MG, an abbreviation for Carbon (C), Hydrogen (H), Oxygen (O), Phosphorous (P), Potassium (K), Iodine (I), Nitrogen (N), Sulfur (S), Calcium (CA), Iron (FE), and Magnesium (MG). Energy is required to blend these elements into useful combination for cell substance, and this energy may be derived in one of two general ways. By directly absorbing energy foods, such as available sugars, and breaking down these substances through the activity of enzymes, energy can be released for cell use. Or the organism may liberate energy through oxidation, a process tied up with respiration in all living cells.

Fulfilling the prerequisite of providing readily available food in a microbiological medium is a great deal more involved than the simple statement might lead you to believe, and unless the provider understands these ramifications of nutrition, healthy, actively growing crops of microorganisms might not materialize.

PREREQUISITE III: THE CORRECT ACID-BASE BALANCE—THE *p*H

If all of the other requirements for a good microbial medium are supplied, but the acidity or the alkalinity is not properly controlled, poor growth of organisms, or no growth at all, may result. The usual ingredients incorporated into common media are acid in character, and we must neutralize at least part of this acidity by adding alkaline substances called BASES. Most organisms prefer a medium that is close to neutrality, but as might be expected, some species require marked deviations from neutrality before they find optimum growth conditions. All organisms have a maximum, an optimum, and a minimum chemical reaction with respect to growth of that species.

We learn in chemistry that it is the concentration of dissociated or ionized hydrogen (H) or hydroxyl (OH) that determines the effective acidity or alkalinity of a solution. The theory of dissociation of electrolytes was formulated in 1887 by the Swedish chemist, Svante Arrhenius (1859–1927), and measurement of the acid-base reaction of a medium in microbiology is determined by the HYDROGEN ION CONCENTRATION, abbreviated *p*H. Equal concentrations of so-called weak acids and strong acids show marked differences in true acidity, or sourness. This difference depends upon the ability of the acid to ionize, or dissociate. Strong acids dissociate into relatively large numbers of hydrogen ions, while weak acids do not ionize to such a degree.

Pure water dissociates very slightly into equal numbers of hydrogen (H) ions and hydroxyl (OH) ions, and hence such water is neutral in reaction. At 22°C. the hydrogen ion concentration of pure water is 1×10^{-7} gram ions per liter. Hydrogen ion potential is expressed as the logarithm of the reciprocal of the hydrogen ion concentration, and this expression was given the symbol *p*H by S. P. L. Sörensen in 1909. In other words, *p*H can be determined by the formula $p\text{H} = \text{Log} \frac{1}{[\text{H}]}$.

Exact determinations have revealed that one liter of pure water (H_2O) contains 0.000,000,000,000,01 (10^{-14}) gram of hydrogen and hydroxyl ions. The hydrogen and the hydroxyl ions are always equal in pure water. It follows, therefore, that the hydrogen ions are found in a concentration of 0.000,000,1 gram per liter. The logarithm of this fraction (0.000,000,1 gram) is minus 7. For convenience we express this as a positive number and call it *p*H 7.0. Numbers less than 7.0 on the *p*H scale, therefore, represent greater acidities. Figures above *p*H 7.0 indicate lesser hydrogen ion concentrations, but greater hydroxyl ion concentrations, or alkalinities. The normality of the hydrogen ions times the normality of hydroxyl ions is a constant number, called the dissociation constant. This number must always be approximately 10^{-14}, so if we know the concentration of hydrogen ions, we can determine the hydroxyl ion concentration by subtraction. Each whole number on the *p*H scale represents a ten-fold difference from the number above or below it. A *p*H of 6.0 would therefore have a hydrogen ion concentration ten times that of *p*H 7.0. We can express these facts about *p*H in tabular form.

TABLE 1

HYDROGEN ION CONCENTRATION AND *p*H

GRAMS OF HYDROGEN IONS PER LITER		*p*H	NUMBER OF TIMES ACIDITY OR ALKALINITY EXCEEDS THAT OF PURE WATER	
1.0	(10^{-0})	0.0	10,000,000	↑
0.1	(10^{-1})	1.0	1,000,000	↑
0.01	(10^{-2})	2.0	100,000	Acid
0.001	(10^{-3})	3.0	10,000	↓
0.000,1	(10^{-4})	4.0	1,000	↓
0.000,01	(10^{-5})	5.0	100	↓
0.000,001	(10^{-6})	6.0	10	↓
0.000,000,1	(10^{-7})	7.0	0	Neutral (pure H_2O)
0.000,000,01	(10^{-8})	8.0	10	↑
0.000,000,001	(10^{-9})	9.0	100	↑
0.000,000,000,1	(10^{-10})	10.0	1,000	↑
0.000,000,000,01	(10^{-11})	11.0	10,000	↑
0.000,000,000,001	(10^{-12})	12.0	100,000	Alkaline
0.000,000,000,000,1	(10^{-13})	13.0	1,000,000	↓
0.000,000,000,000,01	(10^{-14})	14.0	10,000,000	↓

Several rather simple technics have been devised for measuring *p*H in the laboratory. One of these methods, and the more accurate of the two we shall mention, depends upon electrometric devices, called POTENTIOMETERS. The other technic, and the more commonly employed method, depends upon indicator dye solutions, usually weak organic acids or bases, which change color as the *p*H is altered. When attempting to determine the *p*H of colored solutions, or of solutions containing high concentrations of proteins which may absorb dye from the indicator, the electrometric devices must be used, since the color changes of *p*H indicators may be masked under these conditions. However, for most *p*H determinations, the colorometric technics lend themselves well for use in bacteriological laboratories, in spite of the sacrifice of some accuracy. The series of color standards can readily be checked electrometrically before labeling the tubes with the determined *p*H.

The effective range (the useful range) of many indicators commonly employed in *p*H determinations covers 1.6 points on the *p*H scale, but again we find exceptions to the rule, some covering a greater spread on the scale. On page 68 is a partial list of some of the satisfactory indicator dyes, with their color changes and their *p*H ranges.

Notice in Table 2 that thymol blue has both an acid range (1.2–2.8) and an alkaline range (8.0–9.6). This characteristic is true of a few *p*H indicators.

The question may arise as to which indicator one should choose, if several dye ranges overlap on the *p*H scale. It depends upon the particular use to which the dye is to be put, but when attempting to adjust the *p*H of a medium, choose an indicator whose mid-point is close to the final desired *p*H of your medium. The degree of color change at the mid-point with each minor alteration in *p*H is more pronounced than are the color changes at the low end or at the high end of the indicator's effective range.

One of the characteristics of many microorganisms is their ability to attack sugars or proteins present in a medium, and the resulting concentrations of acids or alkalies may be sufficient to kill the very cells that produced them. When attempting to harvest

TABLE 2

INDICATORS AND THEIR *p*H RANGES

NAME OF INDICATOR	EFFECTIVE *p*H RANGE	COLOR ON LOWER SIDE	COLOR ON UPPER SIDE
Thymol blue	1.2–2.8	Red	Yellow
Bromphenol blue	3.0–4.6	Yellow	Blue
Congo red	3.0–5.0	Blue	Red
Methyl orange	3.1–4.4	Orange red	Yellow
Brom cresol green	3.8–5.4	Yellow	Blue
Methyl red	4.4–6.0	Red	Yellow
Chlorophenol red	4.8–6.4	Yellow	Red
Litmus	4.5–8.3	Red	Blue
Brom cresol purple	5.2–6.8	Yellow	Purple
Brom thymol blue	6.0–7.6	Yellow	Blue
Phenol red	6.8–8.4	Yellow	Red
Cresol red	7.2–8.8	Yellow	Red
Thymol blue	8.0–9.6	Yellow	Blue
Cresolphthalein	8.2–9.8	Colorless	Red
Phenolphthalein	8.3–10.0	Colorless	Red
Alizarine yellow	10.0–12.0	Colorless	Yellow
LaMotte sulfo orange	11.0–12.6	Pale yellow	Deep orange

a maximum crop of organisms we must minimize *p*H changes in our medium by incorporating substances called BUFFERS. Buffers may be defined as substances, which by their presence in solutions, increase the amount of acid or alkali that must be added to cause material change in *p*H of the solution. The word is derived from the German word *Puffer* (plug or bung), and the most efficient buffers are mixtures of weak acids or weak bases, in combination with their salts and certain other AMPHOTERIC substances. Amphoteric substances are able to dissociate so that under one set of conditions they yield hydrogen ions, and under another set of conditions hydroxyl ions are liberated. Sometimes both types of ions are released simultaneously. Alkaline dissociation predominates in an acid medium, and acidic dissociation of amphoteric substances can be expected when the medium is alkaline. The hydrogen ion concentration at which this type of dissociation is at a minimum is called the ISOELECTRIC POINT of the amphoteric substances. Buffers do not stop *p*H changes, they merely retard

rapid changes upon additions of *small* amounts of acids or alkalies.

To help visualize degrees of sourness as they correlate with *p*H, the following list of common substances is presented:

TABLE 3

THE *p*H OF COMMON MATERIALS

	*p*H
Hydrochloric acid	1.0
Human gastric contents	2.0
Ginger ale	3.0
Wines	3.0
Sour pickles	3.2
Tomatoes	4.2
Beans	5.5
Human saliva	7.0
Human blood plasma	7.4
Sea water	8.2

It has been mentioned that each organism has its own *p*H range which limits growth of that species, and Table 4 lists a few such organisms.

TABLE 4

THE *p*H LIMITS FOR MICROBIAL GROWTH

1. Molds	1.4 – 9.0
2. Yeasts	2.5 – 9.0
3. Most bacteria	5.0 – 9.0
A. *Escherichia coli*, the common organism found in the intestines of all warm-blooded animals	4.8–10.0
B. *Clostridium tetani*, the etiological agent in lockjaw	5.5 – 8.3
C. *Salmonella typhosa*, the typhoid organism	5.6 – 8.5

PREREQUISITE IV: STERILITY

Physical Methods

MOIST HEAT STERILIZATION. Sterility means one thing to the layman and something quite different to a microbiologist. So often we hear people say that they have sterilized the baby's bottle by boiling it for ten minutes. In some cases this treatment may very well sterilize the bottle, but because of spores, those resistant bodies so useful in helping some organisms withstand periods of unfavorable environment, we cannot always be sure that mere

boiling kills all microorganisms. It is not meant to imply that boiling a baby's bottle for ten minutes is not a safe procedure. Quite to the contrary. This common practice is based upon sound scientific principles. Pathogenic bacteria are not normally able to withstand such heat treatment, and the spore-formers which might survive are not likely to cause any upset in the infant who drinks milk stored under proper refrigeration in such a boiled bottle. To a microbiologist sterility means the killing, or the removal, of all living cells, whether they be plant or animal, microbe or whale!

The living protoplasm of bacterial cells is composed of protein distributed in a well balanced state known as a COLLOID. Matter is said to be in a COLLOIDAL STATE when it is dispersed permanently and so finely that the individual particles cannot be seen with the ordinary microscope, even though the particles may be larger than molecules. Anything that we can do to tip this colloidal protoplasm out of balance is going to adversely affect the organism, and a severe unbalance will result in the eventual death of the cell. We can coagulate cell protein in much the same way that we congeal an egg by heating it, and such disruption of the colloidal balance is enhanced in the presence of water, as will be evident from the figures in Table 5.

TABLE 5

RELATIONSHIP OF MOISTURE CONTENT TO COAGULATION TEMPERATURE OF EGG ALBUMIN IN THIRTY MINUTES

Egg albumin + 50% water	coagulates at 56° C.
Egg albumin + 25% water	coagulates at 74–80° C.
Egg albumin + 18% water	coagulates at 80–90° C.
Egg albumin + 6% water	coagulates at 145° C.
Egg albumin + NO water	coagulates at 160–170° C.

Boiling. Under certain conditions, in the absence of resistant spores, boiling will free liquids of living cells, but as previously mentioned, since one cannot be sure when spores are present, boiling should not be relied upon to insure bacteriological sterility. An interesting and important sidelight is the influence of elevation upon the boiling point. At sea level water boils in an open con-

tainer at 100° C. (212° F.), but as the elevation increases above sea level, the boiling point decreases. On the top of Pikes Peak in the Rocky Mountains of Colorado where the elevation reaches 14,109 feet above sea level, water boils in an open vessel at about 87° C. (187° F.). Persons residing in a locality that is appreciably

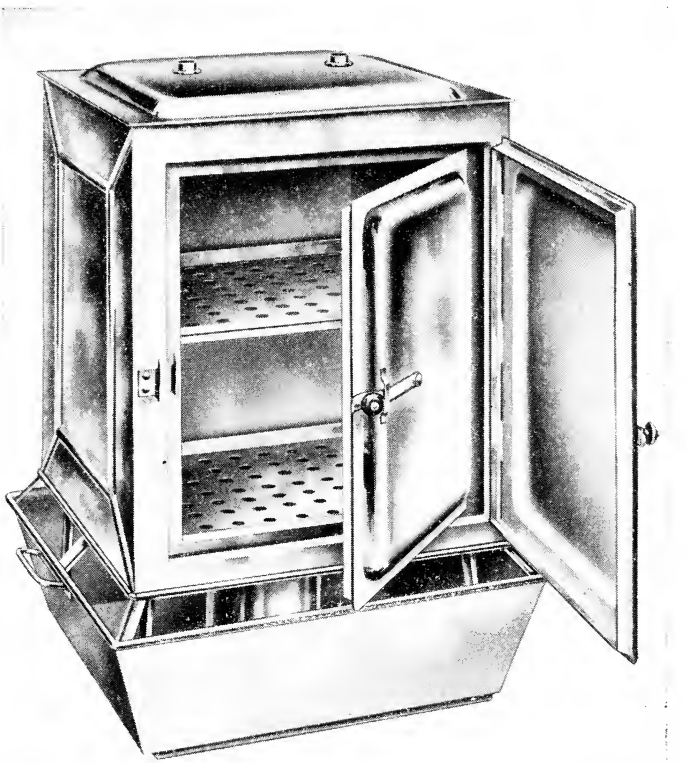


Fig. 15. Arnold Sterilizer. (*Manufactured by Wilmot Castle Company. Will Corporation catalog 25101.*)

above sea level should remember that boiling occurs at such low temperatures that cooking periods often must be double that at sea level where boiling takes place at a hotter temperature. It is not the mere bubbling of water that determines effectiveness of heating, it is the temperature at which this boiling occurs.

Intermittent Heating (Arnold sterilization). This technic is advocated in the sterilization of THERMO-LABILE substances (broken down by heat) which can withstand a temperature of 60 to 65° C., the point where most vegetative protein coagulates. The underlying principle of intermittent heating is to destroy the vegetative (non-spore-containing) cells present in the material to be sterilized, incubate the product at a temperature which will encourage germination of spores, heat once more to destroy the new crop of vegetative cells, re-incubate, etc., until the third heating has disposed of the last germinated spores. This method does have certain advantages, but few microbiological laboratories employ the technic extensively.

Autoclaving. One of the most efficient and reliable methods of sterilizing liquids is the use of steam under pressure. The instruments employed in laboratories for accomplishing this are called AUTOCLAVES, and they resemble home pressure cookers. Water boils at 100° C. at atmospheric pressures of 760 millimeters of mercury, but if we build up the pressure in a sealed container, water can be kept from boiling until the temperature goes well above 100° C. Table 6 reviews the relationship of steam under pressure at sea level to temperatures attained.

TABLE 6

CORRELATION OF STEAM PRESSURE WITH TEMPERATURE

POUNDS OF STEAM PRESSURE	TEMPERATURE DEGREES C.	TEMPERATURE DEGREES F.
0	100.0	212.0
5	109.0	228.2
10	115.5	239.9
15	121.5	250.7
20	126.5	259.6

The usual rule of thumb for operating an autoclave is to subject the liquids being sterilized to 15 pounds of live steam pressure for at least 15 minutes. But as the volume of material to be sterilized increases, sufficient time must be added to allow the

entire bulk to be in contact with 121° C. for at least the minimum 15-minute time period. In general, a liter flask of broth should be kept for 30 minutes under 15 pounds steam pressure, while lesser volumes require shorter sterilization times. Remember, it is not the pressure that kills the organisms, it is the temperature created

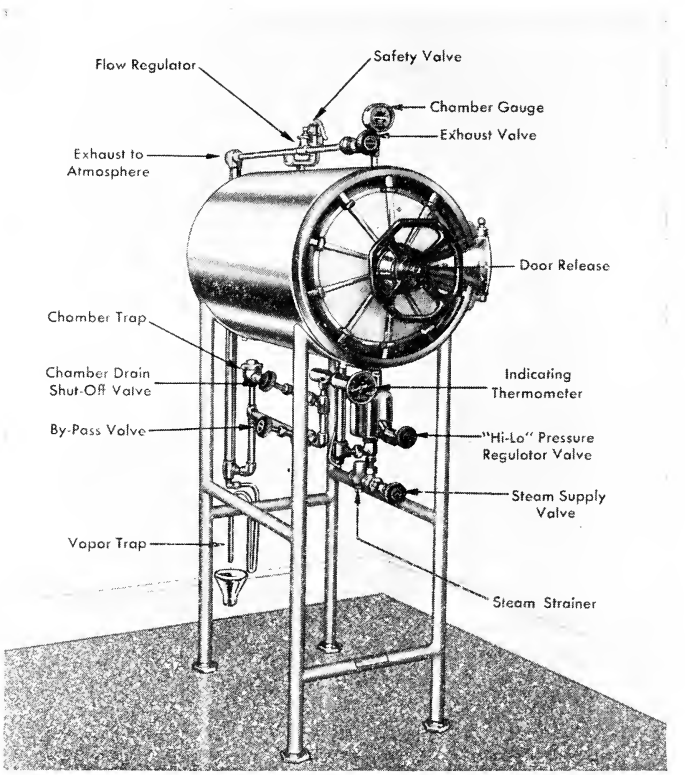


Fig. 16. Steam sterilizer (autoclave). (*Courtesy of the American Sterilizer Company, Erie, Pennsylvania.*)

by raising steam pressure to 15 pounds above normal atmospheric pressure. To insure that the material in the autoclave is being subjected to live steam under pressure, not merely to a mixture of cold air and steam, rely more upon the reading of the temperature gauge than upon the pressure indicator. After securely fastening the door of the sterilizer, drive out the trapped cold air by allowing

steam to enter the chamber and to escape through the drain before closing all outlets. As soon as the temperature approaches 121° C., the pressure gauge, if it is functioning properly, should register close to 15 pounds. At the conclusion of the sterilization period, *do not release the pressure quickly* or the liquids in the autoclave will "blow their tops" and spill the carefully prepared media. For best results, merely shut off the steam inlet valve and allow the pressure to decrease gradually. If the autoclave has no serious leaks in the gaskets or valves, this cooling-off period should reduce the pressure to zero in from 10 to 20 minutes after closing the steam valve.

Tubes or flasks of liquids being autoclaved are commonly stoppered with plugs of non-absorbent cotton to allow ready access to live steam. Under no circumstances should absorbent cotton be substituted, since once the cotton becomes soaked with moisture, it loses its capacity to retard the entrance of contaminating organisms into the sterile fluids. The importance of the introduction by Schröder and von Dusch of cotton plugs to microbiology cannot be overemphasized.

Dry Heat Sterilization. *Indirect Heat.* Most of the glassware, such as pipettes and petri dishes, used in microbiological laboratories must be dry after sterilization, and this precludes the wet autoclave as a means of sterilizing much of this equipment. However, a common baking oven, heated with gas, kerosene, or electricity, serves as a useful instrument for dry heat sterilization. In addition to glassware, all oils and greases should be subjected to dry oven treatment to sterilize them. A number of hospitals still mistakenly "sterilize" such things as mineral oil and vaseline gauze in the autoclave, forgetting the old adage that oil and water do not mix. Only the outer exposed portion of such greasy substances which come in intimate contact with the live steam are being subjected to moist heat at 121° C. The rest of the material is practically water-free, and 121° C. dry heat is not sufficient to sterilize in the usual 15-minute contact period. The chart in Table 5 indicates that protein in the absence of moisture does not coagulate until the temperature approaches 160° C. Therefore, when

employing oven heat in sterilization, the temperature is raised to 170° C. (a 10-degree temperature margin) and is maintained for *at least one hour*, with two hours' contact being more common in many laboratories.

Open Flame Sterilization. In transferring growths from one container to another, the usual practice is to handle these organisms by means of sterile wires or wires fashioned into loops. The most convenient method of sterilizing these needles and loops is to plunge them into the direct flame of a bunsen burner or an alcohol lamp. Incineration insures sterility. Surgical instruments cannot, however, be subjected to such rough treatment.

Filtration as a Means of Sterilization. Some liquids cannot be sterilized by heat because the elevated temperature will coagulate the material or will adversely affect the chemical structure of the compound. Various types of filters have been developed to overcome the objections to heat treatment, and today we employ filters to sterilize blood sera, to separate exotoxins from their parent cells, to separate viruses from bacteria and other organisms, to aid in the isolation of enzymes, and to free thermo-labile liquids from contaminating organisms. It is not the mere physical removal of organisms by the minute pores in the filters that accomplishes sterilization; adsorption phenomena associated with electrical charges are also instrumental in this filtering process. Liquids may be drawn through filters at a rate faster than gravity would normally allow by applying positive pressure as the liquid is introduced into the filter, but a more common practice is to provide negative pressure (suction) to the receiving flask. Too much positive or negative pressure, however, will create an undesirable differential which may nullify the surface action of the filters by drawing the bacteria away from the sides of the pore spaces where they have been trapped. A differential of 150 to 200 millimeters of mercury is usually recommended to speed up filtration without sacrificing dependability. We shall mention but four of the commonly-employed filters. Those readers interested in more complete discussions of the subject are referred to advanced textbooks in the field.

Chamberland Candles. These are composed of mixtures of silicon and kaolin, and are unglazed porcelain filters shaped like a hollow candle open at one end. The candles can be sterilized in the autoclave, attached to suction flasks, and the liquid to be freed of organisms can be introduced into the open end of the candle

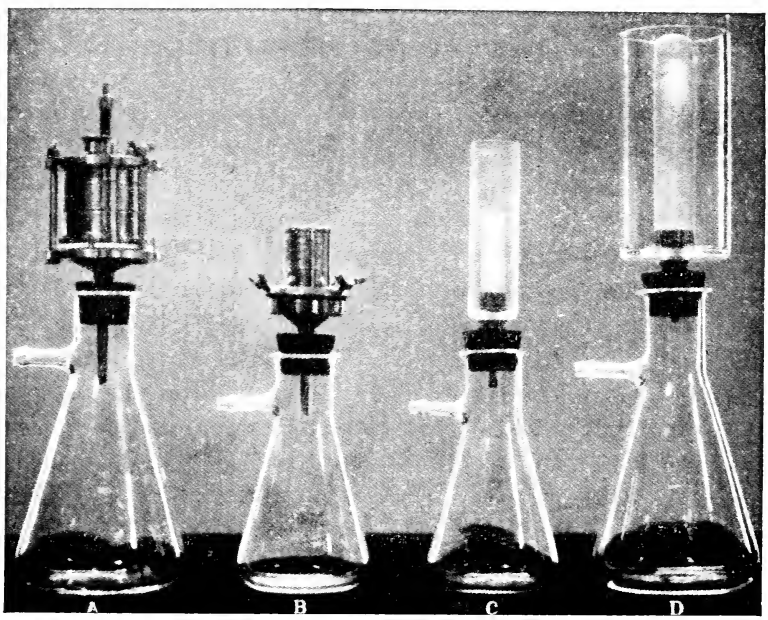


Fig. 17. Some of the common filters used by bacteriologists. (A) Seitz filter designed to operate with external pressure; (B) Small Seitz filter, suction model; (C) and (D) Berkefeld filter candles mounted in glass mantles. (*From* Textbook of Bacteriology, *E. O. Jordan, and W. Burrows, 14th ed. Copyright 1945, W. B. Saunders Company, Philadelphia.*)

and allowed to pass through the filter with the aid of negative pressure applied to the side arm of the receiving flask. Bacteria normally carry a negative charge. Chamberland candles possess a positive charge. Great care must be exercised to insure that the candles are not cracked. A minute crevice in the filter can allow organisms to squeeze into the filtrate.

Berkefeld Filters. These filters of varying porosities are composed of diatomaceous earth which is obtained from the silica-like skeletons of marine or fresh-water algae. These are coarse Berkefeld *V* (German *viel*), medium *N* (German *normal*), and fine Berkefeld *W* (German *wenig*) filters, but the effectiveness of any of these devices may be materially impaired unless the filters are kept scrupulously clean. The *V* filter may allow some of the smaller bacteria to pass through, but it serves a useful purpose as the first step in filtering masses of organisms from fluids when centrifugation is not convenient. The *N* filters usually remove 100% of the suspended bacteria, and the *W* filter, having a fine porosity, can usually be relied upon to free fluids of all cells. It is a wise precaution, however, to run sterility checks on all filtrates whenever freedom from microbes is essential.

If the pores become coated with grease, the adsorptive power of the pore walls may be neutralized, and organisms may slip through the filter. Stuart Mudd has calculated that Chamberland and Berkefeld filters that are "tight" to bacteria have mean pore space sizes in the range of 3 to 4 microns.

Cleaning of filters can sometimes be accomplished by merely passing a continuous stream of clear water through them for several hours. Heating in ovens is usually discouraged because the extreme temperature changes may cause the filter to crack, often inside where the eye cannot readily detect the fissure. A suggested cleaning technic involves passing a five-tenths per cent solution of potassium permanganate through the filter, followed by a 5% sodium bisulfite solution before allowing streams of clear water to wash out all traces of these cleansing agents.

Seitz Pads. Discussion of these filters can be dismissed by saying that they are made by compressing shredded asbestos into pads which can be inserted into special chambers, sterilized in the autoclave, attached to side-arm flasks, and used to filter liquids with the aid of negative pressure to speed up the process.

Sintered Glass Filters. Germany led in the production of these devices made from Jena glass, but American pyrex glass is now being manufactured into excellent sintered glass filters. This spe-

cial glass is ground up very fine, placed in molds, and heated just to the point where the glass particles adhere to each other, leaving pore spaces through which liquids may pass. The fineness of the filters is directly correlated with the size of the glass particles

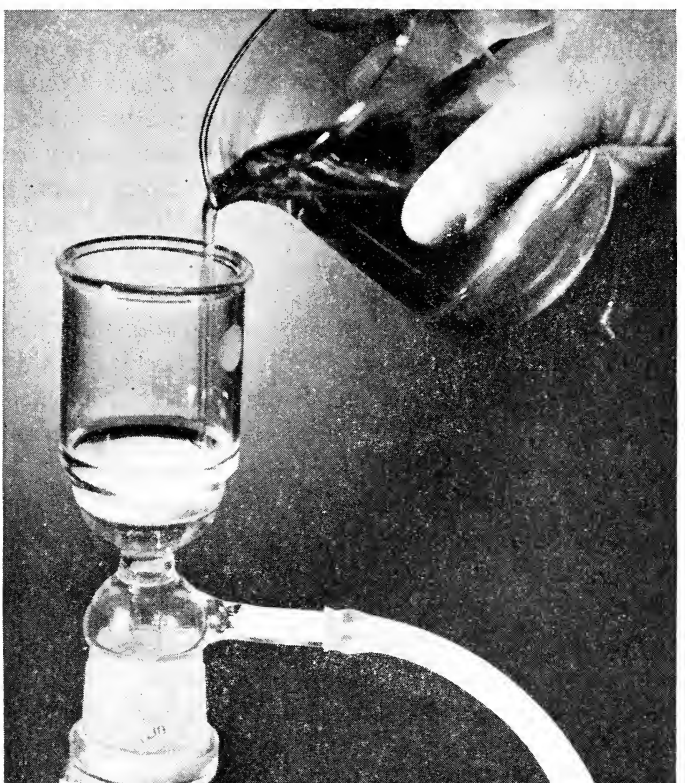


Fig. 18. Sintered glass filter. (*Courtesy of Corning Glass Works. Corning, New York.*)

employed in their manufacture. Cleaning such sintered glass is accomplished by passing sulfuric acid containing 1% sodium nitrate through the filter. Dichromate cleaning fluid should be avoided since it is adsorbed on the glass and may adversely affect future filtrates.

In brief, we autoclave all liquids except those which are thermolabile. Such fluids are usually filtered or sterilized by intermittent selective heating over a period of days. All glassware that must be dry after sterilization and all oils and greases should be oven-sterilized. Glassware that need not be dry before use may be autoclaved, which is a shorter process than the oven treatment.

Chemical Methods of Sterilization

Chemical sterilization of bacteriological media is impractical. Any chemical used to free a medium of organisms will nullify the usefulness of that substrate for the subsequent cultivation of cells. Selected chemicals are being employed for the sterilization of surgical instruments which cannot undergo the rigors of heat treatment, but the choice of chemical and the length of the contact period are prime considerations. In a later chapter the use of chemicals as antiseptics and as disinfectants will be discussed, pointing out the limitations as well at the strong points in favor of representative chemical agents.

PREREQUISITE V: DESIRED PHYSICAL PROPERTIES

Every medium blended in the laboratory has a definite use, and the physical properties of that medium should help to fulfill specific requirements. In studying motility of organisms the culture should be grown in a liquid medium which encourages flagella to develop. Organisms picked from a colony growing on a solid medium cannot be expected to exhibit active motility, if they show locomotion at all. In nutritional studies where development of turbidity is to be the criterion of growth, it is imperative that the culture medium be as clear as possible before inoculation with the test organisms. If characteristics of colonies are to be studied, our microbes must be cultivated on a medium containing some solidifying agent. In brief, a liquid medium, a clear medium, or a solid medium is provided depending upon the ultimate use to which the substrate is to be put. The effect of such factors as surface tension, concentration of ingredients, etc., will be discussed in a later chapter.

GELATIN VS. AGAR

Of all the solidifying agents employed in microbiology, agar is by far the most common. Robert Koch introduced gelatin as one of the first substances, but because of undesirable characteristics of this solidifying agent, it was replaced by agar. A brief comparison of gelatin and agar is presented in Table 7.

TABLE 7

CHARACTERISTICS OF GELATIN AND AGAR

	GELATIN	AGAR
1. Food value.	May be used by some bacteria.	No food value.
2. Chemical composition.	An incomplete protein.	A hemicellulose.
3. Melting point.	About 25° C.	About 90° C.
4. Solidifying point.	About 23° C.	About 40° C.
5. Other properties.	Forms no water of condensation.	Produces water of condensation.
	Clear in appearance.	Clear when melted, but slightly opalescent upon solidification.

Agar is a complex sugar, a POLYSACCHARIDE, that for all practical purposes is not attacked by organisms in their quest for food, but gelatin is not very resistant to attack by microbial enzymes. If gelatin is being employed as the solidifying agent in the attempted isolation of bacterial colonies, and if the organisms are capable of liquefying the gelatin through enzymatic activity, much of the value of the gelatin has been sacrificed. It should be emphasized, however, that nutrient gelatin serves as a useful medium in the study of physiological reactions as part of the procedure in the identification of organisms, since some organisms can attack gelatin while others cannot.

In the isolation, study, and identification of organisms derived from warm-blooded animals, it is oftentimes imperative that the cultures be incubated at body temperature or above. Since gelatin becomes liquid without any physiological activity when the temperature approaches 25° C., it is obvious that this solidifying agent

cannot be used in such a medium. Some bacteria, including those which live and thrive in such locations as hot springs, cannot be cultivated unless the optimum temperature for the growth is provided. This may mean incubation of the cultures at 50° C. or higher. With agar as a base, the medium can be relied upon to remain solid even at these elevated temperatures, since agar normally does not melt until the temperature approaches 90° C. Gelatin has a narrow differential between the melting point and the solidifying point, about 2 degrees, while agar has a 50-degree spread, which is an advantage under some circumstances.

When incubating cultures in special plates called PETRI DISHES, such plates must be inverted if agar is the solidifying agent. The water is not bound the way it is in gelatin media, and the heat of the incubator allows moisture to escape from agar media and to condense on the lid of the petri dish. When the drops of condensed moisture become large enough, they can drop onto the agar surface and link up the growths from well-isolated colonies, nullifying the otherwise effective isolation procedure. Gelatin does not produce water of condensation, and hence it does not require incubation in an inverted position. In fact, gelatin plates should be kept upright in the incubator because of the liquefying ability of some organisms. The slight opalescence imparted to solidified agar does not interfere with its usefulness in distinguishing colonies of organisms growing either in or on the medium, while the clarity of gelatin is completely overshadowed by the serious disadvantages previously discussed.

CLASSIFICATION OF MEDIA

We can conveniently arrange the types of media under four major headings:

I. Natural Media—Substances occurring in nature.
   1. Milk.
   2. Eggs.
   3. Blood and other body fluids and tissues.
   4. Extracts of plant and animal tissues.

II. Derived Media—Comprised of known substances but the exact chemical composition of which is not known.
   1. Nutrient broth.
   2. Nutrient agar.
   3. Nutrient gelatin.

III. Synthetic Media—The exact chemical composition is known.

IV. Special Media—Combinations of the other three types of media.

PREPARATION OF STANDARD NUTRIENT AGAR

There are literally hundreds of different combinations of ingredients comprising the workable list of media employed by microbiologists. Trying to remember the formula for very many media is generally a waste of a person's time. But it doesn't seem too much to ask students, even in an elementary course, to remember the constituents of the most commonly used medium, *Standard Nutrient Agar*. During a single semester each student may use a hundred or more tubes of media. In many bacteriology courses one of the early laboratory exercises is devoted to having students prepare a batch of standard agar. Such an exercise should accomplish two objectives. First, the very fact that the operation involves "doing" will help the student to remember the constituents and the technic for blending them much better than merely hearing the steps presented in a lecture. But even more important, perhaps, the student should be impressed with the amount of time, effort, and expense involved in the preparation of even one tube of culture medium.

STANDARD NUTRIENT AGAR

	GRAMS/LITER
Bacteriological peptone (0.5%)	5.0
Beef extract (0.3%)	3.0
Agar (1.5%)*	15.0
Distilled water Up to 1 liter	(1000 ml.)
Final *p*H of medium—6.8 to 7.0	

*A firmer medium can be obtained by adding a higher concentration of agar —usually up to two per cent.

The peptone and the beef extract are heated in the distilled water just sufficiently to dissolve them. After determining the *p*H of the broth, either electrometrically or colorimetrically, 10% sodium hydroxide (NaOH) is added to raise the *p*H to the proper level (about neutrality), before the carefully-weighed agar is introduced. The percentage of agar added to the broth varies from 1½% to 2%, depending upon the hardness desired in the finished medium. Several minutes of a rolling boil (avoid burning or boiling over) will be required to dissolve the agar. The hot medium is transferred to flasks or to tubes, depending upon the ultimate use of the medium, and each container is plugged with non-absorbent cotton. After autoclaving to sterilize the nutrient agar, the tubes or flasks are kept in the refrigerator until ready for use. If the medium is to be used within a few days, storage at room temperature is satisfactory, but refrigeration will materially reduce dehydration during prolonged storage.

The refinements of blending the ingredients, adjusting the *p*H, plugging the tubes, and autoclaving the finished product are covered more fully in laboratory manuals, and hence will not be discussed in detail here.

From these observations on the preparation of a suitable medium, it should become evident that feeding microbes is not a simple matter of throwing them a bone, the way one might satisfy the desires of a hungry canine blessed with sharp incisors. Running a restaurant for microorganisms is a scientific endeavor based upon table service rather than cafeteria style of mass feeding. In devising a menu for microbes, the microbiologist attempts to provide an adequate supply of available food, served in the same style to which the organisms are accustomed in nature where they are eking out their own livelihood in a highly competitive environment.

CHAPTER 5

Microbial Structures and Staining Reactions

REPRODUCTION OF BACTERIA
THE METRIC SYSTEM
SIZE
SHAPE
ARRANGEMENT
WEIGHT
COLLOIDAL NATURE OF PROTOPLASM
INDIVIDUAL STRUCTURES OF BACTERIA
STAINING OF MICROBES

To think in terms of living things as minute as bacteria is more difficult for some individuals than it is for others, and becoming familiar with the concept of a world of microscopic plants and animals requires orientation. A few students never make this transition and their laboratory technic is a direct reflection of this maladjustment. When the instructions call for inoculating a medium with a *few* bacteria, the idea of transferring a barely visible amount of microbial growth cannot be fathomed by persons whose world is entirely populated with humans, horses, elephants, the neighbor's dog, etc.

REPRODUCTION OF BACTERIA

Higher plants and animals possess specialized organs for carrying on the process of reproduction, just as they are endowed with

structures particularly adapted for breathing, digestion, and excretion. Bacteria, however, must metabolize and reproduce within the confines of a single microscopic cell. It appears that reproduction in living things as small as bacteria cannot be too involved, and when compared with multicellular organisms, bacteria do employ a relatively simple reproductive process. At a given signal, best comprehended by bacteria, the single cells split in half across their short axis (at right angles to the long axis), in a process called BINARY FISSION. It is this simple equal division which gives bacteria their class designation of *Schizomycetes* (*schizo*, split; *myket*, fungus). Strictly speaking, growth means enlargement or increase in cell substance, but in bacteriology the terms "growth" and "reproduction" are sometimes loosely used as synonyms.

Just prior to cell division the protoplasm appears to gather at opposite ends of the organism. A cell wall puts in its appearance across the middle of the cell, with or without a visible constriction, and separation into two smaller cells takes place. Each offspring thus retains a part of the parent cell in this asexual type of reproduction. Recent evidence, particularly by geneticists, leads us to believe that sexuality in bacteria has been demonstrated. This is not meant to imply that "female" and "male" bacteria exist in the same form that we think of females and males in higher planes of life. But it has been shown that when different strains of a given species of bacteria are grown together in a test tube (*in vitro*), selected isolations from these mixtures can be demonstrated to have nutritional requirements different from those exhibited by either of the two original strains. Some type of conjugation has apparently occurred, and this type of combination strongly suggests sexual reproduction in contrast to simple binary fission.

When a bacterial cell divides, the two small offspring waste little time growing to a predetermined maximum size. These two new cells are then ready to divide, and under ideal conditions binary fission can take place on an average of once every 20 or 30 minutes for many species. Generation times longer than this are not uncommon, however, among some of the slower-growing bacteria. Fortunately for mankind this multiplication of microorganisms does

not continue indefinitely at this alarming rate. A few examples of what could conceivably occur should microbial growth go unhampered at this optimum rate might be of general interest. A sphere that normally grows in a chain formation (called a *streptococcus*) in forty-eight hours would extend over a mile and a quarter in length, according to one calculation. When you consider that the progeny of a single microbe may amount to over 300,000,000,000 in just 24 hours, Löhnis and Fred have figured that in thirty-six hours the bulk of organisms would fill 200 trucks of five ton capacity each. After a full week at this rate of multiplication the microbial volume would exceed that of the world itself!

These are rather revealing theoretical numbers, but why are they prevented from becoming reality? Unless food is available in the immediate vicinity of the microbes, multiplication of the organisms is prevented. This food shortage when coupled with the accumulation of waste products by the growing bacteria and the natural antagonisms existing between microorganisms in their fight for survival explains in large measure the inability of organisms to multiply indefinitely at the optimum rate of speed.

THE METRIC SYSTEM

Because of the minuteness of bacteria, the use of decimals to express fractions of an inch they represent would be too cumbersome, so we speak of their size in terms of MICRONS (designated by the Greek lower case letter mu, abbreviated μ). It is to be hoped that some day the Congress of the United States will take productive steps to replace the antiquated system of weights and measures we adhere to so tenaciously with the more logical *metric system.* It seems odd indeed, to a scientist, that a progressive country like ours has allowed itself to be saddled with such an outmoded system, but it has become so firmly entrenched in our way of life that getting in step with a great segment of the rest of the world's population will not be an easy matter. From time to time feeble attempts have been made to initiate steps to have America convert to the metric system, but progress on a national scale can be reported as nil. Education is admittedly a slow process. Scientific publica-

tions, however, have led the way by expressing weights and measures in that metric language understandable to fellow scientists throughout the world.

Since this is the first contact some students will have with the formal field of science, they would do well to gain a mental picture of the metric equivalents of some of our weights and measures. A review for other individuals might be profitable. A cubic centimeter (cc), also commonly referred to as a milliliter (ml), should come to mean about twenty small drops, just as a liter (1000 ml) should be visualized as a volume slightly greater than a quart. An object described as being ten millimeters (mm) long should be thought of as about two-fifths of an inch in length. Beginners in science are cautioned not to confuse millimeters (mm) with milliliters (ml). The former is a linear measurement (length), while the latter term refers to a volumetric determination.

It is unnecessary to outline the complete metric system in a book of this type, but the mention of a few of the more commonly employed terms seems justified.

Prefix	*Meaning*
deci-	tenth
centi-	hundredth
milli-	thousandth
micro-	millionth
kilo-	thousand
mega-	million

Length: One United States yard is $^{3600}/_{3937}$ meter. Or to express this in another way, 1 meter equals 39.37 inches.

1 meter = 10 decimeters
1 decimeter = 10 centimeters
1 centimeter = 10 millimeters
1 millimeter = 1000 microns
1 micron = approximately 1/25,000 of an inch.

In measuring light waves we employ the Ångstrom unit, which is 1/10,000 of a micron, or 1/10,000,000 of a millimeter. Ordinary light-type microscopes cannot distinguish objects smaller than

about 0.1 micron in size. As an object for comparison, a human red blood cell measures 7.5 microns in diameter.

Area:

1 square meter	= 100 square decimeters
1 square decimeter	= 100 square centimeters
1 square centimeter	= 100 square millimeters
1 square millimeter	= 1,000,000 square microns

Capacity: One liter is defined as the volume of pure water at 4° C. (point of maximum density) and 760 millimeters pressure which weighs one kilogram (1000 grams).

1 liter	= 1000 milliliters or 1000 cubic centimeters
1 liter	= 33.8 fluid ounces (32 ounces in one quart)
1 cubic centimeter	= 1000 cubic millimeters

Mass:

1 gram	= 10 decigrams
1 decigram	= 10 centigrams
1 centigram	= 10 milligrams
1 gram	= 1000 milligrams

SIZE

While the exact size of bacteria does vary from one species to another, in general these organisms lie within the range of 1 to 5 microns. Variations in size within this group of microorganisms, when they do occur, tend to be reflected in the length rather than in the width of the bacteria.

One of the smallest microbes classified as a bacterium is found in the nasopharynx of certain persons in the early stages of influenza, and it has the scientific name *Dialister pneumonsintes* (from the Greek *pneumon,* lung, and *sintor,* murderer or devastator). This tiny organism measures only 0.15 μ by 0.3 μ, and it is pathogenic for rabbits and guinea pigs. On the other end of the scale may be cited *Bacillus büitschlii,* originally isolated from the cockroach, *Periplaneta orientalis.* This bacillus measures 3.0 μ to 6.0 μ in width and up to 80 μ in length. Most cocci fall within the range

of 0.5 μ to 1.0 μ in diameter, while most spirals are larger than either the rods or the cocci. Some spirals have been reported to be as long as 500 μ, but the general length is under 16 μ. The syphilis germ, *Treponema pallidum*, measures 0.3 μ in width and from 6 to 14 μ in length. The spirochete associated with trench mouth, *Borrelia vincentii*, is very similar in size to the syphilis organism, but the nature of the spiral twists in the two organisms readily differentiates them.

SHAPE

Although variations in morphology (size, shape, and structure) of bacteria may be induced by altering the environment in which the organisms are growing, in general only three principal shapes of bacteria exist: spheres, called *cocci*, (singular *coccus*); rods, called *bacilli* (singular *bacillus*); and twisted rods, called *spirilla* (singular *spirillum*).

It might be well to briefly mention the Latin endings used to designate singular or plural nouns in scientific terminology. Many Latin nouns ending in *-us* form the plural by converting the word ending to *-i* (as bacillus to bacilli). Many words ending in *-a* become plural by changing to *-ae* (such as sarcina to sarcinae). Other nouns ending in *-um* form the plural by changing the ending to *-a*. Occasionally persons prefer the word *mediums* for the more correct plural *media*, and these liberties with Latin may be adopted in time and become part of the accepted terminology.

ARRANGEMENT

After binary fission has taken place, many bacteria separate from their twins and go about their carefree way, but with some species the tendency to remain attached after division has occurred results in an arrangement of the cells which is a characteristic aiding in the identification of the species. To best determine this typical arrangement, cultures should be cultivated in liquid media for from 18 to 24 hours prior to being examined in a so-called HANGING DROP preparation under the high dry objective of the compound microscope. Thus a living culture can be studied and typical arrangement for the species can be determined.

Since multiplication of true bacteria occurs at right angles to the long axis (although there is evidence that at least some spirilla multiply by longitudinal division), it should become apparent that if complete separation of cells does not materialize after fission, the possible arrangement of the cells will depend upon the original shape of the organism. Since cocci possess no long axis, division can take place at any angle with the result that the following arrangements can and do exist:

SPHERES

Coccus—Individual spheres

(Coccus comes from the Greek *kokkos,* meaning berry.)

Diplococcus

This is formed by simple division of a sphere into two cocci which remain attached. Such an arrangement is relatively common and includes such organisms as the pneumococcus (*Diplococcus pneumoniae*), the gonococcus (*Neisseria gonorrheae*), and the meningococcus (*Neisseria intracellularis*). While all three of these examples happen to be pathogens, it is not the intention to imply that all diplococci are disease-producers.

Streptococcus

Continued division of a diplococcus in the same plane forms a chain of spheres resembling a strand of beads. The organism (*Streptococcus lactis*) which plays such an important role in the souring of milk and dairy products is an example of this beaded arrangement. *Streptococcus pyogenes,* which is the etiological agent in a number of human and possibly animal infections, is another example of chain formation.

Tetrads

When a diplococcus divides in such a manner as to form a group of four spheres in the same plane, rather than in a straight line, we

call the resulting formation a tetrad. *Micrococcus tetragenus* is a common skin contaminant.

Sarcina

If a tetrad divides to form four spheres back to back with four other spheres, this packet of eight cocci is a sarcina. An organism commonly found floating on dust particles in the atmosphere is *Sarcina lutea.* The specific name *lutea* is derived from the yellow color this organism produces when grown on a solid medium under aerobic conditions.

Staphylococcus

Haphazard multiplication in all planes resulting in masses of cocci in grape-like clusters gives us an arrangement called a staphylococcus. Representative members of this group include *Micrococcus pyogenes* variety *albus* (formerly called *Staphylococcus albus*), a pus-producing inhabitant of normal skin, and its golden-colored cousin *Micrococcus pyogenes* var. *aureus,* the common cause of pimples, boils, carbuncles, osteomyelitis (a bone disease), and blood poisoning. Both the *albus* and the *aureus* varieties of this organism may be implicated in cases of food poisoning, but the golden variety is more often the causative agent. Many workers in the field of microbiology, probably from force of habit, prefer the term *Staphylococcus* food poisoning to *Micrococcus* food poisoning.

The typical arrangement of mature cultures of bacteria is characteristic of the species and aids in the identification process. While it is true that a single sphere placed in a liquid medium must first pass through the diplococcus stage before it can become a tetrad, a streptococcus, a sarcina, or a staphylococcus, if that single sphere was destined to be a staphylococcus, it will eventually mature and have that arrangement. If a coccus is destined to be a diplococcus, it will stop at the diplo stage. However, a diplococcus may temporarily appear as a tetrad just at the time that it is dividing into another diplococcus, but the characteristic arrange-

ment of the spheres in pairs will eventually predominate in the mature culture.

RODS

In contrast to the cocci, rod-shaped bacteria can only divide one way—at right angles to the long axis, and this limits the number of possible arrangements such rods can have.

Diplobacillus

If the single rod divides and the two newly formed rods adhere to one another, the result is a diplobacillus.

Streptobacillus

Should the diplobacillus in turn divide and the four rods form a chain, we speak of this arrangement as a streptobacillus.

SPIRALS

Spirals do not ordinarily remain in close proximity after the signal for them to divide has been relayed to the proper trigger mechanism. It is relatively uncommon, therefore, to see spirals in any arrangement other than singles and occasionally pairs. The looseness or the tightness of the twists in a spiral are also of some diagnostic significance. The tight cork-screw appearance of a living syphilis germ (*Treponema pallidum*) as it spins its way through the fluid obtained from a suspected lesion being examined under the darkfield microscope is so characteristic that a trained observer can confirm a clinical diagnosis of this terrible disease. In trench mouth the appearance of long, loose spirals found in association with *fusiform* bacteria (rods pointed at each end) in a stained smear made from material swabbed from the gum line of the teeth is a laboratory confirmation of a clinical diagnosis of this troublesome affliction.

THE WEIGHT OF BACTERIA

A curious individual might be tempted to speculate as to the weight of a single bacterium. To dismiss the inquiry by stating that they don't weigh very much is avoiding the issue. People

have faced this problem and have arrived at definite answers confirmed by other workers applying similar or different technics. It is true that when you compare a mouse to an elephant, the mouse really isn't very big. But to an inquisitive bacterial cell, a mouse is a monstrous thing, while a virus undoubtedly looks extremely tiny to this same bacterial cell. Everything is relative.

Bearing in mind that weight depends upon size and density, it has been calculated that it would take five billion bacteria to weigh one milligram. To express this in another way, a single organism weighs about 0.000,000,000,000,2 gram. A moderate sized drop of milk can easily harbor a number of bacteria exceeding the entire human population of New York City, and there would still be plenty of room to spare in that drop of milk. A farmer, when informed that the milk he had shipped to the creamery a few days previously had a bacterial count exceeding one million, struck his forehead in amazement and remarked, "I wonder how I ever managed to get the lid on the can!" His surprise would have been still greater had he been told that every twenty drops of milk in that forty quart can contained over a million bacteria. It is true that bacteria don't weigh much, nor do they occupy much space, if we are thinking purely in terms of macroscopic rather than of microscopic objects.

COLLOIDAL NATURE OF PROTOPLASM

PROTOPLASM (Greek *protos*, meaning first, and *plasma*, meaning formed substance) is the basis of all life, and it is still a biological mystery. Even though scientists have compounded materials apparently identical with protoplasm, the synthetic material lacks the spark of life. Hugo von Mohl in 1846 originated the word protoplasm to describe that colorless or gray, translucent, semifluid, colloidal substance found in all cells. As our knowledge of cells increased, other words were introduced to describe definite structures within the protoplasm. The term NUCLEOPLASM (sometimes called karyoplasm) was applied to the material within the nucleus, and CYTOPLASM for the material surrounding the nucleus. Protoplasm has certain characteristics in general, but specific

cells have their own chemical configuration. Protoplasm has been defined as a system of chemical compounds held together in a colloidal suspension and containing carbon, hydrogen, oxygen and nitrogen, among other elements, in the form of proteins, carbohydrates and fats. The English physicist, Thomas Graham, introduced the word COLLOID in 1861 to describe substances that will not diffuse through a membrane. Those substances which can diffuse are called CRYSTALLOIDS. The minute colloid particles are suspended in a liquid, and there is a constant interplay and exchange of various atoms, molecules, electrical forces, physical and chemical stresses and attractions—all in a delicately balanced state of equilibrium. Any abnormal alteration of any of these forces will affect the functioning of the cell and may result in its death. Microbiologists employ this knowledge in their attempts to create as favorable conditions for growth as possible, and also in their attempts to destroy microbes by chemical and physical forces when destruction is desirable.

The properties of protoplasm are those which distinguish living from dead substance: movement, irritability, reproduction, metabolism, and death. The latter term, *death,* is the final distinguishing characteristic of protoplasm, in a given stage, but to define death is not easy. Just to say that it is cessation of life is hardly satisfactory, because a definition of life is equally difficult. We do know that the colloidal structure of protoplasm is markedly altered when death steps in, but other than that, our knowledge is fragmentary.

INDIVIDUAL STRUCTURES OF BACTERIA

CELL WALL AND CELL MEMBRANE

The cell walls of plants are more rigid than the walls normally found on animal cells. This membrane varies in its permeability between organisms, and the permeability within a given species can be materially altered by application of chemical or physical forces. Most evidence points to the conclusion that the wall functions principally as a protective device for the underlying structures and gives the cell its shape. If it were not fairly rigid,

bacteria would all tend to assume a spherical shape. When it is said that the wall is firm, it should also be pointed out that it is somewhat elastic. Ordinary staining usually fails to distinguish the wall from the rest of the cell unless the organism has been grown in a concentrated solution which causes the cytoplasm to shrink away from the outer wall, leaving a ghost-like shell.

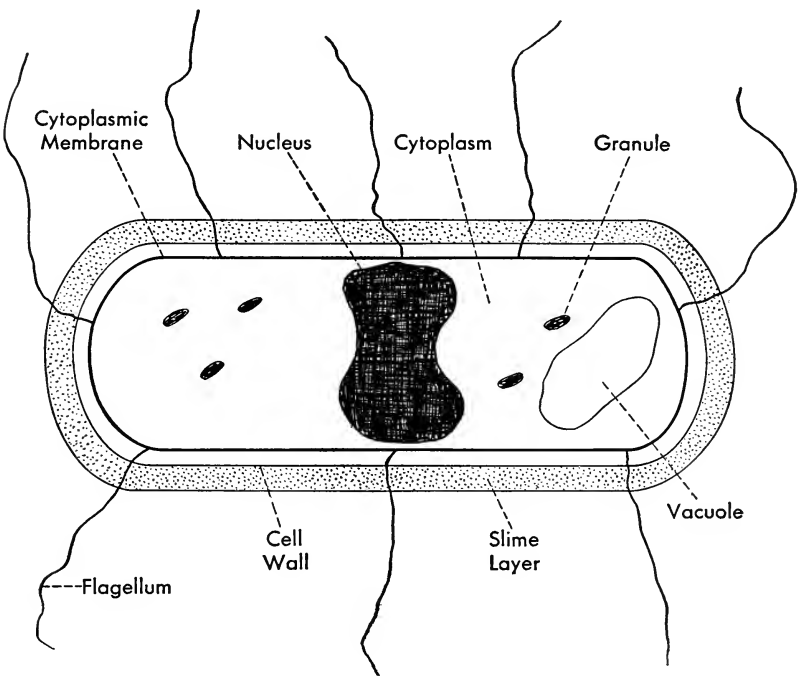


Fig. 19. Schematic drawing of a hypothetical bacterial cell.

Cellulose, hemicellulose, or mucin (nitrogen-containing compounds) are the common constituents of bacterial cell walls.

The cell wall proper is probably not as discriminatory as is the underlying CELL MEMBRANE, which limits the cytoplasm of the organism. Because of its selectivity we refer to this membrane as being SEMI-PERMEABLE in nature. Theories have been proposed to explain this selectivity, and these explanations vary from a simple mechanical sieve theory to complex physico-chemical re-

actions. There is little doubt that a number of forces are active in the process, and to try to pinpoint a single explanation is probably not feasible.

NUCLEUS

A typical cell contains a NUCLEUS, usually rather spherical in shape, enclosed within a thin membrane, and surrounded by material called CYTOPLASM. But bacteria, just to be different, do not usually possess a well-defined nucleus such as we observe in most other cells. Among other things nuclear material consists of CHROMATIN, a substance believed to be vital to all living cells. The name chromatin is derived from the strong affinity it has for certain coal-tar dyes, and the nucleus of cells stains more deeply than other parts of the protoplasm.

Some investigators go so far as to claim that the primitive nature of bacteria makes a well-defined nucleus unnecessary, but others hold the opposite view, and they claim that a bacterial cell is extremely complex. They feel that it has to be, in order to cope with all of the life processes within the borders of a single cell. The nucleus is the heart of the cell—the control panel of a complex mechanism.

A nucleus is usually considered to be the determiner of hereditary characteristics, and to some persons the nucleus is a single chromosome which must undergo division before new cells can be formed. If each offspring is to resemble the parent cell, there must be some mechanism for the transmission of these hereditary characteristics, usually a function of chromatin material.

When we subject bacteria to nuclear dyes, the entire cell becomes stained, suggesting that the nuclear material may be diffuse. There is some evidence to support the concept that at certain stages in the growth of bacteria the nuclear material may undergo a local concentration.

Common beliefs relative to the bacterial nucleus include the following:

1. The entire bacterial cell is composed of nuclear material, with little or no cytoplasm.

2. The entire cell is composed of cytoplasm with no nucleus.
3. There is a definite nucleus in bacterial cells but the usual staining technics do not reveal a nucleus except when the cells are grown under specific conditions, and stains are made at a precise stage in their growth.
4. Chromatin granules are spread out in the cytoplasm and are abundant enough to give the impression that the entire cell is composed of chromatin material.

This latter theory of a diffuse nucleus has considerable support in the bacteriology profession. Blue-green algae have such a nuclear structure, but because these bodies are larger than in bacteria, they are more readily observed under the microscope. Electron microscope studies have revealed distinct nuclear-like material in some bacterial species and undifferentiated nuclear matter in still other cells. By adhering to the structure definition of a nucleus, it must be admitted that bacteria probably have no nucleus. But if function is considered, all cells must have a regulatory body, and bacteria are probably no exception. From the functional point of view, the structure of the nucleus is of relatively little importance.

CAPSULES AND SHEATHS

Although it is not possible to demonstrate capsules on all bacteria, it may be a safe assumption that all bacteria possess a slime layer, sometimes of a thickness not detectable by the usual technics employed for their demonstration. There is evidence that capsules serve as a protective material for some organisms, by slowing down or preventing penetration of chemicals and body juices. Most capsular material is carbohydrate or carbohydrate-like in nature, but lipoid material predominates in a few selected species. The capsule may be a mere thickening of the outer membrane or more probably a secretion or excretion which deposits itself around the cell. Capsule formation can be enhanced by animal passage of certain organisms or by growing them in high carbohydrate media.

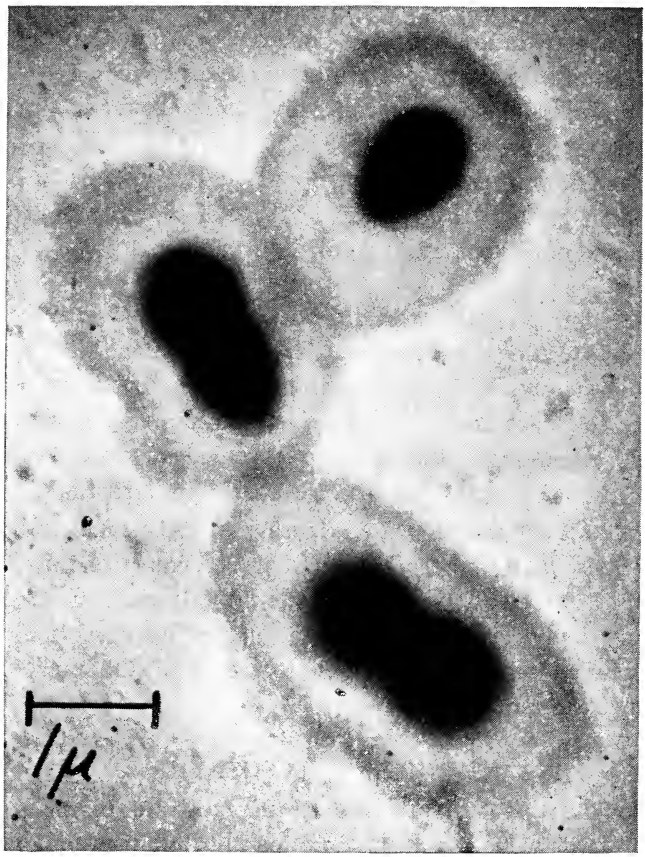


Fig. 20. Electron micrograph of *Diplococcus pneumoniae,* Type I. The capsules are swollen as a result of exposure to Type I antiserum. (*From Mudd, S., Heinmets, F., and Anderson, T. F.* The Journal of Experimental Medicine, *1943, 78, 327–332.*)

Capsules are of more than just passing interest to bacteriologists. They have played an important role in medicine particularly with respect to pneumococcus pneumonia. Before the advent of the sulfa drugs and the more recent antibiotics, it was necessary for a physician to know which of the many (seventy-five or more) types of pneumonia a patient was suffering from before antiserum could be administered. Type III pneumococcus infections were par-

ticularly difficult to treat because their capsule is so much thicker than we find in other types of pneumococci. Treatment with the antibiotics is not dependent upon the specific type of capsular material; all pneumococci react about the same to these newer drugs. Now that pneumococcus typing by a qualified technician

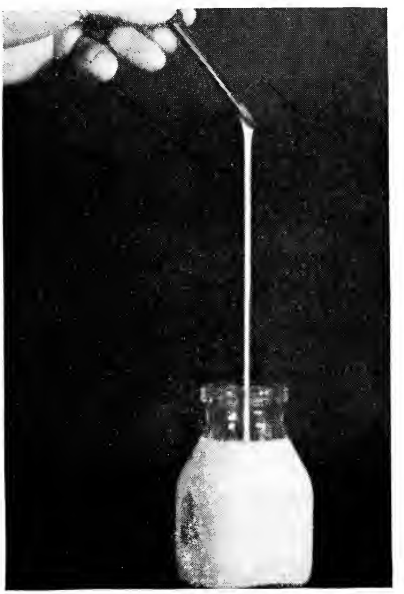


Fig. 21. Ropy milk caused by the growth of *Alcaligenes viscosus*. (*From* Microbiology, *W. B. Sarles, W. C. Frazier, J. B. Wilson, and S. D. Knight. Copyright 1951, Harper and Brothers, New York.*)

is unnecessary, the time saved before specific treatment can be initiated can mean the difference in the outcome of the disease.

Another interesting sidelight on capsules is their role in producing slimy, or ropy, milk. Some organisms (*Alcaligenes viscosus*) commonly found in swampy areas, among other locations, are endowed with greatly enlarged capsules, and if these bacteria become established in a milk processing plant, they can cause the milk to become stringy. The relatively high resistance of these encapsulated forms to the normal heat and chemical treatments em-

ployed by milk-processing plants makes them difficult to eliminate, and by the time the situation has been cleared up, many customers have found new sources of supply for their milk.

Ropy bread will result when the flour from which it is made becomes contaminated with encapsulated organisms. Ropiness is due to a breakdown of starch by the organisms and the synthesis of gums from the resulting carbohydrates. Should these forms of bacteria become established in a sugar-refining plant, they are capable of interfering with normal crystallization of the sugar, a costly affair for a large plant. Constant vigilance and strict sanitary measures must be the watch-word in food-processing plants.

Many pathogenic bacteria lose their virulence when they are stripped of their slime layer, but *Bacillus anthracis*, the etiological agent in anthrax, becomes encapsulated after it gets into a susceptible animal, and it may eventually destroy the host. It is also possible for sheep to ingest spores of the anthrax bacilli, and these spores can germinate and become encapsulated *in vivo* with similar fatal results for the host.

Certain bacteria, particularly the iron bacteria, are capable of secreting or excreting a substance called a SHEATH, which becomes quite firm in contrast to the slimy nature of representative capsules. Iron compounds may be deposited in these sheaths, and when their volume builds up, these bacteria are capable of completely occluding water pipes.

The importance of transfusions with whole blood or with blood plasma in saving human life has been amply demonstrated during World War II and in the Korean conflict, as well as in civilian hospitals. With the source of supply of these vital fluids relatively limited, research has been undertaken to provide needed substitutes. A substance called DEXTRAN has been found to be such a possibility. While dextran cannot be considered to be a complete substitute for either whole blood or plasma, its use as a so-called "extender" is proving valuable in restoring the balance so essential in the blood stream in cases of shock.

Dextran is produced by the action of certain species of the bacterial genus *Leuconostoc*. These organisms are gram positive

spheres occurring in pairs and in chains, and when they are cultivated in sucrose solutions, the chains are surrounded by a thick, gelatinous, colorless membrane consisting essentially of dextran. The dextran is removed by selective chemical action and is purified for use in human transfusions.

GRANULES

Ordinary staining technics commonly employed with bacteria reveal at times deeply stained bodies called *metachromatic granules* within the cells. Definite agreement as to the origin and the function of these bodies is still not available. Some workers feel that they are particles of reserve food material. Others call them waste products since the granules in some bacteria do not appear until the twilight of the microbe's life, and granules tend to disappear when active microbial multiplication is encouraged by subculturing in a new medium. Chemical analysis reveals these particles to be fat, carbohydrate, or complex nitrogenous compounds. Pronounced development of granules is a characteristic feature aiding in the identification of the etiological agent in diphtheria (*Corynebacterium diphtheriae*). The arrangement of granules at the ends (poles) in the plague organism (*Pasteurella pestis*) is in contrast to the bands and scattered dots seen in the diphtheria organisms. Sulfur and iron granules may be seen in some of the higher bacteria.

SPORES

When bacteria are gradually subjected to increasingly unfavorable conditions, including lack of water, depletion of available food, and marked temperature deviations from the optimum, many organisms will die. Certain rod-shaped species classified under the family *Bacillaceae* are able to develop structures called ENDOSPORES (spore within a cell), which can withstand relatively undesirable environmental conditions. On the other hand, few cocci or spirals exhibit spores. Bacteria lacking these structures are termed VEGETATIVE CELLS. Bacterial spores might be considered to represent a resting stage similar, perhaps, to the hibernation of some higher animals and the encysted stage present among protozoa. Since

but one spore is formed by a single cell, there is reproduction without multiplication. This is in contrast to mold spores which are reproductive bodies, many of which are formed by a single mold plant.

Frequent transplantation under ideal growth conditions may prevent spore-formation, and some strains so cultivated may lose their ability to sporulate even when conditions become unfavorable for the cell. The cause of sporulation is unknown, but it is not necessarily a response to marked unfavorable conditions, since some bacteria sporulate early in their lifetime when conditions are apparently still favorable for microbial growth and multiplication. After the danger has passed, the "possum-like" microbes emerge from their dormancy, or resting stage, and revert to their original vegetative state. The first visible change when spores are placed in a suitable medium is an enlargement of the spore, probably due to the absorption of water. After losing its refractive nature, the spore elongates. Some shed their "skin" as they germinate, while others appear to absorb the spore material. There is some evidence that the "shedding" type of spore can withstand more unfavorable environmental conditions than can the "absorbing" type.

A given species of organism will exhibit a constant size, shape, and location of the spore (*terminal, subterminal,* or *central*) within the cell, and in some cases this structure aids in the tentative identification of an organism. Should the swelling occur in the center of the rod, it gives a spindle-shaped appearance, and if the bulge is located terminally, the cell takes on a drumstick shape.

Not all spores are equally resistant to chemical and physical forces, but the most resistant spores are the basis for the time and temperature relationships employed in sterilization technics. Were there no spores, we could drastically revise our sterilizing temperatures downward. The common soil organism *Bacillus subtilis* has been found to withstand 100° C. dry heat for three hours, and *Clostridium botulinum*, the causative agent in a highly fatal type of food poisoning to be discussed in a later chapter, may withstand four or five hours of boiling. The spores of some

THERMOPHILIC (heat-loving) bacteria are much more resistant to heat, whereas vegetative bacteria are killed by an exposure to a temperature of 65–80° C. moist heat for only a few minutes.

Fortunately, few spore-formers are capable of causing disease in man. But the most important ones are probably *Clostridium tetani,* the cause of lockjaw, *Clostridium perfringens,* the etiological agent of gas gangrene, the previously mentioned *Clostridium botulinum,* and *Bacillus anthracis,* the biological agent in anthrax.

FLAGELLA

Rapid locomotion by bacteria is not uncommon, and this movement is brought about principally through the influence of hair-like projections termed FLAGELLA ("little whips"). All spirals are motile, probably half of the rods are motile, and practically none of the cocci exhibit independent movement. The position and the number of flagella will vary between species, but constancy of arrangement of flagella is an aid in the identification of species.

Since bacteria are single-celled organisms, we must consider the flagella to be a continuation of the cell with a direct connection to the underlying cytoplasm. The electron microscope substantiates this claim. Electronographs reveal many flagella to be much longer than the cells from which they arise, so it is not too surprising to find free flagella floating around in a medium, since flagella are delicate and can be broken off by even gentle agitation.

Motility is a valuable asset to bacteria, just as it is important to man and to other animals. The chances of survival are greatly enhanced when an organism has an opportunity to go out and look for food if the supply begins to run low. Being trapped in an unfavorable locality without means of escape can be as critical for a bacterium as it is for a helpless bedridden patient in a locked room that has caught fire when no one is around to offer assistance.

One of the topics of the day in active bacteriological circles is this entire question of flagella. Do bacteria have them, or are they artifacts which appear as a result of locomotion? Are flagella the cause or the effect of motility? Until definitely proven otherwise,

we will continue to speak of flagella as the cause of bacterial locomotion.

If we consider the time in which bacteria can cover a distance equal to their own length, their speed becomes jet-like in character.

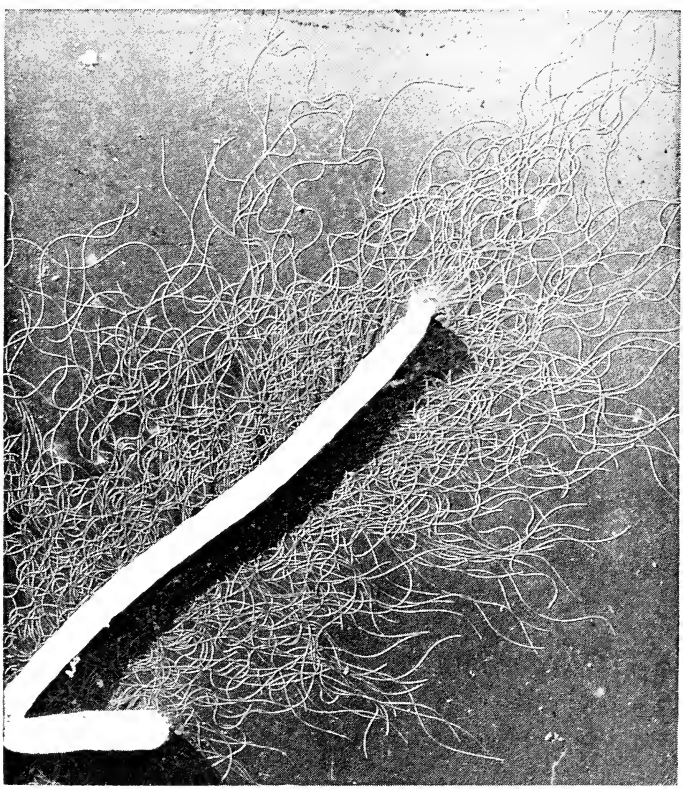


Fig. 22. Electronograph of a *Proteus* species prepared by a shadowing technic, showing flagella. (*Courtesy of C. F. Robinow and J. Hillier.*)

It has been calculated that a car traveling at comparable speed for its mass as some of our more active microbes would have to go over 1000 miles an hour! What induces a microbe to change direction is still not known, but a car attempting to alter its course

at the same relative speed would turn over. Perhaps bacteria do execute a "barrel roll" as they change direction. After all, which side is up for a bacterium?

When objects are very small they are bombarded by molecular forces external to the cell, and the particles undergo a vibrating, trembling-like motion called BROWNIAN MOVEMENT. This phenomenon was reported in 1827 by Robert Brown, a botanist. As the particle size diminishes, Brownian movement increases. To distinguish this reaction from true movement caused by forces from within the cell, more than mere vibration must be detected. True motility means making progress through liquids—actually getting somewhere rather than merely vibrating in one spot. Motion and motility, in this sense, are not synonymous. The sluggish movement of some bacteria makes the distinction between the two quite difficult unless the culture is in the active stage of growth (less than twenty-four hours old), and is examined at the optimum temperature under the high dry objective of the microscope in a preparation called a HANGING DROP. Drifting with the tide is often mistaken for motility; bacteria must buck the tide and swim upstream, as it were, before we can call the motion true motility.

The natural slow movement of some microorganisms makes distinction between motility and Brownian movement difficult, but speed of the bacteria may be enhanced by examining hanging drop preparations on a warm microscope stage with the culture in the active phase of growth. Not all rods are flagellated, but rods exhibiting motility have all been shown to possess flagella. Some of the spiral forms of bacteria appear to move more by a twisting of their elongated cells than by flagella, although this point is still debatable.

Because of their narrow width (about 0.03 μ in diameter), flagella are not visible when the usual staining technics are employed. The extreme fragility of these structures is another factor complicating their observation.

Spirals tend to have flagella located at the ends of the cells but not on the sides. With straight rods, however, we find singles, tufts, and dispersed flagella arranged around the cell's perimeter.

The following terminology has been commonly accepted to describe arrangement of flagella:

Monotrichic	a single flagellum at one end.
Lophotrichic	a tuft of two or more flagella at one end.
Amphitrichic	one or more flagella at each end.
Peritrichic	flagella around the entire perimeter.

When flagella are lacking, the organism is said to be atrichic. It has been suggested by some workers that amphitrichic flagellation as such does not exist. What is being viewed is two monotrichic or two lophotrichic organisms about to divide but not as yet completely separated into individual organisms. Others classify motile bacteria into but two categories: Those having only lateral flagella, and those possessing only terminal organs of locomotion. Any finer breakdown, to these scientists, seems unjustified.

Movement of flagella is not mere thrashing about. An apparent rotating motion propels them through fluids. Periodic contractions run around the flagella from one end to another, giving the appearance of a rotary motion. These rhythmic contractions are capable of forcing organisms through liquids at a rate exceeding 100 microns per second, although 25 to 30 microns per second is more common. The cholera organism has sped through a measured course at a registered speed of 8 inches per hour, and this speed can apparently be maintained for considerable time. The angle formed by the flagella with the cell body determines the direction of movement.

These protoplasmic threads, as they are referred to by some investigators, have a constant arrangement for each species on which flagella are found, and this can usually be relied upon in the identification of organisms. We can slow down the speed of organisms by growing them in a semi-solid medium which is more viscous than nutrient broth.

STAINING OF MICROBES

Bacteria stain well with basic (in the sense of *p*H) dyes because of the dye affinity of the nucleic acids contained in the

organisms. These stains belong to the group of aniline (coal-tar) dyes and include crystal violet, basic fuchsin, safranin, methylene blue, eosine, etc. After flooding a properly prepared smear on a glass slide with the particular dye and allowing a contact of from a few seconds to a minute, enough dye will be taken up by the cell contents to make the organism readily visible under the microscope. Stained slides are usually best examined under the oil immersion objective, which allows magnifications of one thousand or more diameters. Different stains and combinations of stains have been devised for specific purposes. A brief discussion of a few of these will follow.

An important prerequisite for any staining operation is to have a clean slide on which the smear is to be prepared. Greasy slides do not allow even distribution of the test material, and much of the bacterial film may wash off during the staining processes. Beginners in bacteriology have a tendency to prepare films much too crowded with organisms for satisfactory examination of individual cells—the ultimate goal of any bacterial staining process. With a little practice the novice soon learns that a little bacterial culture will go a long way.

Once the organisms have been introduced into the drop of liquid on the slide, the film should be spread out to provide thick as well as thin areas of material to be stained. Drying is usually best accomplished by allowing the smears to air-dry, but if more rapid drying is desired, no more heat should be applied to the slide than would be comfortable for your own fingers. It it is too hot for your hand, it is certainly too hot for the bacteria in the wet smear where abundant moisture intensifies the heating action. Bacteria can tolerate higher temperatures of dry heat than they can withstand moist heat. It's not the heat, it's the humidity! Over-heating wet smears is undesirable, otherwise staining may not be characteristic. Some persons advocate a quick passage of the dried film through an open flame to "fix" the smear more firmly to the slide. The value of this technic is debatable.

Stains may be classified into two major groups: (1) *General Stains* in which basic dyes are usually employed to make bacteria

more readily observable, and (2) *Differential Stains* in which technics are used to divide bacteria into groups or to bring out some specific structure of organisms.

The idea of staining microbes was first introduced into microbiology by Carl von Weigert in 1871 when he first stained bacteria with carmine and later aniline dyes. Dozens of dyes have come into common use since those early experiments of Weigert. A dye is a colored organic compound which has the ability to combine with certain substances and to impart color to them. It is possible to enhance staining ability by adding *intensifiers*. For example, if a basic dye solution is made more alkaline, bacteria tend to stain more intensely. The introduction of surface tension depressants (wetting agents) may allow more intimate contact of dye with protoplasm. By applying heat and by adding carbolic acid (phenol) it is possible to intensify the dye-protoplasm union. Staining is a chemical or a physical union between the dye and components of the cell. If it is a chemical reaction, a new compound is formed, and simple washing in water does not liberate the bound dye. But if it is mere physical union, it is easier, as a rule, to decolorize such organisms. Many staining reactions are undoubtedly a combination of physical and chemical unions.

NEGATIVE STAINS

Negative stains, including India ink, nigrosine, and Congo red, do not have an affinity for bacterial protoplasm. Negative staining is also known as RELIEF STAINING, since the background material retains the dye while the bacteria stand out in relief as unstained areas. To prevent bacterial growth in these dyes during storage—a common occurrence and a troublesome one—the addition of 0.5% formalin is advocated.

India ink is a fine suspension of carbon particles in an aqueous gelatinous medium, but because it lacks uniformity this dye is not as popular as some of the others, particularly nigrosine which is a colloidal suspension. When Congo red is the dye being employed, some persons recommend that the completed preparation be dipped in acid-alcohol (1% hydrochloric acid in ethyl alcohol)

for a few seconds. This will convert the Congo red to a blue color, which appears to be easier on the eyes for some individuals. Sharp boundaries of organisms are characteristic when negative dyes are used, and in studying sizes and shapes of bacteria, particularly the spiral forms, this is a decided advantage. Students should be cautioned not to confuse negative staining with a gram negative reaction; they bear no relationship to each other.

GRAM STAIN

The gram stain is by far the most important differential stain employed in bacteriology. The technic was introduced in 1884 by Christian Gram, a Danish scientist, after a chance observation that tissues stained by his method could be made to release the dye but the bacteria embedded in the tissue retained their color. Further experimentation revealed that organisms could be divided into GRAM POSITIVE bacteria (those bacteria retaining the dye) and GRAM NEGATIVE bacteria (those organisms giving up the dye) when the slide was placed in alcohol. Gram's original method was to treat the smear with crystal violet followed by exposure to a dilute solution of potassium iodide. In order to distinguish the gram negative bacteria which have lost their color after the alcohol treatment, a counterstain of safranin or of carbol fuchsin is employed. Thus, a mixture of gram positive and gram negative organisms after having been subjected to this differential staining procedure will reveal purple cells (the gram positive bacteria) and pink cells (the gram negative organisms).

The binding effect of the iodine with the crystal violet is less pronounced with the protoplasm comprising the gram negative organisms, and subsequent decolorization in alcohol is more easily accomplished. It should be pointed out, however, that excessive exposure to alcohol will also decolorize gram positive bacteria. The contact periods in each solution vary with the preference of individual laboratories, and procedures designated to acquaint students with the gram stain will outline specific methods.

There are degrees of gram positiveness and gram negativeness. To minimize the number of stained preparations that are difficult

to call one reaction or the other, it is important that young, actively growing cultures be employed as source material. After twenty-four hours of growth on laboratory media many gram positive bacteria tend to lose their dye-holding capacity, and they will yield a gram negative or a gram variable reaction.

TABLE 8

SOME ORGANISMS THAT EXHIBIT A GRAM NEGATIVE STAINING REACTION

NAME OF ORGANISM	FUNCTION, SOURCE OR HABITAT
Aerobacter aerogenes	Found in the soil, on plants and grains.
Azotobacter chroococcum	Fixes atmospheric nitrogen non-symbiotically in the soil.
Brucella abortus	The cause of contagious abortion in cattle and undulant fever in man.
Escherichia coli	Inhabitant of the intestines of warm-blooded animals.
Hemophilus pertussis	The cause of whooping cough.
Neisseria intracellularis	The cause of epidemic meningitis.
Proteus vulgaris	Found in certain infections and in putrefying material.
Salmonella typhosa	The cause of typhoid fever.

TABLE 9

SOME ORGANISMS THAT EXHIBIT A GRAM POSITIVE STAINING REACTION

NAME OF ORGANISM	FUNCTION, SOURCE OR HABITAT
Bacillus anthracis	The cause of anthrax.
Bacillus cereus	Found in soil and dust.
Corynebacterium diphtheriae	The cause of diphtheria.
Clostridium tetani	The cause of tetanus, or lock-jaw.
Diplococcus pneumoniae	The cause of lobar pneumonia.
Sarcina lutea	Found in air, soil, water, and on skin surfaces.
Streptococcus lactis	Found in milk and milk products. Plants may be the natural habitat.

When attempting to identify a bacterial culture, the gram stain is one of the first tests conducted on the organisms. If the stain reveals a gram positive cell, the investigator can immediately

dismiss the hundreds of gram negative organisms described in *Bergey's Manual of Determinative Bacteriology.* Identification of organisms is an elimination procedure, and the gram stain represents a very important early step in this process.

Without discussing complex chemical and physical theories proposed to explain this staining difference of bacteria, a few broad concepts will be mentioned. Some persons believe that there is a difference in the permeability (intactness) of the cell walls, with gram negative cells being more permeable than the gram positive bacteria, both for the entrance and for the egress of the crystal violet dye. The chemical composition of the surface of the cells is another theory put forth to explain the difference in gram staining reactions between cells. Gram positive bacteria contain a chemical compound called magnesium ribonucleate at or near the cell surface. When these organisms are stripped of this chemical, they become negative in their staining reactions. If the magnesium ribonucleate is "replated" on these stripped cells, they revert to their gram positive status. Attempts to convert true gram negative cells by this plating technic had met with failure until recent studies indicated that if a viscous ribonucleate is employed, it is possible to change gram negative bacteria into gram positive organisms. That the gram stain depends upon surface phenomena is fairly well agreed, but the exact mechanism of the reaction is still in doubt.

CAPSULE STAIN

It is sometimes difficult to demonstrate capsules on bacteria, and some failures undoubtedly are due to the ease with which some slime layers can slip off the cells during the staining process. In fact, slime can be demonstrated in some liquid cultures, but the bacteria in that culture may fail to show capsules around the cell wall.

A generally accepted technic for staining capsules employs India ink, nigrosine, or Congo red (so-called negative stains) as background material against which the unstained organisms stand out. By counter-staining with a basic dye like crystal violet, the bacterial cell will take up the dye while the capsule stains only

faintly, if at all. This type of staining can be enhanced if the organisms are grown or suspended in blood serum or in milk. Many workers in the field are reluctant to accept the relatively wide halo seen around such cells as representing the true size of the capsule. Some shrinking occurs in the staining process and capsules are exaggerated. By employing mordants and by examining undried preparations, more reliable results should be obtained.

A rather widely accepted technic for demonstrating capsules is the *Hiss Stain.* This method recommends serum or ascitic (body) fluid as background material in the preparation of the smear. After allowing the slide to dry rather slowly at room temperature, the smear is flooded with a crystal violet solution and heated just enough to make steam visible. By washing off the excess dye with a solution of copper sulfate (use no water anywhere in the process), draining away the excess fluid and blotting gently until the slide is dry, the capsule will be seen as a faint purplish halo about the more deeply stained underlying organism when the oil immersion objective is employed.

ACID-FAST STAIN

As the name suggests, this stain remains fast even in the presence of mineral acid. There are limits to this fastness, however, and just as timing is important in the various steps of the gram stain, we must understand the limits of exposure of acid-fast bacteria to acid.

A relatively small group of organisms, principally members of the genus *Mycobacterium,* possess chemical or physical properties which make the cells difficult to stain, and once stained they are difficult to de-stain by ordinary methods. Robert Koch's difficulty in finding the causative agent in "consumption" (tuberculosis) may be traced to this peculiarity in staining of the organisms. The fatty-waxy nature of the capsule surrounding these organisms has been the usual explanation of why the tuberculosis organism is difficult to penetrate with dyes. Once this tough barrier has been breached, the reverse process of removing the dye is equally

difficult. By applying heated dye, such as carbol fuchsin (composed of basic fuchsin dye, alcohol, and phenol), the permeability of the capsule is increased, possibly due to the softening effect of the heat on the capsule. A common procedure employed in staining acid-fast material is that method proposed by Ziehl-Neelsen in which hot carbol fuchsin is applied to the dried smear for about 5 minutes. Decolorization is carried out using a mixture of ethyl alcohol and 2 or 3% hydrochloric acid. This is called acid alcohol. The length of exposure to this decolorizer depends upon the thickness of the preparation, but in general a few seconds are sufficient to remove the color from everything except the acid-fast organisms. The decolorization is stopped by plunging the slide into water, and the preparation is then counterstained in methylene blue or some similar dye. The blue background facilitates locating the pink acid-fast organisms.

Paul Ehrlich noted this acid-fast phenomenon in 1882 when he was studying the tuberculosis organisms. Recent evidence points to mycolic acid, a constituent of the waxy material, as the cause of acid-fastness. When mycolic acid is in combination with complex sugars (polysaccharides), it is even more acid-fast than when it is tested alone. However, just to make the problem more interesting, some workers have shown that not all acid-fast bacteria possess mycolic acid. Undoubtedly, more than one explanation of acid-fastness probably exists, and the full story is yet to be revealed.

In recent years acid-fast bacteria have been stained with a dyestuff called *auramine,* which possesses properties of fluorescence. When such stained organisms are examined with ultaviolet light, using a yellow filter to block out the blue light, the field is dark but the auramine-stained organisms stand out as luminous yellow bodies. This technic has value in the diagnosis of tuberculosis.

Persons suffering from leprosy harbor acid-fast bacteria within their diseased tissues, and these organisms resemble the tuberculosis microbes. *Mycobacterium leprae* is the name given to the leprosy acid-fast cells. To date these organisms have not been isolated and grown on artificial media, but the fact that they are

seen in diseased tissue of lepers leads us to believe that they are the etiology of the disease.

Saprophytic acid-fast bacteria are troublesome and can lead to false conclusions unless the clinician understands the problem. An organism known as *Mycobacterium smegmatis* is a normal saprophytic inhabitant of the prepuce of males and the external labia of women. If urine specimens are collected without taking proper precautions, such as the use of a catheter which permits the collection of urine directly from the bladder or from the kidneys, acid-fast bacteria found in urine specimens might lead to a false diagnosis of tuberculosis of the genito-urinary tract.

GRANULE STAINS

Specialized stains have been developed to detect more easily granules in bacteria. Loeffler's alkaline methylene blue encourages the irregular staining so characteristic of typical diphtheria organisms. Ponder's stain and Gohar's technic have also come into popular use for similar studies. The ingredients of all these stains and the technics for their use may be found in bacteriology laboratory manuals.

SPORE STAINS

Bacterial spores are usually refractile to the common technics employed for staining bacteria, but once the spores have stained, they retain the dye longer than the vegetative protoplasm. Malachite green is a very satisfactory dye to be forced into spores with the aid of heat. A counterstain of safranin will yield a pink vegetative cell in which the green-stained spore can be observed. Carbol fuchsin is another satisfactory dye for primary staining with methylene blue as a counterstain. Many combinations have been suggested for spore-staining, and a number of these methods are quite satisfactory. The low permeability of the spore walls to dyestuffs is the most common explanation of spore resistance to staining and to subsequent decolorization. Without the use of heat, a staining time of from 2 to 4 hours is not uncommon. In ordinary staining without the application of heat the spore stands out as a clear refractile body within the cell. At times it is

difficult to demonstrate the actual spore, but the resistance of a culture to 80° C. for 10 minutes suggests the presence of spore-forming organisms or the presence of cells that are unusually resistant to heat.

FLAGELLA STAINS

Before attempting to demonstrate flagella by staining reactions, the culture must be grown under conditions optimum for the encouragement of the formation of these structures. One theory, which in practice has been shown to possess considerable merit, is to make the organism go hunting for food in a large tube of distilled water after the bacteria have been grown in the condensation water of an agar slant and serially transferred each twenty-four hour period for several days. By dashing about in the distilled water in search of food, development of flagella is theoretically encouraged. Some persons, on the other hand, believe it is the slow growth or static condition of the cultures which permits development of flagella to take place. Flagella apparently become thicker and longer with age and are more readily stained than when they are in the active stages of their development. By treating the dry film on the slide with a mordant (a complex colloidal solution), the diameter of the flagella is built up to within microscope range by the packing-on of mordant. Subsequent staining with either methylene blue or carbol fuchsin will usually reveal the thread-like flagella, particularly at the edge of the stained smear. Strict attention to details is of the utmost importance in this delicate staining procedure, and scrupulously clean slides free of scratches are essential. The presence of even minute amounts of organic matter interferes with good staining, because the debris may react with the mordant and absorb some of the dye.

The discussion of staining and staining technics in this chapter has been brief. Definite procedural details vary so greatly from one laboratory to another that it seems wise to leave the fine details of specific staining to the individual instructors as part of their lecture or laboratory discussions.

CHAPTER 6

Cultivation and Identification of Bacteria

INTRODUCTION

Because microbiology is an entirely new field to most persons enrolled in a survey course of this type, the student must be introduced to a new language. With a little effort, a student can readily expand his vocabulary by several hundred scientific words, and if he has studied Latin or has been exposed to some Greek, many terms used in microbiology will be familiar to him.

An agreement as to the meaning of common terms seems appropriate at the outset of this chapter. When bacteria are transferred from one medium to another, the material being transferred

is called the INOCULUM and the resultant growth, whether it be in a liquid or in a solid medium, is termed a CULTURE, or SUBCULTURE. If the transfer is made to a solid medium, the visible growth which appears after a suitable INCUBATION period is a COLONY. If but one species of organism is involved in this cultivation technic, it is said to be a PURE CULTURE. A single kind of organism may be isolated

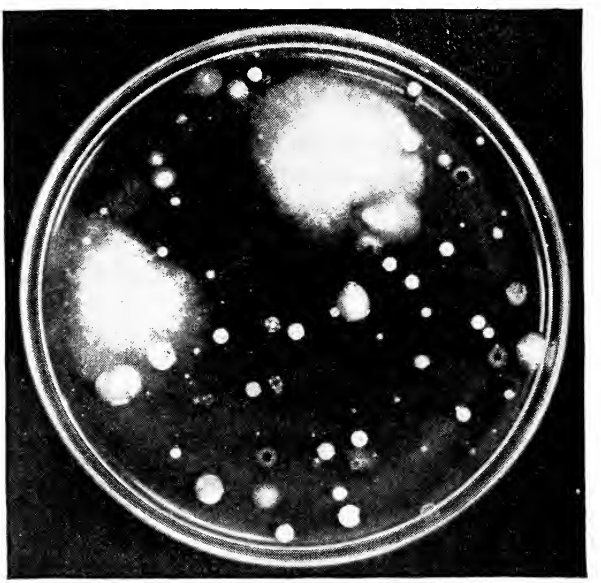


Fig. 23. Colonies developing on nutrient agar exposed for ten minutes to the air in a classroom. (*By permission from* Introduction to the Bacteria *by C. E. Clifton. Copyright, 1950. McGraw-Hill Book Company, Inc.*)

from mixtures of organisms by a purification process to be discussed in this chapter.

Attempts to study the metabolism of a single bacterial cell are impractical, in fact, probably impossible. But by cultivating masses of organisms, as long as they arise from a single cell or from a group of like organisms, the results of a study can be relied upon, and reproducible reactions are possible. It therefore becomes important to grow volumes of bacteria by employing the prerequisites of a good microbiological medium discussed pre-

viously in Chapter 4. To be assured of a good harvest a farmer not only has to plant good seed in fertile ground, he must also rely upon Providence to furnish the necessary warmth, sunshine, and moisture to allow these seeds to germinate and to develop into mature plants. Once the plants begin to grow, the care-taker must control inevitable pests—the insects and the diseases to which these growing plants are susceptible.

A bacteriologist in cultivating microbes is able to control these factors of heat, moisture, and contaminants. If he knows the food requirements of his proposed crop, and if he understands the elementary principles of aseptic technic (not allowing undesirable organisms to get into his cultures), the unwanted "weeds" can be eliminated, and he can be assured of harvesting a good crop of pure culture. Controlled cultivation is within the reach of all laboratory workers who follow the fundamental rules of the game.

No reliable physiological determinations can be made on mixed cultures of bacteria. The purity of the microbial culture is just as important, if not more so, than it is in growing grain crops. In the latter case, should an undesirable seed find its way into the batch, the growing plant can readily be pulled up and discarded prior to the harvest. But in the bacteriology laboratory, once the contaminant gains entrance into a culture, especially liquids, it may be necessary to plant a new crop, sometimes repeatedly, until the culture has been purified.

Bacteria are ubiquitous, and if technicians will keep that thought constantly in mind, proper technics for handling and cultivating pure bacterial masses are more likely to be employed. Careful attention to what may seem like exacting details will pay dividends in the long run. Not only will contamination be minimized, but the worker may save himself the painful experience of contracting a laboratory infection when he handles pathogenic organisms.

PURE CULTURE ISOLATION TECHNICS

There are very few places where pure cultures of organisms exist in nature. As long as bacteria are found practically every-

where, it must be expected that they will occur as mixed cultures. A number of technics designed to separate bacteria from their neighbors are available, but before much can be done in the way of identifying these organisms, it is necessary that the culture be pure.

BROTH DILUTION

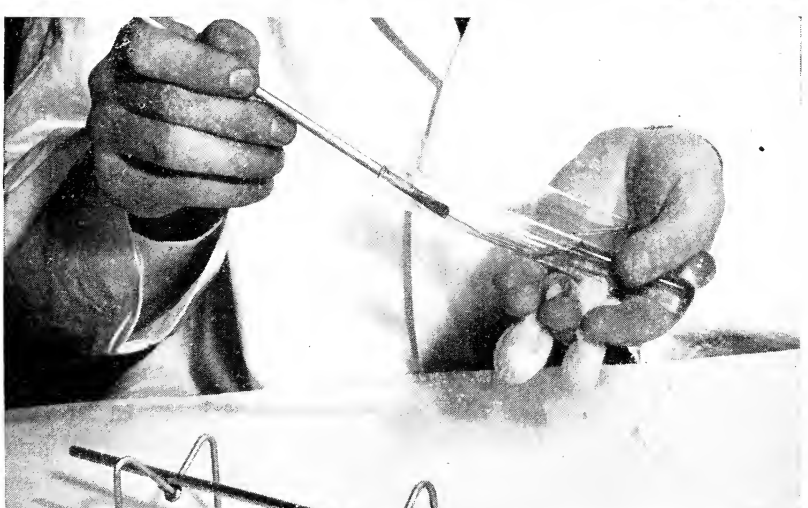
This method represents one of the earliest attempts to secure pure cultures of organisms. The first in a series of broth tubes is inoculated with the material containing the bacteria, and after thorough mixing, a small quantity (a loopful or a drop) from tube #1 is transferred to tube #2. After mixing the contents of tube #2, a transfer is carried from #2 to tube #3, etc., in series. A decreasing number of organisms is carried over into each succeeding tube of broth, and if this dilution procedure is carried along through a sufficient number of transfers, the point is eventually reached where the inoculum consists of only one or a very few cells. The species predominating in the original material logically would be found in pure culture in the last tube of the series showing visible growth after a suitable incubation period.

A little contemplation should make it obvious that this broth dilution technic has several serious drawbacks. First of all, one can never be sure that the last dilution tube will always contain a pure culture. If two species of organisms are found in about equal numbers in the original test material, they might both be carried over into the last dilution tube and yield a mixed culture. But a serious disadvantage of the method is that it denies the opportunity for isolating those species which happen to be in the minority in the original microbial mixture.

AGAR DILUTION

By incorporating a solidifying agent in the broth to be employed in the serial dilutions, it is possible to anchor the organisms in the solid medium. If the melted agar is cooled to between 45–50° C. before inoculation and is poured into a culture dish before the medium thickens, the poured agar will solidify when the temperature approaches 40° C., and the organisms are trapped

A



B

Fig. 24. (A) One of the accepted technics for holding test tubes and cotton stoppers during transfer of cultures. (B) A close-up of the same technic.

in the solidified agar. Each separate cell develops in the medium producing a visible growth, called a COLONY, and these usually represent pure cultures which arise from either a single cell or from a group of like cells. If the dilutions are carried out in series, the developing colonies will be far enough apart to facilitate their being picked from the agar with the aid of a sterile wire or loop. By subculturing these isolations to tubes of sterile broth or to solid media, many different isolations are possible from different colonies on a single culture plate, and they will represent those organisms found in low numbers as well as the predominating species in a given mixed culture.

This solid medium technic is a decided improvement over the serial broth procedure, but it still has certain undesirable features which can readily be overcome.

One of the principal disadvantages of the method is the difference in the appearance of colonies of the same species when the organisms develop at different oxygen tensions. Colonies growing on the surface of agar plates and having full access to atmospheric concentrations of oxygen are usually larger than subsurface, imbedded colonies. In the discussion of chromogenesis in the previous chapter it was emphasized that only in the presence of an abundant oxygen supply can pigmentation by chromogenic organisms be assured. Upon examination of an agar dilution plate made of such organisms, it would appear on the basis of differences in pigmentation that more than one species of organism was present in the plate. By inhibiting chromogenesis and reducing colony size of sub-surface colonies, one is faced with serious diagnostic disadvantages. Plates over-crowded with colonies is another undesirable feature of this culturing technic.

STREAK PLATES

It is possible to overcome the above criticisms by a simple expedient. Instead of mixing the bacteria with the liquefiable-solid medium before transferring it into a petri dish, the agar can be poured into the dish first, allowed to harden, and then the culture can be smeared or streaked on the hardened surface of the medium.

It may seem incredible to a novice that it is possible to take a loopful of culture containing hundreds or thousands of bacteria, and by simply streaking that loop back and forth in an orderly manner over the surface of nutrient agar in a 65 square centimeter area of a petri dish, one can deposit in certain areas on that agar surface single, well-isolated bacteria which are capable of growing into

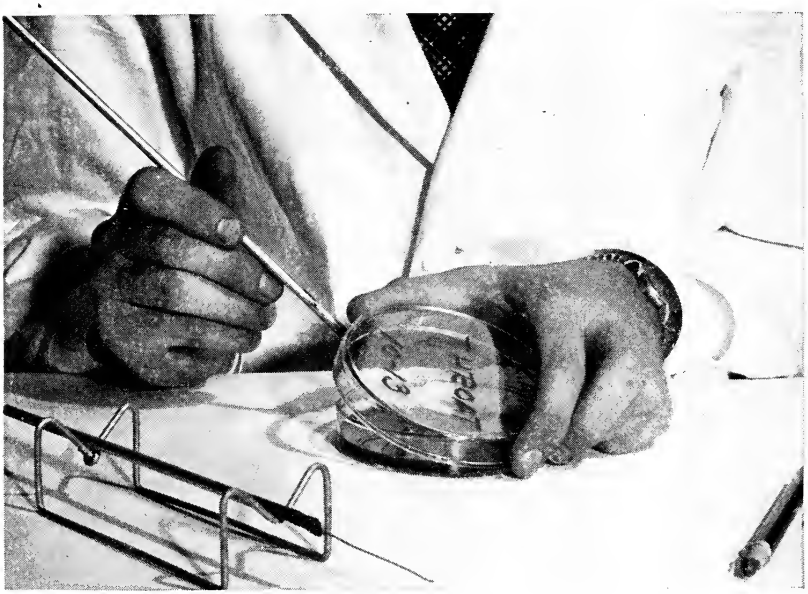


Fig. 25. When making streak platings, the lid of the petri dish should be held in a position that will protect the surface of the agar in the plate from outside contamination.

distinct colonies. There are probably almost as many modifications of technics for streaking plates as there are teaching institutions, but most methods will produce satisfactory results if the prescribed directions are carefully followed. Rather than outline any one technic to the exclusion of others, it seems best to leave that specific detail to the individual instructor who undoubtedly has a method he has previously found to be satisfactory.

The streak plate is the most universally accepted procedure for obtaining isolations from mixed cultures, and if a reasonable

amount of inoculum is used, isolated colonies representing even some of the minorities in mixed cultures can be examined for differences in their size, shape, elevation, and pigmentation—the common criteria for identifying different species on a streak plate. At times, however, it becomes necessary to employ enrichment (selective diet) technics to encourage the growth of certain organisms found in small numbers in a given mixed culture. Final identification of these isolated species involves a systematic elimination procedure to be discussed later in this chapter.

SELECTIVE AND ENRICHMENT MEDIA

The studies of Churchman and others have been instrumental in the development of selective media for the isolation of specific bacteria from mixtures. Certain dyes have been found to exert a growth-inhibitory effect upon gram positive bacteria as a group, while other dyes act similarly toward gram negative organisms. In general, the gram positive species are more susceptible to dye action than is true for gram negative bacteria, and differences within a given staining group also exist. By incorporating one or more dyes into a medium, the task of isolating selected bacteria is simplified. This principle has decided value in clinical laboratory work, particularly in the detection of intestinal (enteric) pathogens, including typhoid and dysentery organisms. Such dyes as malachite green, brilliant green, gentian violet, and others, can be added in low concentration to various media for retarding growth of selected organisms.

Escherichia coli is one of the more numerous gram negative species found in feces of warm-blooded animals, and because of its aggressive characteristics, it might readily overgrow the enteric pathogens one is trying to isolate by cultivation of feces organisms in a clinical laboratory. Selective media provide a valuable means of retarding the growth of *Escherichia coli.* Another gram negative species, *Proteus vulgaris,* has the peculiar characteristic, especially on primary isolation, of spreading over the entire surface of a culture plate, and this may cover up the growth of other organisms being sought in clinical material. By adding chloral hydrate to

the medium, the spreading of *Proteus* can be prevented and discrete colonies may be more readily examined for some of the cultural characteristics mentioned previously.

Bacteria, like humans, vary in their food demands. A person who has been on a diet of filet mignon finds it difficult to switch to a steady diet of pork and beans. Some bacteria are also very fastidious about what they will eat, and if they are deprived of their high living, they would prefer not to put up a struggle to change their mode of life, and in other instances they may be prevented from doing so. The "vampire-like" *Hemophilus* (blood-loving) group of bacteria, including the organism which causes whooping cough (*Hemophilus pertussis*), is incapable of growing unless it has access to blood and blood derivatives. The more parasitic an organism becomes, the more exacting it may be in its food demands. When viruses are discussed, you will discover that not only must viruses have cells upon which to grow, but they must have living cells, and sometimes even particular living cells from designated tissues. Such extreme dependency limits chances for survival of many parasitic organisms when the environment is altered.

MICROMANIPULATORS FOR SINGLE CELL ISOLATION

At times, particularly in research investigations involving the genetics of bacteria, it becomes desirable to isolate single bacterial cells to insure the purity of a given strain of organism under investigation. This type of isolation is not practical on a routine basis, but its mention should be included in a discussion of technics employed for obtaining pure cultures of bacteria.

By placing a series of small drops of diluted liquid culture on a slide and examining them with the aid of a microscope to find a drop containing only a few bacteria (or only one), it is possible with the aid of a micromanipulator to isolate an organism from its fellow microbes in that fluid drop. Without going into the minute details as to the operation of this microscope attachment, the technic involves drawing isolated organisms into a fine capillary pipette and transferring the trapped cells into a suitable broth

medium. Not all such isolated organisms survive, but if growth occurs in the broth, the offspring can all be traced back to a single cell, and studies relative to nutrition and genetics will have more significance when they are based upon cultures originating from known single cells.

SELECTIVE HEATING

It has been pointed out that spores resist higher temperatures for longer periods of time than do vegetable cells. To separate a spore-former from a mixed culture, the culture is subjected to varying degrees of heating, and the surviving spores can then be separated. Not all spores are equally resistant to heat, so heating of parts of the suspension at different temperatures may be necessary to separate mixtures of spore-formers.

USE OF LABORATORY ANIMALS

Some animals are known to be extremely susceptible to the action of specific organisms. The mouse, for example, can be injected with a mixture of many organisms, but if in that mixture is found a virulent pneumococcus, it is quite possible for the natural destructive forces of the mouse to dispose of all of the injected bacteria except the pneumococcus. This organism and its progeny may eventually destroy the mouse, oftentimes in less than 24 hours. Other organisms, however, are also capable of being pathogenic for mice. An examination of the peritoneal cavity of the mouse immediately after death will frequently reveal a pure culture of the pneumococcus. The guinea pig serves as a similar filter for separating the tuberculosis microbes from materials such as sputum. This purification process may take from 4 to 6 weeks, however, because of the slow metabolism of the tuberculosis organisms. Autopsy examination of the lungs, liver, spleen, and other organs will generally reveal pure cultures of packed organisms in tubercles —visible growths of the pathogenic agent.

When Koch's postulates were discussed it was made clear that unless a *susceptible* animal is used, it is not possible to prove the etiology of all diseases by his postulates. This is true for some

viruses, and the number of animals that can be used for such studies is more limited. Mice are susceptible to the action of some viruses, rhesus monkeys to others, and ferrets to still others, but many animals are completely refractory to some viruses.

IDENTIFICATION OF PURE CULTURES

After primary isolation procedures have assured that the cultures so obtained are *pure* (only one species), the next step is to subject the pure culture to an orderly sequence of morphological, cultural, and physiological tests.

Trying to determine the accepted name of an organism involves an elimination procedure, each test narrowing down the possibilities. *Bergey's Manual of Determinative Bacteriology* is the standard reference work used in the final identification of bacteria.

Since the number of organisms studied in a course of this nature is usually not very extensive, many institutions incorporate into their laboratory manuals simplified reference charts to which students may refer, rather than have the beginning students laboriously thumb through BERGEY'S MANUAL. By employing a chart similar to the one which follows, all pertinent information for the organisms under investigation can be tabulated in an orderly fashion for handy reference. It seems appropriate to discuss in some detail the theory and the significance of some of these tests commonly used in describing the growth and activities of microorganisms. This should aid the student to understand better the why and the wherefore of what might appear to be hocus-pocus practiced in bacteriology laboratories.

MORPHOLOGY

Gram Stain

A carefully prepared gram stain will immediately place the organism in question into one of two major groups—the GRAM POSITIVE bacteria or the GRAM NEGATIVE bacteria. This is the first important step in the elimination process. The value of a reliable gram stain cannot be over-emphasized. Much time and effort can be needlessly wasted by trying to identify an organism that doesn't

DESCRIPTIVE CHART FOR BACTERIA

I. Morphology

VEGETATIVE CELLS:

Gram staining reaction:_____.
Form: *spheres, short rods, long rods, filaments, spirals.*
Size:_____Sketch:_____.
Motility: *present, absent.*
Arrangement: *singles, pairs, chains, tetrads, clusters, cubical packets.*
Spores: *present, absent.*
Location: *central, terminal, subterminal.*
Sporangia: *swollen, not swollen.*

II. Cultural Characteristics

COLONY: Medium_____Age:_____.
Form: *punctiform, circular, rhizoid, irregular.*
Surface: *smooth, rough, dry, moist, dull, glistening.*
Elevation: *flat, raised,*
Edge: *entire, wavy, filamentous.*
Growth: *slow, moderate, rapid.*

SLANT: Medium:_____Age:_____.
Form: *thread-like, beaded, root-like, spreading.*
Consistency: *butyrous, viscid, brittle.*
Medium: *grayed, browned, greened, unchanged.*
Optical characters: *translucent, opaque, irridescent.*
Color: _____ *water soluble, water insoluble.*

NUTRIENT BROTH: Age:_____.
Surface growth: *ring, pellicle, none.*
Clouding: *slight, moderate, heavy, none.*
Amount of sediment: *none, scanty, abundant.*
Type of Sediment: *flaky, granular, viscid on agitation.*

ENDO OR EOSIN METHYLENE BLUE AGAR:

Growth: *present, absent.*
Color: *present, absent.*
Metallic sheen: *present, absent.*

III. Physiology

FERMENTATIONS:

Glucose: *acid, gas, negative.*
Age:_____.
Lactose: *acid, gas, negative.*
Age:_____.

BROM CRESOL PURPLE MILK:

Reaction: *acid, neutral, or alkaline.*
Age:_____.
Curds: *acid, rennet, none.*
Proteolysis: *none, slight, moderate, complete.*
Age:_____.

METHYLENE BLUE MILK:

Reduction: *none, slight, moderate, complete.*
Age:_____.

GELATIN LIQUEFACTION:

None, moderate, complete.
Slow, moderate, rapid.

INDOLE TEST: positive, negative.

METHYL RED TEST: positive, negative.

VOGES-PROSKAUER TEST: positive, negative.

CITRATE TEST: positive, negative.

UREA TEST: positive, negative.

IV. Additional Data

Name of organism concluded from the above reactions:

_____

Student:

Dates of study:

exist, and this is exactly what might occur if false conclusions are drawn from improperly prepared gram stains. A microscopic examination, usually with the oil immersion objective, will reveal the form and the size of an organism, in addition to its gram reaction. By underlining or circling the applicable terms that appear on the descriptive chart, a quick glance will point out to the observer the outstanding characteristics of the organism.

Hanging Drop

By examining a hanging drop of a young (less than twenty-four hours) broth culture under the high dry objective of the microscope, motility and typical arrangement of the species can be determined.

Spores

Spores fail to stain during ordinary gram staining, but by the application of heat with dyes such as malachite green or carbol fuchsin, spores can be stained and their size and location can be ascertained after suitably counter-staining the preparation with a contrast dye such as safranin or methylene blue which colors the vegetative cells and the non-spore components of spore-bearing bacteria.

CULTURAL CHARACTERISTICS

To a trained observer the cultural characteristics can supply valuable clues as to the possible genus, and sometimes even as to the species of the test organism. It is unwise, however, to allow these criteria to be the only studies made, since closely related, yet distinct, species may have similar cultural characteristics.

Streak Plate

Because a streak plate allows all colonies to develop on the surface of the medium, such considerations as form, surface appearance, elevation, edge of the colony, and the speed of microbial growth can be compared. Again, these characteristics in themselves are not conclusive evidence as to the genus or species name

of an organism, but these data do add weight to the other factors considered in the identification process.

Slant Cultures

Media containing a solidifying agent such as agar can be dispensed in tubes while the media are still hot and in a liquid state. By placing these tubes at an angle during the solidifying process, the material will harden into what is termed a SLANT. Such a preparation provides more surface area for inoculation. Care must be exercised to avoid wetting the cotton stoppers with the medium during the slanting operation, otherwise the natural filtering ability of the cotton will be lost and contamination of the tube's contents might well be expected.

In addition to furnishing information as to the form, consistency, and optical characters of the bacterial growth, the agar slant also provides information relative to the color-producing capacity (chromogenesis) of the species. Some media are better than others for stimulating pigmentation of cultures; meat infusion agar is one such medium. If the color produced by organisms is water soluble, the pigment will diffuse throughout the agar. Most bacterial pigments, however, are of the water-insoluble type, and fail to leave the cell, at least not in detectable amounts.

Nutrient Broth

A young broth culture, in addition to providing information about motility and natural arrangement of organisms, also displays cultural characteristics, such as surface growth, clouding of the medium, and sediment formation. A sudden jarring of the tube will suspend the sediment, and the type of sediment can be determined and recorded under the appropriate heading on the descriptive chart.

Selective Media

This has been discussed earlier in this chapter and needs little more elaboration than to point out that organisms which do grow

on these media may exhibit characteristic cultural appearances, some of which are of diagnostic significance to a trained eye.

PHYSIOLOGY

In simple terms, physiology involves the enzyme systems possessed by bacteria and the effects these enzymes have on the substrates employed for the cultivation of these organisms. The reactions to be discussed represent only a few of the fundamental considerations in the identification of bacteria.

Fermentation

In advanced courses in microbiology it is not uncommon to study the fermentation of a dozen or more substances. Two sugars you can expect to find in all such lists are glucose (also known as dextrose) and lactose (milk sugar). Glucose is a simple sugar—a monosaccharide, while lactose is more complex—a disaccharide.

Different species of bacteria attack some sugars and not others, and the type of physiological reaction produced is also variable. The fermentation process may produce, among other things, various acids, and to detect the presence of acid it is customary to incorporate a *p*H indicator dye in the broth to which the sugar has been added. The indicator chosen varies from one laboratory to another depending upon individual preferences. Brom thymol blue, the same indicator employed in the *p*H adjustment of standard nutrient agar, is commonly used in fermentation studies. Andrade's indicator is another. As the growing organisms attack the sugar in the broth, the acid produced depresses the *p*H level to the point where color changes are brought about in the dye indicators.

In addition to acids formed, various gases, notably carbon dioxide and hydrogen, may be evolved. A gas trap is included in these fermentation tubes by placing a small test tube, or vial, in an inverted position within the tube containing the sugar broth. Gas being liberated during the breakdown of the sugar rises in the

medium and some of it is caught in the gas trap. Displacement of the liquid in the inner inverted tube is evidence of gas formation. Five visible changes may take place in tubes of sugar broth undergoing microbial action. If the bacteria do not possess the enzymes required to attack specific sugars, growth in the broth will occur, as evidenced by clouding of the medium, but no color change will take place in the medium and no gas will be liberated to be trapped in the inverted vial. Acid production without gas formation is a second possibility, and acid together with gas evolution represents the third visible change in the sugar broth. Alkali production is a fourth possible change, and alkali production coupled with gas formation is the fifth reaction.

Brom Cresol Purple Milk

If brom cresol purple indicator is added to milk, physiological changes taking place in the inoculated milk can be detected and interpreted. Lactose, the natural sugar found in milk, may be attacked by some organisms, and if the amount of acid produced is great enough to push the *p*H down to the curdling point, the milk will exhibit a hard acid curd. Coagulation of milk protein (casein) may also be brought about in the absence of acids, however, by an enzyme called *rennet.* This enzyme may be extracted from the stomach of calves, and it has been purified and sold on the market as a constituent of custard-like desserts. Some bacteria also produce rennet, and their physiological action may cause sweet-curdling of milk. The curd is called "sweet" because it normally occurs at or near neutrality. Such curds may be easily distinguished from the acid curds which are harder and form only at a low *p*H.

Another reaction that may take place in milk is the digestion, or proteolysis, of the milk protein. The breakdown of casein results in the appearance of a clear liquid lacking the opaqueness of ordinary milk. The liquid formed in such proteolysis occurs at or near neutrality and should not be confused with the whey that separates from an acid curd at a low *p*H.

Methylene Blue Milk

Methylene blue dye imparts a robin's egg blue color to milk. In contrast to brom cresol purple, brom thymol blue, and other indicators, methylene blue provides no information about *p*H. But this dye does respond to different levels of oxygen tension. If methylene blue milk has its dissolved oxygen removed, either mechanically with the aid of a vacuum pump or biologically by organisms which produce the enzyme *reductase,* the indicator will change from blue to its white form. The latter is termed *leuco* methylene blue.

The color change with methylene blue dye is a reversible one. That is, if we shake a tube of reduced methylene blue milk and reincorporate oxygen, the milk will revert to its former blue color and will remain blue until the critical low oxygen level is once more attained, at which time the blue color will fade into the leuco form.

Gelatin Liquefaction

While nutrient gelatin is not a satisfactory medium for routine streak platings because of the disadvantage outlined in the chapter on media making, gelatin does serve a useful function in the study of physiological reactions of organisms. If the bacteria secrete the enzyme *gelatinase,* this digestive enzyme can alter gelatin to the extent that even though the medium is chilled below its normal solidifying point (about 23° C.), the gelatin will remain liquid. Since some organisms produce the enzyme gelatinase and others do not, we have one more reaction in the battery of tests employed to identify microbial species. The speed and the degree of liquifaction are other variables between organisms. When a bacteriologist refers to a physiological reaction as being rapid, moderate, or slow, this usually means the reaction takes place in one day, two days, or longer than two days, respectively.

Indole Reaction

This test is based upon the ability of certain microorganisms to produce an enzyme capable of attacking a substance called *trypto-*

phane and releasing the part of this molecule known as the indole fraction. Proteins are constructed of building blocks known as amino acids, and tryptophane is one of these substances.

Paul Ehrlich found that by adding an indicator substance, which has the interesting designation of *paradimethylamidobenzaldehyde* (Ehrlich's aldehyde for short!), the presence of free indole could be detected in the medium. As long as the indole is still attached to the parent tryptophane molecule, the aldehyde indicator will not turn pink. If free indole is present, the Ehrlich's aldehyde will turn a pink color. This is a positive indole test.

In addition to aiding in the identificaton of a number of bacteria, the indole test has particular significance in water analysis where sewage organisms (*Escherichia coli*) can be distinguished from the closely related soil organisms (*Aerobacter aerogenes*) by the indole reaction when it is used in conjunction with other tests.

Methyl Red Test

When the *pH* drops to 4.7 or below, methyl red indicator turns red from the normal yellow color it exhibits above *p*H 4.7. By cultivating bacteria in a highly buffered glucose broth (Clark and Lubs broth), if the bacteria have generated enough acid to depress the *p*H of this buffered broth to the critical level, the broth will turn red when methyl red indicator is added to the tube. This test also has significance in water analysis.

Voges-Proskauer Test

This is the third of four tests employed to separate *Escherichia coli* from *Aerobacter aerogenes*. Without going into the intricate chemistry involved in this reaction, suffice it to say that if bacteria are cultivated in Clark and Lubs broth, *Aerobacter aerogenes* is capable of forming an intermediate breakdown product of glucose. When a strong alkali (sodium or potassium hydroxide) is added, the intermediate breakdown product is converted into a pink-colored compound. A positive Voges-Proskauer test means that this series of reactions has occurred, whereas no change in color is a negative V.-P. test. It happens that a typical *Aerobacter aero-*

genes culture is V.-P. positive and *Escherichia coli* is V.-P. negative.

Citrate Test

In the chapter on media making various substances were classified into natural, derived, and synthetic media, and it was pointed out that a synthetic medium is one of which we know the exact composition. Koser developed a synthetic mixture in which the sole source of carbon is sodium citrate.

All living cells require carbon for growth and metabolism, but unless the carbon is in an available form, the organisms cannot utilize this element. *Escherichia coli* is incapable of growing in Koser's citrate medium while *Aerobacter aerogenes* flourishes with sodium citrate as its sole source of carbon. An organism which grows is said to be citrate positive in contrast to the citrate negative species which are incapable of growing in this synthetic medium.

There is at least one precaution that should be stressed relative to Koser's medium. It should be inoculated very lightly with the test organisms. If too many cells are transferred to the broth, nutrients may be carried over and false interpretations may result. Bacteria might also cannibalize each other for their carbon. It is recommended that before transferring the bacteria to citrate broth, a light suspension of the organisms should be prepared in sterile distilled water, and a small loopful of the water blank suspension should be used as the inoculum for the citrate broth.

These last four tests are the accepted reactions for separating the closely related *coli* and *aerogenes* species, and for convenience we refer to these tests as the *IMViC Reactions* tabulated as follows:

	ESCH. COLI	AEROB. AEROGENES
I—Indole Test	+	−
M—Methyl Red Reaction	+	−
V—Voges-Proskauer Test	−	+
C—Citrate Test	−	+

The small letter *i* in the word IMViC is inserted purely to make the word more pronounceable; it does not refer to any particular

diagnostic test. A typical strain of *Escherichia coli,* therefore, is IMViC ++−−, while a typical *Aerobacter aerogenes* is IMViC −−++. There are fourteen other combinations of these four reactions, and organisms exhibiting these in-between reactions are called *intermediates.* The significance of these intermediates will be discussed in the chapter on water.

Urea Test

Many organisms have the power of converting urea (the most prominent nitrogen compound present in urine) to ammonium carbonate and finally to free ammonia and carbon dioxide. The enzyme capable of attacking urea is *urease.* This reaction is an important one in the soil where fertility is dependent to a large measure on microbial activity. By growing bacteria in a highly buffered urea broth with phenol red as a *p*H indicator, if urease is produced by the test organisms, the liberated ammonia will raise the *p*H of the medium to the point where the indicator will turn deep red—a positive urea test. In studying the gram negative rods we find, conveniently enough, that of the fermenting organisms only the *Proteus* genus is capable of giving a positive urea test, and this is an important physiological reaction employed in screening cultures of *Proteus* (usually considered to be non-pathogenic) from pathogenic gram negative rods.

SEROLOGY

Even after running through the usual morphological, cultural, and physiological reactions, some closely related organisms cannot be separated from each other unless serological tests are employed. This situation would not ordinarily exist in an introductory course in microbiology the way it might in advanced pathogenic bacteriology. Serology is a study of the test tube (*in vitro*) reactions using blood serum containing antibodies in an attempt to determine the nature of some chemical components in the cell. Practical applications of serology will be considered in the discussion of blood types and blood groupings later in the book, but serology is

mentioned here merely for completeness of the discussion on bacterial identification.

It bears repeating that the reactions discussed in this chapter are only a few representatives of the untold numbers of physiological tests employed in microbial identification. Individual laboratories have their own media preferences, and additions to or deletions from the list of reactions presented here should not be surprising.

CHAPTER 7

Bacterial Multiplication

GROWTH CURVES

The speed with which a microorganism will multiply under a standard set of conditions is predictable, but minor variations in any factor affecting growth of organisms will alter the growth rate. Such considerations as the concentration and availability of food, *p*H, temperature, moisture, presence of accessory growth-stimulating substances, surface tension, and the accumulation of waste products have a direct bearing on growth curves.

Growth of higher forms of plant and animal life involves an increase in size which accompanies an increase in the number of cells comprising a given tissue or structure of the body. Bacterial growth, on the other hand, is usually used as a synonym for multiplication. Bacteria grow to a predetermined size and stop if

growth conditions are not favorable, or they divide into two small cells if the environment permits. Each time that a cell divides it carries over some of the parent cell, and this leads Frobisher to ask the question, "Are bacteria immortal?"

There appears to be an interesting correlation, with some exceptions, between the size of a living thing and its generation time and life expectancy. Going up the scale with respect to size we can tabulate the speed of multiplication, or the gestation period, of a few living things as follows:

Bacteria	20 minutes up to several hours
Mice	20–21 days
Albino rats	21–25 days
Rabbits	30–34 days
Guinea pigs	68–71 days
Humans	270–295 days
Horses	330–380 days
Elephants	Indian 607–641 days African 641 days.

Bacteria have such a relatively short life span that they are not confronted with retirement problems. They pack a great deal of living into a short time, but in spite of this fact Pasteur once made the statement, "Gentlemen, it will be the microbes who have the last word."

When we speak of growth curves of bacteria it should be borne in mind that they refer to pure cultures living without all of the competition with which they are faced in nature where many species may be growing in a given environment under highly competitive conditions. The curve to be discussed in this chapter represents laboratory conditions and cultures of single species of bacteria.

If we plot against time the number of living cells of a pure culture growing in broth under optimum conditions, a line characteristic of a skewed frequency curve is obtained. The curve expresses the general law of growth by Pearl. Whether we are dealing with the population of organisms in a limited environment, with people in a country, or with fruit flies in a bottle, the same

sort of curve can be expected. At first the amount of food is in excess of the needs of the organisms and they grow more and more rapidly until they reach their peak, called the LOGARITHMIC GROWTH PHASE. As the quantity of available food becomes insufficient to maintain this rate of multiplication, growth slows down at an increasing rate. Things which cannot grow usually die, and that is the fate of organisms. How long it takes a culture to become free of living cells depends upon a number of factors having to do with

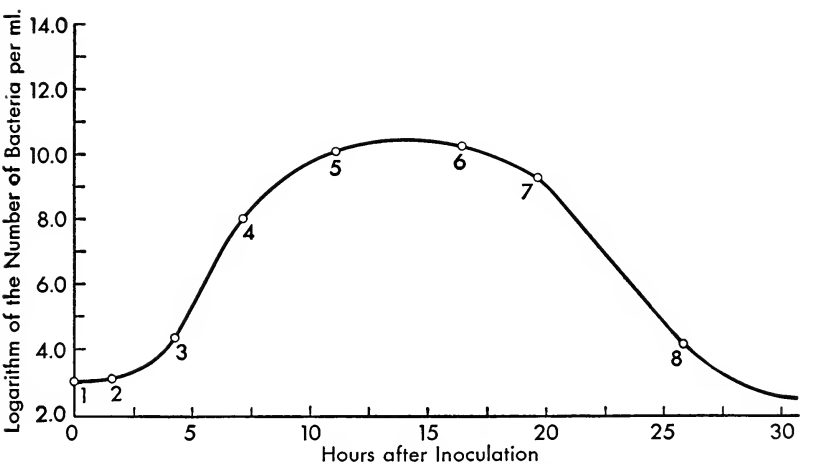


Fig. 26. Bacterial growth curve. (*Reprinted with modifications by permission of Porter*, Bacterial Chemistry and Physiology, *Copyright 1946, John Wiley and Sons, Inc., New York.*)

the environment and with the nature of the organisms; it may take days, weeks, or months.

Growth curves can be divided into four major phases: the LAG PHASE, LOGARITHMIC GROWTH PHASE, MAXIMUM STATIONARY PHASE, and the PHASE OF DECLINE. Buchanan, however, felt that seven stages could be described, each phase being distinct from the others. A brief discussion of these seven steps in the growth pattern of a typical pure culture of bacteria transferred to a fresh broth medium will clarify the nature of the changes taking place under such conditions.

1. The *latent* or *initial stationary phase,* from 1 to 2 on the growth curve, is a period during which there is no detectable increase in the number of bacterial cells. In fact a few cells may die due to lack of adjustment to their new environment.

2. *The lag phase,* from 2 to 3, represents a period during which the cells are beginning to show signs of multiplication. This lag phase plus the initial stationary phase are combined by some persons into what they prefer to call the adjustment period.

The length of the lag phase can be altered in a number of ways. A large inoculum, for instance, will usually shorten this period in the growth curve, while spore-formers or bacteria transferred to this fresh medium from another culture in its death phase will tend to prolong the lag phase. Any deviation from the optimum with respect to the temperature of incubation or the type of food provided will influence the length of this adjustment phase in the growth curve.

It was discovered by Valley and Rettger that carbon dioxide gas greatly influences the growth of bacteria. If this gas is completely removed from an otherwise ideal environment, growth of organisms will cease. The reincorporation of even minute traces of carbon dioxide promptly initiates growth once again. Perhaps a heavy inoculum helps to provide enough of this vital gas to shorten the lag phase of growth.

3. *The logarithmic phase,* from 3 to 4, is that stage in the growth cycle when multiplication of the cells is at its maximum speed and is occurring at regular intervals. The increase in numbers of cells is in a geometric progression, and if the logarithms of the numbers of cells are plotted against time, they will fall along an ascending straight line.

4. *The negative acceleration phase,* from 4 to 5, is the period during which multiplication slows down and the straight ascending line begins to bend to the right, indicating fewer cell divisions per unit of time.

5. *The maximum stationary phase,* from 5 to 6, is a segment during which the numbers of viable bacteria remain almost con-

stant. A balance exists between the number of new cells being created and the number of bacteria dying.

6. *The accelerated death phase*, from 6 to 7, is the period during which cells are beginning to die at an increased rate, with a net loss in viable cells.

7. *The logarithmic death phase*, from 7 to 8, indicates that cells are dying at about a geometric rate, a reverse of the logarithmic growth phase. There is some evidence that the death rate is not logarithmic, and many persons prefer to designate this period in the curve as *the phase of decline.* The segment from 8 on in the above growth curve has no technical designation, but were the phase of decline accepted as the proper terminology, the latter phase in the curve could be included.

Examination of the organisms during the senescent phase of decline will frequently reveal the presence of weird sizes and shapes of bacteria, and these unusual morphological types are called *involution forms.* Their formation is probably induced by extremely unfavorable conditions in their environment—the accumulation of waste products of metabolism. This is another reason for examining young, actively growing cultures if typical morphology of the species is to be ascertained by use of the gram stain or by examination of hanging drop preparations.

METHODS FOR DETERMINING TOTAL BACTERIAL NUMBERS

Whereas many bacteriological procedures are aimed at determining the number of living cells present in a given material, there are occasions when total counts—living plus dead cells—are of interest and importance. For example, a sample of pasteurized milk may give a relatively low colony count, which is a reflection of the viable cells present, but unless technics were available for determining total cell counts, it would be difficult to say with any assurance what the quality of that milk was before pasteurization. Heating of milk is designed to make it safe for consumption; it is not an attempt to make good milk out of a poor raw product. Pasteurization may kill better than 90% of the bacteria present in

milk, and this figure may even rise to 98% if spore-forming organisms and heat-resistant species are present only in small numbers. Total bacterial counts, as found by the *Breed Smear* to be discussed later, will reveal not only the total bacterial count, but the smear will also point out whether leucocytes (pus cells) are present—an indication that an infection exists in the animal from which the milk was drawn.

When preparing bacterial vaccines it is necessary to standardize the number of organisms within prescribed limits. Since these suspensions are to be employed as killed cells, a technic must be available for determining the total bacterial count.

BREED SMEARS

The Breed Smear has its greatest application in the milk industry. By spreading 0.01 ml. of milk over a 1 square centimeter area on a clean slide, and staining the dried and fixed preparation with an appropriate dye (usually methylene blue), it is possible to count the number of cells present in the preparation and to convert the information into terms of cells per milliliter of milk.

Standardization of the microscope is necessary. This will determine the area you are viewing when you look through the oil immersion objective, which is the power of the microscope employed during such examinations. There are available slides upon which are etched lines spaced 10 microns apart; such slides are called STAGE MICROMETERS. By focusing the oil immersion objective on these etched lines, the diameter of the microscopic field can be determined. For example, if the field is found to be 16 stage micrometer spaces across, the diameter of the microscope field is 160 microns (16×10). You may recall that the area of a circle is determined by the formula: Area $= \pi r^2$, where pi is 3.1416 and the radius is one-half the diameter.

To continue with the above example, the area of the microscope field is 3.1416×6400 (the radius is 80 μ and it is squared by multiplying it by itself—80×80, or 6400). By multiplying these figures, the area of a single microscope field is 21,106 square microns. Since the milk was spread over an area 1 centimeter square (100,-

000,000 square microns), and since each field of the microscope has an area of 21,106 square microns, it would be necessary to examine 4738 different fields of the microscope (21,106 divided into 100,000,000) to see the entire area of milk spread out on the slide. Since bacterial counts are expressed "per milliliter" of liquid being examined, and only 0.01 ml. of milk was placed on the slide, it would be necessary to count the organisms in one hundred such areas of milk, or 4738×100, before a full milliliter would be examined microscopically. In round numbers the *Microscope Factor* in the above problem would be 475,000 (4738×100). For every cell, or clump of cells, seen in an average microscope field there should be about 475,000 similar cells or clumps of cells, in the entire milliliter of milk.

Obviously, examining that number of fields is impractical. But experience has shown that by counting the average number of organisms, or clumps of organisms, in thirty, forty, or fifty fields, a fairly representative count can be obtained for the entire smear. The lower the bacterial count, the greater is the number of fields that should be examined for increased accuracy. If the smear is to be representative of the entire batch of milk, uniform, adequate mixing of the milk samples is imperative.

Because individuals have different ideas about what is meant by adequate mixing, it has become necessary to standardize this process for greater accuracy in determining bacterial counts. If one person shook a milk sample ten times in a half-hearted fashion, and someone else vigorously shook that same sample twenty times, one would not expect to get the same results. Bacteria tend to grow in clumps, and the more these aggregates are broken up by shaking, the more individual organisms or smaller clumps can be expected. The standard shaking technic calls for twenty-five strokes (up and down constituting one stroke), in the space of one foot, in an arc of ninety degrees (a quarter of a circle), in exactly seven seconds. As specific as this description of shaking is, variations do occur between individuals. Ask a dozen persons to hold their hands exactly one foot apart, and the chances are that you would get ten or a dozen different distances. The concept of

a time interval like seven seconds is another variable, even when the individuals have a watch to look at. The ideal situation would be to have the shaking done with standardized machines, but that is not possible for all laboratories.

WRIGHT'S PROPORTIONAL COUNT TECHNIC

Wright in 1902 proposed a method of mixing a known volume of bacterial suspension with a known volume of human blood, spreading a loopful or two of the mixture on a slide, drying, staining, and finding the ratio of organisms to red cells in a given number of microscopic fields. Average healthy male blood contains approximately five million red blood cells per cubic millimeter (five billion per cubic centimeter, or milliliter), and female blood averages about four and a half billion red cells per milliliter. For more accurate determinations using Wright's technic, actual counts should be run on the specific blood to be employed in the test.

Since we know the number of blood cells per unit volume, the ratio of bacteria to red cells allows a comparison to be made, and the count of bacteria per ml. can be calculated. To give a simple illustration, if the average blood cell count is found to be fifty per microscopic field, and if in those same fields the average bacterial count is found to be one hundred per field, there must be twice as many bacteria as there are blood cells per unit volume. If we know there are five billion blood cells per ml., there must be, in this case, ten billion bacteria per ml. The principal error in Wright's technic lies in the difficulty of securing a uniform distribution of organisms and blood cells, but reasonably accurate results may be expected with this method for determining total bacterial counts in liquids.

HEMOCYTOMETER METHOD

By using a special chamber, similar to the type employed by clinical laboratories for determining blood cell counts, bacterial suspensions can be examined and the number of organisms enumerated with an error estimated to be less than 10%. Some workers claim that the error is less than 3%, and that this technic is ex-

tremely reliable for determining total bacterial counts. Various modifications in lighting and in staining of the bacteria have been advocated, and interested students are referred to advanced textbooks for further information relative to these procedures.

The three methods just discussed involve direct counting technics, but there are also some indirect procedures available for the determination of total bacterial counts, and a few of these will be mentioned briefly.

OPACITY METHOD

As bacteria grow in liquid media their protoplasm produces an opaqueness, or turbidity. A given strain of organism of known numbers will impart a standard opacity to the medium. Therefore, by terminating bacterial growth at a given level, and by killing the cells to prevent further multiplication, this suspension of cells can be employed as a standard to which can be compared unknown suspensions of bacteria. If we have a series of known standards of different densities, it is possible to match an unknown with one of the opacity standards and to determine with reasonable accuracy the number of cells in the unknown suspension. As with the other technics previously discussed, the opacity method has its limitations. For one thing, not all organisms are of the same size, and one large cell may produce an opaqueness greater than one small cell. But in a culture containing millions, or even billions, of organisms this factor is probably compensated for. Trying to match densities introduces the human error. But if more accurate determinations are desirable, such aids as *photometers* (light-measuring devices) or *photoelectric densitometers* (density-measuring devices) are available.

Bacterial suspensions, even though inactivated with heat, may undergo physical change upon prolonged storage. To circumvent this condition, chemical opacity standards may be prepared. When certain chemicals (such as barium chloride and sulfuric acid) are mixed together in controlled proportions, a precipitate (milkiness) will form in a predictable quantity. This turbidity can be standardized to correspond to known numbers of morphological types of bacteria in suspension. Such nephelometers

when sealed to prevent evaporation, can be kept almost indefinitely. Just before use, the precipitate can be resuspended by shaking to distribute the milkiness evenly throughout the test tube. When adjusting the density of unknown bacterial suspensions, it is important to remember that tubes of the same diameter as the standard comparison tubes must be employed.

This method is accepted by some workers as a good index of bacterial mass but not as an accurate means of determining numbers of bacteria. Leise in 1926 reported that opacity of spherical cells is determined by the surface area of the cells and thus varies with the radius. Vaccines, especially autogenous vaccines, are commonly standardized as to numbers by resorting to the opacity technic.

CENTRIFUGE METHOD

While not too accurate a method for indirectly measuring total numbers of cells, the centrifuge technic is a rather ingenious approach to the problem. By centrifuging (spinning down) a broth culture in a special capillary tube and measuring the volume of packed cells, the total number of cells in the packed mass can be calculated, if you know the diameter of a single cell. The volume occupied by an individual cell can be divided into the volume of packed cells in the centrifuge tube, and an estimate of the total number of bacteria can be obtained.

A miscellaneous group of methods available for these determinations of total numbers of cells, including the calculation of the total nitrogen content of the cells, the amount of acid produced by a culture, direct weighing of bacterial masses, etc., are interesting, but they are not as commonly used as the ones discussed above.

TECHNICS FOR DETERMINING VIABLE BACTERIAL COUNTS

BROTH DILUTION METHOD

The dilution method discussed under isolation technics for bacteria may also be put to use in determining the number of living bacteria in a suspension. This is also known as *the most probable numbers method*, and it has application in water and sewage analysis where the determination of the most probable numbers of coli-

forms in the samples will aid the technician in determining the degree of pollution.

If a bacterial suspension is diluted 1:10, 1:100, 1:1000, 1:10,000, 1:100,000, 1:1,000,000 and 1:10,000,000, etc., in nutrient broth tubes, these tubes may be incubated for a suitable time at the optimum temperature and examined for evidence of growth. Should the tubes up to and including a dilution of 1:100,000 show growth, but the 1:1,000,000 tube fail to show growth for example, it would indicate that the original bacterial suspension probably contained about 100,000 bacteria but not as many as one million organisms per ml. The difference between these two figures is considerable, and a single viable organism either carried over or left behind in the serial dilution could make a ten-fold difference in the result.

THE PLATE COUNT

An improved modification of the dilution technic is the agar plate count. By substituting a liquefiable-solid medium, such as nutrient agar, for the nutrient broth, the contents of the tubes can be poured into petri dishes, the organisms trapped in the solidified medium can grow, and the resulting colonies can be counted. If the dilutions are carefully prepared, if strict attention is paid to the temperature of the agar medium in the tubes, and if the samples being treated are carefully shaken according to the standard method previously described under the Breed Smear technic, the plate count affords a fairly reliable index of the number of living organisms in a suspension capable of growing under the particular set of conditions provided.

Rather than prepare the dilutions in measured amounts of agar, which must be kept within a limited temperature range during the dilution procedure, it is customary to prepare dilutions in bottles of sterile water. Aliquot portions are pipetted into sterile petri dishes, and sterile nutrient agar at a temperature of between 45–50° C. is poured into the dishes and mixed thoroughly to insure even distribution of the bacteria. Each cell, or clump of cells, will theoretically develop into a bacterial colony, visible to the unaided eye. It is important to have the test material sufficiently diluted

to insure that the number of colonies developing on the plates will fall within the range of thirty and three hundred. Such a plate is called *countable*. If more than three hundred colonies grow on a plate, some bacteria are crowded out by the more aggressive organisms, and colony formation by the weaker bacteria may be inhibited.

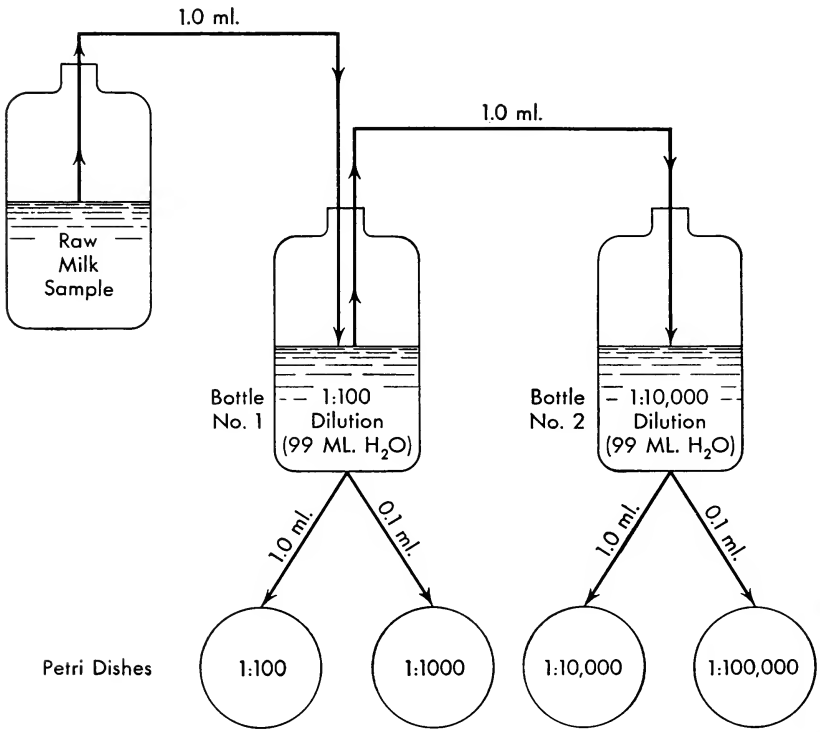


Fig. 27. Method of preparing dilutions.

The method of preparing dilutions is best explained with the aid of a diagram, and the above shows how a sample of raw milk might be diluted for plating purposes. The technic is not restricted to milk, however. Each petri dish should be fully and accurately labeled before diluted material is introduced into it. Aseptic precautions must be practiced throughout the plating process to minimize the entrance of contaminating organisms.

For those who have difficulty following diagrams, the complete steps involved in preparing the above plates will be put into words.

1. Shake the raw milk sample in the prescribed manner.
2. Pipette 1.0 ml. of the sample into 99 ml. of sterile water. (Bottle #1.) This will give a 1:100 dilution of the milk.
3. Shake this water dilution in the standard way.
4. Transfer 1.0 ml. of the diluted milk into a petri dish labeled 1:100.
5. From this same water dilution bottle pipette 0.1 ml. into a second petri dish labeled 1:1000.
6. Take 1.0 ml. from dilution bottle #1 and transfer it into dilution bottle #2 which contains 99 ml. of sterile water. This second bottle gives a 1:10,000 dilution of the original raw milk.
7. Shake bottle #2 in the prescribed manner.
8. Pipette 1.0 ml. from bottle #2 into a petri dish labeled 1:10,000.
9. From this same bottle #2 transfer 0.1 ml. into a petri dish, labeled 1:100,000.
10. Into the 1:100 plate pour one tube of melted and cooled (to between 45–50° C.) agar. Mix the agar and diluted milk by gently rotating the petri dish on a flat surface such as a table top. The mixing should be thorough but not so vigorous that the agar slops over the side of the petri dish.
11. Pour one tube of melted and cooled agar into the 1:1000 plate as you did with the 1:100 plate above. Continue this procedure with each plate prepared. Do not allow more than 20 minutes to elapse between the time the milk sample is originally diluted and the time that the agar is poured into the petri dish.
12. Set the plates aside until the medium solidifies, then incubate them in an inverted position to prevent water of condensation from forming on the lids and dropping back onto the agar surface. At the conclusion of the prescribed incubation period, the plates are examined. Count only the plate having between thirty and three-hundred colonies, and multiply the

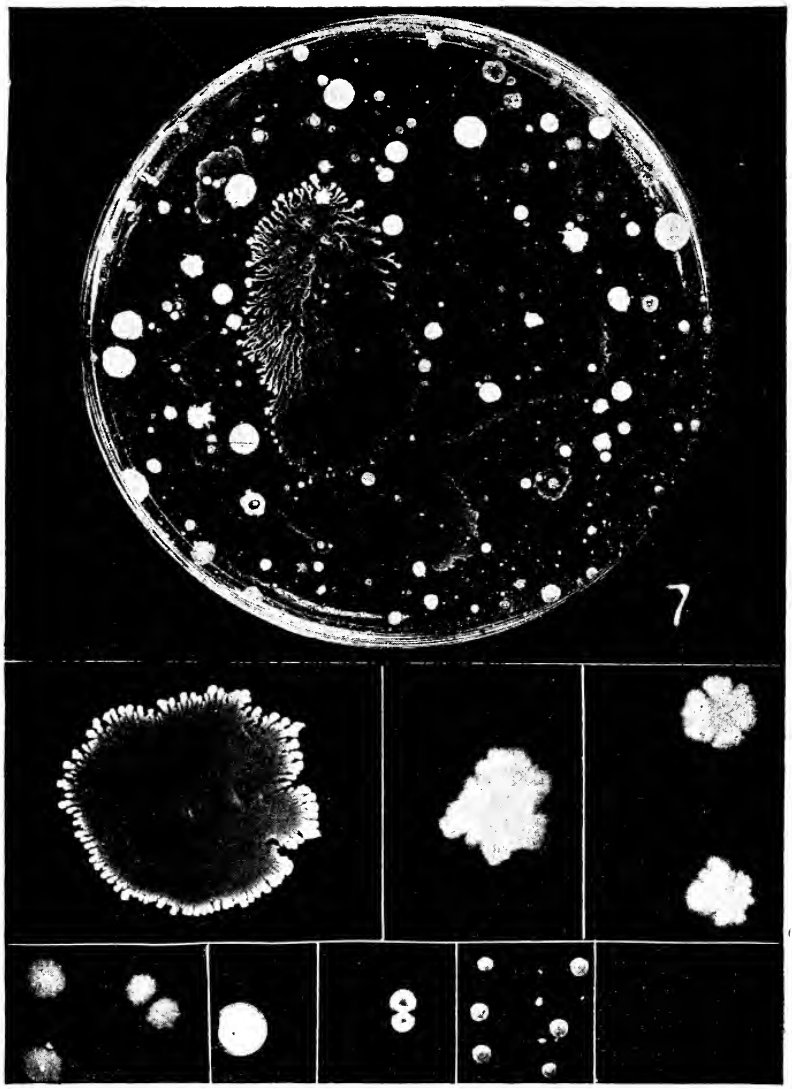


Fig. 28. Bacterial colonies. In the petri dish (above) are two "spreaders." In the corner (lower right) are "pin point" colonies which are very small even when growing on the surface. (*From* General Bacteriology, *D. B. Swingle and W. G. Walter. Copyright 1947, D. Van Nostrand Company, Inc., New York.*)

number of colonies by the figure found on the lid of the dish. For example, if the 1:10,000 plate appears to be countable, count the number of visible colonies and multiply the result by 10,000, since the colonies arose from only one ten-thousandth of a milliliter of the original milk.

It is sometimes difficult for students to understand how it is possible to secure a 1:100 plate and a 1:1000 plate from the same 1:100 dilution bottle. The labels on the petri plate represent the fractions of a milliliter of the original milk actually transferred to the petri dish; it is not necessarily a dilution as such.

Experience shows the number of organisms normally to be expected in a given test material, and the types of bacteria, within limits, may also be predicted. The number of dilutions required and the extent of the dilutions must be estimated, with sufficient plates being prepared to hit the correct dilution range. There are occasions when this estimate may be incorrect, and none of the prepared plates will fall within the prescribed 30–300 limit. In such instances it becomes necessary to employ the plate coming closest to being countable. It is better to get some idea of the viable count than it is to discard the plates because they are too crowded, or because they do not have as many as thirty colonies on them. If the plate coming closest to being countable is very crowded, it is not necessary to count every single colony on that plate. It is permissible to count representative areas and to calculate the total number of colonies on the plate. An average petri dish has an area of about 65 square centimeters. If 5 representative square centimeter areas are counted and multiplied by 13, the total number of colonies on the plate can be estimated ($5 \times 13 = 65$ square cm.). The importance of adequate mixing of the agar with the milk dilution in the plates becomes very obvious when even distribution of colonies is desirable for counting colonies.

Colony-counting devices are available which provide adequate diffused illumination and which have glass plates marked off in square centimeter areas, and smaller areas, to facilitate counting

the colonies on agar plates. The guide squares are clearly visible through the agar in the dish, and by counting colonies in an orderly sequence, square by square and line by line, no colonies should be counted twice nor should any colonies be missed in the operation.

Fig. 29. Quebec colony counter in use. (*Courtesy of the American Optical Company, Instrument Division, Buffalo, New York.*)

There is no doubt that these chambers improve the accuracy of the counting procedure.

It should be emphasized that not all bacteria that are alive in a tested material such as milk or water will develop into colonies in the standard plating technic. There is no universal medium which will allow every species of organism to develop under one set of growth conditions. Temperature requirements, nutritional needs,

oxygen demands, etc., vary tremendously among species of bacteria. However, the standard agar-plating technic is as good a method as is available for routine use in determining viable bacteria counts. As long as each step in the process is standardized, and the two main sources of error are minimized—namely, *dilution error* and *colony distribution error*, the method has merit if correct interpretation of the results are attempted. Technicians should be able to check each other on a given sample within 5 to 10%, and for all practical purposes, this is accurate enough.

CHAPTER 8

Effects of Physical Forces on Bacteria

TEMPERATURE
MOISTURE
PRESSURE
RADIATIONS
ULTRASOUND
SURFACE TENSION
ROCKING AND SHAKING

Bacteria are composed of protoplasm, a living substance existing in a rather delicate state of balance. Methods designed to destroy organisms are aimed at tipping this protoplasm out of balance, either by physical or by chemical forces. Once this unbalance has been accomplished, a vital link in the chain of living events is removed and normal metabolism of the cell is prevented. If the tilt is severe enough and if it is prolonged, the cell will die.

Without going into a great deal of detail relative to theories proposed to explain how physical forces adversely affect bacteria, this chapter will attempt to describe some of the commonly used physical technics. For students who may be interested in further readings on this topic, Porter's *Bacterial Chemistry and Physiology* is recommended. Additional references covering reviews of specific phases of physical forces on bacteria may also be found in this book.

TEMPERATURE

FREEZING

Higher plants and animals are adversely affected when their temperature is reduced, and warm-blooded animals will perish if their heat-producing devices are unable to overcome falling temperature. Because of efficient temperature-control mechanisms, however, extremes in the environmental temperature can be compensated for by physiological adjustments and by the wearing of clothes by man. Fur-bearing animals alter the thickness of their coat with temperature cycles. The tolerance of cold-blooded species varies considerably.

The unique resistance of bacteria to low temperatures with no pronounced change in their survival power when they are once more returned to normal temperatures, may well explain their survival during glacial periods in our earth's history. "Brother and sister" bacteria, as well as those relatives many times removed, vary in their temperature tolerance, however. Some of the more fastidious pathogens, including members of the *Neisseria* genus, fail to survive low temperatures, particularly in the moist state. Most microorganisms, even including pathogens, can be stored at refrigerator temperature (4° C.) for many months, and this is a common laboratory procedure for maintaining a *stock culture collection* for research and for teaching purposes. The life of a bacteriologist would be considerably more involved if he were obliged to isolate fresh cultures every time he needed specific organisms.

The effect of freezing upon bacteria depends upon the speed with which the organisms are frozen. Rapid freezing is much less harmful to microorganisms than is slow freezing. When slow-frozen, ice crystals are larger and their shearing action on the cell substance is materially greater than the cutting, if any, of the small crystals formed during fast freezing. This principle is vital to the frozen food industry. Slow-frozen strawberries, for example, would thaw into shapeless mush because of the breakdown of tissue cells caused by large, sharp ice crystals. Freezing does not sterilize food. You can't take a product with a high bacterial

count and expect to make a good product out of it by merely freezing the material, any more than you can make high quality pasteurized milk from poor raw material simply by heating the milk to destroy the bacteria. It just doesn't work. With the recent popularity of home freezers, the notion that freezing kills microbes is a concept that needs to be dispelled. It must be conceded that freezing may lower the bacterial count, because the "weaker" organisms will not tolerate such a minimal metabolism, if life processes do go on at these low temperatures. An interesting observation along this line, however, is that pork containing living trichina worms can be made "safe" by freezing for 24 hours. Please don't confuse trichina worms with bacteria.

The substrate in which organisms are frozen has a great deal to do with the lethal effects of the temperature. While the typhoid organism normally cannot survive freezing in water for more than six months, this same organism has been found to be still viable after two years at —4° C. in frozen ice cream. When an organism cannot grow, it oftentimes will die, but the length of survival depends upon a number of environmental factors. One suggested explanation of death of microbes in the frozen state is suffocation brought on by too much oxygen, since the oxygen concentration at 0° C. is twice that found at 30° C. in many substrates. The redistribution of materials by freezing is another interesting reaction. A 10% sugar solution when frozen may be found to have as low as 2% sugar around the edge which is frozen first, while the core may have as high as a 35% concentration of the sugar. Perhaps a similar redistribution of protoplasm within the cell occurs, and if the condition is not relieved in time, the cell perishes. Cold, in general, should be considered as a bacteriostat, not as a germicide. It has been shown that bacteria grow at —8.89° C. (16° F.), and while metabolism may be at an extremely low rate, life processes, nevertheless, have been reported. When materials are frozen the moisture is not available, or at least it is not as readily available, and this might be compared to drying a culture. Since water usually is considered to function as a carrying agent for food and

waste products, many cells cannot efficiently metabolize, and the weaker members of the species give up the struggle.

Lipman exposed organisms to a temperature of *minus* 270° C. without causing death of all the cells. Absolute zero is only three degrees colder than this, and at —273° C. all chemical activity is believed to cease.

While extremely cold temperatures do not ordinarily destroy bacterial cultures in short periods of time, the combination of repeated freezing and thawing is very harmful to microbes. Experiments with *Salmonella typhosa* bear out these statements.

FROZEN SOLID		ALTERNATE FREEZING AND THAWING	
Before freezing	40,896	Before freezing	40,896
Frozen 24 hours	29,780	Frozen 3 times	90
Frozen 3 days	1,800	Frozen 5 times	0
Frozen 4 days	950	Frozen 6 times	0

Lyophilization. If an aqueous suspension of bacteria is frozen rapidly by such materials as solid carbon dioxide (dry ice), and the frozen product is dried by high vacuum sublimation, the dried bacteria kept sealed in this vacuum remain viable for years. This is called the LYOPHIL process, or LYOPHILIZATION. Lyophilic is a designation given to colloidal substances, such as proteins, which have an affinity for water. *Lyo* comes from the Greek and means to dissolve, while *philus* means loving. When literally translated the word means to dissolve readily. One of the earliest devices for drying bacteria was the *Chryochem Process* of Flosdorf and Mudd.

Lyophilization has many practical applications. The American Type Culture Collection, the central agency from which pure cultures of organisms may be purchased, maintains its stock culture collection in part by lyophilizing the organisms. It is possible to keep many bacteria for a number of years in their original state when they are so treated. We should consider this technic as a combination of freezing and drying, rather than either process alone.

HEATING

The destructive action of heat was discussed under media making in an earlier chapter. Suffice it to say here that dry heat is less destructive than moist heat at the same temperature, since coagulation of protein is readily induced when moisture is present to aid in the process. Hence, sterilization in the autoclave ordinarily requires only 121° C. for 15 minutes, while dry oven sterilization is accomplished only after subjecting the material to about 170° C. for a minimum of one hour.

GROWTH RANGE OF BACTERIA

Every organism has three cardinal points with respect to temperature—a maximum, an optimum, and a minimum. The maximum growth temperature may be considered as the highest temperature at which an organism may live and carry on any of its life processes. The maximum for growth may be different from the maximum for fermentation, but in general we think in terms of growth. Too high an environmental temperature results in "malaise" of the bacteria, if we may use that expression for microbes. Prolonged exposure of an organism to a temperature exceeding its maximum growth temperature will eventually cause the death of the organism. Naturally, the higher the temperature, the more rapidly will death occur. For most rapid growth, other factors being equal, an organism should be kept at the optimum temperature. This is the point where organisms do their best work. We humans also have our likes and dislikes with respect to temperature. In general the temperate zone encourages more efficient use of our bodies, both mental and physical, than we find true in either the tropics or the arctic zones.

The minimum growth temperature is the lowest point on the thermometer at which growth occurs to a measurable degree, and this is above the freezing point. There are indications that minimal growth and metabolism are possible below freezing, which makes an exact demarcation difficult for some organisms. Some pathogenic bacteria have an extremely narrow growth range, with the minimum

temperature close to that of the maximum temperature for growth. It should be made clear that the growth temperature range does not necessarily coincide with the resistance range of organisms, particularly on the low end of the temperature scale.

We can classify bacteria into three general groups on the basis of their temperature ranges. Again, the lines between these groups are not sharp, and some overlapping occurs. But for the sake of convenience microbes are designated as *psychrophilic, mesophilic,* and *thermophilic.* Scientists do not agree as to the temperature limits of each group. The following summary, however, represents a cross-section of accepted limits for growth temperatures of bacteria.

	DEGREES CENTIGRADE		
	MINIMUM	OPTIMUM	MAXIMUM[1]
Psychrophilic	0	15–20	30
Mesophilic	15–25	25–40	50
Thermophilic	25–45	45–55	55–85

The PSYCHROPHILIC (cold-loving) bacteria are those organisms which play an important part in the spoilage of foods in your refrigerator and in large cold-storage plants.

MESOPHILIC (middle-loving) organisms include the bulk—that great middle group—of organisms, including the pathogens for man and lower animals as well as the soil and water forms.

A group of organisms which grow well at elevated temperatures lethal to most bacteria are termed THERMOPHILIC (heat-loving). Proteins usually coagulate at between 55–60° C., but these organisms thrive in such localities as hot springs where the temperature may go as high as 85° C., or more. The nature of protein able to withstand such elevated temperatures needs further investigation.

The term MICROPHILIC is a designation sometimes applied to those organisms having a narrow growth range. Some of the highly pathogenic species, including the gonococcus and the meningococcus, fall into this category.

A great deal of trouble can be caused in the food canning industry and in milk processing plants by THERMODURIC bacteria—

those organisms capable of withstanding high temperatures. They usually do not multiply at high temperatures.

The probable temperature limits between which bacterial life is possible are absolute zero (—273° C.) and 160° C., according to some authorities in the field, but the time factor must be borne in mind. Life activities, however, are confined to a narrower range—about 0° C. to 90° C., and in general, a growth temperature cooler than optimum will increase the life span of microbes, while temperatures higher than optimum tend to "burn up" the cells more quickly. This same observation has been reported for fruit flies. When kept at 10° C. they survived much longer than flies kept at 30° C. where they lived at a killing pace.

THERMAL DEATH POINT

Because heat is relatively cheap and most readily controlled, it is quite commonly employed for killing microorganisms. Most non spore-formers are found to have a thermal death point between 55 and 60° C., while spore-formers may withstand boiling for hours. The *thermal death point* (abbreviated T.D.P.) is determined by exposing a 24-hour nutrient broth culture to increasing degrees of heat for a constant time period of 10 minutes. The lowest temperature which sterilizes the culture during the stated time period is called the T.D.P.

THERMAL DEATH TIME

Thermal death time is an important consideration in food industries where undesirable flavors and colors may be imparted to the food by excessive exposure to high temperatures. It may be more desirable to heat at a lower temperature for a longer time period to accomplish the same effect as higher temperatures for a shorter time period. Each product has its own optimum conditions based upon bulk, the nature of the product, etc. About seven billion #2 cans of food are processed annually, and the importance of heat in destroying undesirable organisms is only too apparent.

All cells of a given species of bacteria do not perish at once when exposed to heat. Some of the more resistant strains in a

given culture may outlast the others. It becomes a sort of contest like trying to hold your breath, and individuals vary in their abilities. The heat resistance of bacteria depends upon (1) temperature, (2) length of exposure, (3) degree of moisture present, (4) *p*H of the menstrum in which the bacteria are being heated, (5) the type of medium in which the organisms are suspended, and (6) the innate nature of the organisms. It is easier to heat-kill bacteria in water than it is to accomplish the same end in milk or in cream, which tend to protect the organisms from the lethal effects of the heat.

MOISTURE

A cartoon character was once pictured as drinking copious amounts of water, and when pressed for an explanation he admitted that he had just eaten some dirt and he was swallowing the water to drown the germs. Unfortunately, bacteria are not killed that easily, in fact they thrive in the presence of a high percentage of water. Bacteria are more aquatic than terrestrial, but in a water-logged soil where oxygen is not sufficiently soluble, some aerobic bacteria have to give way in favor of anaerobic organisms which thrive when free oxygen is lacking. Some oxygen can diffuse into liquids exposed to the air, and motile bacteria can congregate at the level where oxygen concentrations are optimum.

While most bacteria thrive in high concentrations of moisture, it is surprising how little water is necessary to keep organisms alive. Like a goldfish removed from his bowl and exposed to the drying effects and the oxygen differences in the air, bacteria dried in the air will eventually die. If the exposure is not prolonged, both the goldfish and the microbe might recover when returned to their normal habitat.

Delicate pathogens are more susceptible to desiccation than are most saprophytes. This is an important point for clinical bacteriologists to bear in mind. Swabs delivered to the laboratory for bacteriological culturing should not be allowed to lie around before being placed in or on appropriate media. Negative results may occur where positive findings are indicated. The lethal effect of desiccation depends upon (1) the medium in which the bacteria

are dried. Thick materials like pus or sputum will retard drying and exert a protective effect on the organisms. (2) Drying films in the light is more destructive to bacteria than is drying in the dark. (3) The higher the drying temperature, the more destructive to living cells. (4) Drying in air is more lethal than desiccation in a vacuum or in a gaseous atmosphere like inert nitrogen. (5) The nature of the organism, whether it be a spore-former or just a resistant form like some cocci, directly affects resistance to drying. Complete desiccation seems to suspend bacterial action, and in time the cells perish. The drying of foods as a means of preservation is predicated upon the need of moisture for microbial activity. Deprive a bacterium of water for a long enough period and the organism will usually not be a factor in the spoilage of food as long as the moisture level is kept below a certain point. There is some evidence, however, that the enzymes of organisms may continue to act even in the absence of bacterial growth.

People have dried meats and fish for centuries as a means of preservation, but they didn't know the bacteriological laws underlying the success of their methods. Such foods as dried figs, dried apples, prunes (dried plums), and raisins (dried grapes) keep well because of a combination of physical changes. As moisture is removed, the relative concentrations of sugar go up, and this increased sugar concentration adds to the unfavorable conditions for bacterial growth as we shall see in the discussion of osmosis.

The possibilities of dehydration were given adequate field trials during World War II, as many GI's will attest. Entire meals of so-called reconstituted foods were quite common. Dehydrated milk, eggs, potatoes, etc., were used in tremendous quantities for feeding the troops. The space and the weight conserved by dehydration are considerable, and during time of war, such factors are of paramount importance. Why ship heavy, bulky water to far-flung areas where water is readily available? It is like carrying coals to Newcastle. While the principles of dehydration are sound, a steady diet of dried powder and a glass of water can become very tiring, even though it may be nutritious.

Drying may not kill some bacteria, and upon hydrating de-

hydrated foods, the same precautions with respect to refrigeration must be observed or the food will undergo spoilage the same as the original product before the water was removed. Filterable viruses are quite resistant to drying. Just as slow freezing is more destructive to bacteria than is fast freezing, we find that slow dehydration acts in a similar way. Lipman reported finding bacteria in desiccated adobe bricks where he felt they might have remained in suspended animation for centuries.

The role of moisture in sterilization technics has been discussed in a previous chapter.

PRESSURE

MECHANICAL

At sea level the atmosphere exerts a pressure of 14.7 pounds per square inch (one atmosphere), and with increases in elevation above sea level, this force is reduced as the air becomes more rarefied. In recent years with the advent of stratosphere and super-stratosphere flying in commercial airlines as well as in jet planes, man has been forced to develop ingenious devices to allow the occupants of these planes to survive not only the rarefied oxygen but the tremendous changes in atmospheric pressure from those found at sea level. One need not be a jet pilot, however, to experience in a mild way the effect of changing atmospheric pressure on the mind and on the body. Transport yourself to the top of a high mountain, such as Pike's Peak (elevation 14,109 feet), and the atmospheric effects become only too apparent. The reduced oxygen per unit volume of air makes some folks giddy, others sleepy, and still others ill. Even mild exertion calls for materially increased breathing. Water boils at about 187° F. at this elevation and cooking foods like potatoes in an open vessel may require twice the time that it does at lower elevations.

Man can adapt himself to these atmospheric changes, and one of the evident alterations he makes is in the number of oxygen-carrying cells in the blood. A normal red blood cell count of five million per cubic millimeter may rise to six million or more at higher elevations. Since each cell can carry just so much oxygen,

and since the amount of oxygen per unit of air is reduced, nature takes a simple way out by producing more oxygen-carrying cells. Until these added cells are manufactured and put into operation, however, deeper breathing can partially aid in relieving the lack of the oxygen. Going back to sea level from higher elevations requires a reverse adjustment phase, namely, a reduction in red blood cells to the number more commonly found at sea level. The length of the adjustment period varies with different people, and during the transition period, it is not uncommon for individuals to complain that they lack "pep."

Deep sea divers cannot be returned too quickly to the surface after being submerged at great depths, or the sudden release of pressure on their bodies will cause the release of gas bubbles, principally nitrogen, in the blood, causing the dreaded "bends" or "caisson disease." Decompression chambers must be employed to gradually return such divers to normal sea level pressures. Man can endure remarkable changes in pressure if the changes are made gradually.

Getting back to bacteria, how much pressure can these microbes endure without being killed? A great deal of conflicting information exists on this point, but a direct pressure of 6,000 atmospheres has been shown to kill non spore-bearing bacteria in 14 hours. Some spores required twice this pressure for a similar time period to be inactivated, but 20,000 atmospheres were required for still other endospores to be killed. Anyone planning to stop the fermentation of grape juice by the application of pressure would be obliged to exert 100,000 pounds pressure per square inch for 10 minutes to accomplish this feat. Obviously there are more practical ways of inhibiting microbial fermentation. There is evidence that sudden osmotic changes are responsible for the death of organisms subjected to extremes in pressure. A pressure of 12,000 atmospheres may reduce the volume of water to 80% of normal, and this same type of condensation of bacterial protein followed by sudden release of the pressure would materially alter the colloidal nature of the cells. Just as the sudden release of steam pressure in an autoclave causes cotton-plugged tubes of media to "blow their

tops," bacteria under gas pressure may expand to the point of disintegration. Some bacteria found at great depths in the sea, however, show remarkable powers of adjustment to changes in air pressure. Gram negative organisms are much more susceptible to sudden release of carbon dioxide pressure than the tougher gram positive species. The latter organisms may be killed in the process but visible disruption of the cells is not evident. Fish have been recovered from the ocean depths where the pressure may reach 300 atmospheres. A unique pressure bladder allows these fish to adjust to pressure changes within limits.

If oxygen is the gas employed, a compression of 100 atmospheres above bacterial cultures may increase the amount of dissolved oxygen in the cells by as much as one hundred times the normal values. Many cells "suffocate" from so much oxygen, a toxic effect comparable to chemical sterilization. Inert gases, such as hydrogen and nitrogen, do not have this same effect, but they may alter staining characteristics of the organisms. Acid-fast bacteria may lose their acid-fastness, and gram positive bacteria may lose their gram positiveness. Carbon dioxide, however, may aid in the death of cells exposed to increased pressure of this gas. The amount of carbon dioxide in carbonated drinks is no small factor in holding down the bacterial count in these products. Many of these popular drinks have a very low *p*H which is unfavorable for bacteria, and it may be harmful to the enamel on the teeth of persons who drink these carbonated beverages to excess. Many fish and meats that would ordinarily undergo slow decomposition as the result of action by bacterial psychrophiles at or near 0° C., can be preserved for extended periods when stored in a carbon dioxide atmosphere. A high oxygen pressure (8 to 10 atmospheres), when coupled with low temperatures, has been successfully employed on an experimental basis for the preservation of milk in the *Hofius Process.*

OSMOTIC PRESSURE

Osmotic Pressure is defined as the diffusion pressure of a solvent passing through a semi-permeable membrane. This is an equaliz-

ing process. Nature strives for equality in the biological as well as the physical world. Equal opportunity for all was a law of nature long before man applied the principle in democratic forms of government.

Solutions having equal osmotic pressure are called ISOTONIC, while solutions of high concentrations are called HYPERTONIC, in contrast to HYPOTONIC solutions which have a low concentration. Cells thrive best under isotonic conditions; hypertonic solutions cause cells to shrink, while swelling occurs when cells are placed in hypotonic solutions. These changes are readily demonstrable with red blood cells which have more elastic cell walls than are commonly found with bacteria. Observing bacteria with the microscope under these conditions of high and low salt concentrations of solutions, very little, if any, visible change in cell size can be observed. Bacterial cell walls are more rigid than the walls of blood cells, and this lack of elasticity prevents marked changes in cell size.

Claude Zobell has shown that marine bacteria adapted to the salinity of sea water (approximately 3.5%) are quite sensitive to lower salt concentrations; in fact, many of them fail to grow below a salt level of 2%. Conversely, a few organisms whose natural habitat is fresh water, will be inhibited by salt concentrations above 1%. The water of the Great Salt Lake in Utah is made up of 27% salt, yet many bacteria can be isolated from these waters, and these organisms cannot be cultivated in the presence of less than 13% salt. Such salt-loving organisms are called OSMOPHILIC.

A point oftentimes not fully appreciated is that the *solute* as well as the *solvent* tend to diffuse from the region of high concentration to one of less concentration. The speed of this particular reaction and the ultimate result are influenced by (1) the dissociation of the solute, (2) the rates of diffusion of various substances in solution, (3) the molecular weight of the solute, and (4) the nature of the membrane through which the diffusion is taking place.

Sugar in a sealed parchment bag surrounded by water will build up pressure within the container. The greater the number of sugar molecules, the greater will be the difference between the

incoming and the outgoing water, and the greater, therefore, will be the osmotic pressure within the bag. If the bag is connected to an upright tube of mercury, called a MANOMETER, the pressure built up within the bag will be reflected by a rise in solution in the manometer. This pressure is expressed in terms of millimeters of mercury.

Protein molecules and colloidal particles are generally large and occupy considerable space compared to crystalloids. Consequently in equimolar concentrations of crystalloids and colloids, there will be fewer colloids per unit volume. This will be reflected in a lower osmotic pressure for colloids.

The statement is often made that some bacteria, particularly pathogens, when transferred to distilled water will die in a relatively few hours. The inference is made that death is due to osmotic pressure changes. While it is true that water will enter these suspended cells and will build up some pressure, there is more evidence that death of these cells is caused by the presence of toxic trace elements in the water originating in the still, or by the alkali dissolved from the soft glass of the container in which the bacteria are being held. Freshly distilled water is neutral in reaction, but upon standing it absorbs carbon dioxide from the atmosphere, and in the absence of a buffer, the water may become quite acid. Some bacteria, on the other hand, may be shown actually to multiply in distilled water.

Imbibition is the word used to designate the taking in of water by protoplasm or gels. Is this different from osmosis? Gortner (1937) reported that dry seeds are able to withdraw water from a saturated solution of lithium chloride (osmotic pressure of 1,000 atmospheres), although the seeds' salt content is only sufficient to account for but a few atmospheres of osmotic pressure. It is probably by this or some similar mechanism that bacterial spores take up water and germinate, and that certain bacteria are able to survive and multiply in concentrated brine solutions.

Every organism has its maximum, optimum, and minimum concentration of salt in which it may live and grow, just as each cell has similar limitations with respect to temperature, *p*H, etc.

RADIATIONS

VISIBLE SPECTRUM

The portion of the light spectrum with wavelengths large enough to be visible to the human eye is called the visible spectrum. Wavelengths detectable only with the use of special instruments are called ULTRAVIOLET at the blue end of the scale and INFRA-RED at the red end of this band. These wavelengths are measured in Ångstrom units—one ten-millionth of a millimeter in length. The eyes of man are able to see light with wavelengths from 3900 Ångstroms at the violet end to about 8000 Ångstroms at the red end of the band. Different eyes have different seeing abilities, so these upper and lower limits are only approximations.

In general, the visible spectrum has little adverse effect on bacteria, but certain bands of invisible light are decidedly germicidal. Most research has been directed at the ultaviolet rays, which leaves a fertile field of research for persons interested in studying the action of other bands of the light spectrum on microbes.

SUNLIGHT OR SOLAR RADIATION

The destructive action of sunlight on bacteria was first pointed out in 1877–1878 by Downes and Blunt. The lethal effect of sunlight is attributed to the ultraviolet band, and its mode of action appears to be a denaturation of the protein in bacteria. In the absence of chlorophyll, which allows cells of higher plants to convert simple materials into plant substance, bacteria cannot prevent the accumulation of energy within the cells, and the organisms are harmed by this type of light. Strong sunlight coming in direct contact with bacteria exerts a destructive effect, just as we find human skin adversely affected by prolonged contact with sunlight—sunburn. Water purification and treatment of milk with ultraviolet light have been advocated through the years, but the poor penetrating power of ultraviolet light and the expense limit its usefulness.

The strongly bactericidal rays are those between 2500 and 2800 Ångstrom units, and the effect of sunlight in helping to destroy

potentially dangerous bacteria emanating from the nose and throat of infected individuals and from healthy carriers must be considerable. Applications of ultraviolet light have found their way into biological supply houses where such rays are employed to reduce possible contamination of vaccines; in banks where ultraviolet "screens" supposedly protect the tellers from the multitude of organisms sprayed at them during the course of a single day; in school rooms; in military barracks; in the treatment of bottles in beverage plants; in infants wards of hospitals, etc. Commercial ultraviolet lamps give off light with a wavelength of about 2600 Ångstrom units.

Interesting mutations have been observed in bacterial cultures exposed to ultraviolet radiation for controlled periods. Certain combinations of dyes and light may exert effects on organisms that neither dye nor light can produce alone—a synergism. This is called a photo-dynamic action, and it takes place only in the presence of oxygen.

ROENTGEN OR X RAYS

While X-ray therapy may be used in the treatment of certain skin disorders, particularly deep-seated fungus infections inaccessible to ordinary topical applications of drugs, just what effect X rays have on bacteria is a rather sketchy bit of our knowledge. They appear to be lethal, however, and interesting mutations may occur in bacteria exposed to X rays.

RADIOACTIVE ELEMENTS

Uranium, thorium, etc., may give off radiations called alpha, beta, and gamma rays. Alpha rays of radioactive substances are positively charged helium atoms given off at a high velocity. The beta rays are negatively charged particles of uncertain nature traveling at high velocities. Beta particles have more penetrating power than do alpha rays, but they tend to pass directly through small objects like bacteria. Five mm. of aluminum or one mm. of lead will hold back beta particles, in contrast to alpha rays which are retarded by a thin sheet of paper, glass, or aluminum. Gamma

rays are true electromagnetic waves similar to X rays but of a shorter wavelength. They have great penetrating power but their destructive action on bacteria is apparently very slight or nil. The present state of our knowledge indicates that bacteria may be affected by alpha and by beta rays, but the lethal action appears to be slight, at best.

ELECTRICITY

The harmful effect of electricity on bacteria apparently is a combination of a heat reaction and liberation of ozone, nascent oxygen, or chlorine from the bacterial substrate. Attempts to treat water with the use of electric currents has been shown to be too expensive in some localities, to say nothing of some of the technical difficulties involved. But pasteurization of milk using the electropure process is a common practice that is growing in popularity.

In some localities where electric power is reasonable, sewage may be freed of harmful organisms by passing 2.5 volts of electricity through the effluent. Chlorine gas appears at one electrode and alkalies appear at the other electrode, and the process is feasible.

CATHODE RAYS

These rays may be generated by Coolidge electron tubes and have been found to be destructive to bacteria, but too limited investigation has been carried out to date to make any further claims for these cathode rays.

ULTRASOUND

The word SUPERSONIC, to a physicist, means frequency in cycles per second of sound waves pitched too high to be audible to the human ear. ULTRASONIC, on the other hand, is anything beyond the speed of audible sound, which is about 760 miles an hour at sea level.

Sonic wavelengths in the higher frequencies (about 8,000) may be employed to kill bacteria. The action of high frequency sound waves appears to be similar to the action of sudden release of pressure from bacterial cultures, namely a disruption of the cell

wall. As the frequency increases, the death rate rises. Spheres are more difficult to kill than are rods, with the large rods being most easy to kill. This type of cell disruption is useful because it does not appear to denature the protein so readily as application of chemicals and heat. It has particular usefulness in immunological studies of such materials as endotoxins which are intimately bound with the cell's protoplasm. Cells that are not visibly disrupted by ultra-sonics are undoubtedly killed by a combination of physical and chemical forces. Cavitation by dissolved gases aids in cell disruption.

Short exposures to sub-lethal doses of high frequency sound waves are capable of inducing interesting genetic changes in plants. There is some evidence that orchids can be shaken out of their lethargy and germination speeded up when subjected to ultra-sonics. Chemicals that normally settle out of solution can be made to remain in suspension after exposure to high frequency sound waves. Here is another field for fruitful investigation.

SURFACE TENSION

Surface tension is the force that operates in each square centimeter of cross section of the surface to hold it together, while surface energy is the work required to increase the surface area when measured at constant temperatures. Liquids are always bounded by surfaces which have some of the characteristics of membranes. Liquids tend to maintain a minimum surface area, and this is why a drop of water in the air tends to be spherical. The surface is under tension and the sphere has the least surface area for a given volume. The drop becomes spherical for the same reason that a toy balloon becomes round when it is inflated—surface forces.

Surface tension may be measured by means of a Du Noüy Tensiometer, which determines the force required to separate a platinum ring from the surface of a liquid. This force is measured in *dynes*, and a dyne is defined as the force which will produce a velocity of 1 centimeter per second in gram mass.

Surface tension affects microbial growth in terms of clumping, morphology, staining properties, cultural characteristics, and even virulence. The lower the surface tension, the easier it is for a substance to come into intimate contact with bacteria. We make use of this knowledge in the preparation of disinfectants when we suspend the active principle in alcohol (a tincture) instead of in water (an aqueous solution). Alcohol may be said to be wetter than water, in this regard. At 18° C. pure water has a surface tension of 73.0 dynes, glycerol has 65.2 dynes, and ethyl alcohol has only 21.7 dynes of surface tension. Any substance that lowers surface tension tends to gather at the surface, including the surface of bacterial cells. If the surface tension is lowered, some bacteria are killed. In fact, pneumococci are dissolved, and this principle is employed as a test for separating pneumococci from other cocci.

Since bacteria are practically all surface, and surface reactions play such an important part in their metabolism, any change in the nature of the bacterial surface will influence growth of the organism. A man weighing 70 kilograms has a total surface area of about 1.5 meters. The ratio of surface area to weight is about 1:50 in this instance. Bacteria have a surface area to weight ratio of 9000:1. It is not surprising, therefore, that small cells can accomplish such a great deal of work. *Escherichia coli* has been calculated to have a surface area of 0.000,004,5 square millimeters, and a mass of 0.000,000,000,5 milligrams.

ROCKING AND SHAKING

Gentle rocking and slow aeration will stimulate bacterial growth in liquid cultures, possibly due to the fresh food supply made available to organisms as waste products are removed from their immediate vicinity. The rocking may also cause more rapid separation of organisms after binary fission has taken place. Vigorous agitation, however, is definitely detrimental to bacteria. A constant trembling motion exerted by the running of heavy machinery has been reported to kill some bacteria after four days exposure to the vibrations. The destructive action caused by violent agitation is apparently due to denaturation of cell

proteins, unbalancing of the colloidal state, or actual cell disruption. Vegetative cells exhibit greater sensitivities to these forces than do spore-forming organisms. Shaking a liquid culture with glass beads in a mechanical shaker, or rotating a culture in a ball mill, are common practices employed for the disruption of bacterial cells.

CHAPTER 9

Effects of Bacterial Growth on the Environment

Just as physical and chemical forces affect bacterial growth, microorganisms are capable of influencing their environment in visible and measurable ways. Such products of metabolism as heat, light, pigment, toxin, and other miscellaneous reactions will be discussed in this chapter.

THERMOGENESIS (HEAT PRODUCTION)

Heat is liberated during the growth and metabolism of most, if not all, cells—both plant and animal. But because this generated

heat is dissipated before it has an opportunity to build up to a measurable degree, the exothermic reactions are not usually observed. Grain and vegetable cells during their metabolism can add heat to that produced when the moisture content is sufficiently high to support microbial growth. Spontaneous combustion of haystacks can be attributed to an initial build-up of heat by these metabolic processes, and when the temperature reaches a certain level, purely chemical reactions may step in and spark the ignition.

As early as 1884, Schloesing claimed that the heating of haystacks and manure piles was a combination of the work of organisms and chemical oxidation. He held that bacteria are the major cause of temperatures up to 70° C., and from this point upward chemical processes carry on the thermal reaction. Ferdinand Cohn (1888) remarked that temperatures up to 35° C. are due to plant cell respiration, and that heating from 35° C. to 45° C. is brought about by molds. It was reported by Rabinowitsch (1896) that thermophiles develop rapidly after an initial heating process, and these organisms cause an increase in ammonia which facilitates the heating of manure. Distillation products mixed with the air, according to Laupper in 1927, produce a detonating gas which is the immediate cause of spontaneous combustion in the presence of pyrophoric iron.

In a study of the heat produced in grains by single pure cultures of organisms as well as by mixed cultures, James, Rettger, and Thom (1928), and later Wedberg and Rettger (1941) devised insulated chambers with automatic aerating devices. By employing cracked corn as a substrate and by adjusting the moisture content to about 30%, temperatures as high as 67° C. were recorded with mixed cultures, and close to 60° C. was registered with a pure culture of *Bacillus subtilis* growing on sterile cracked corn. This organism is also popularly known as the *hay bacillus*. Temperature values of between 45° C. and 55° C. were quite common for many of the ordinary soil organisms tested.

Adequate drying of hay and of grain prior to storage is of paramount importance in the prevention of harmful and dangerous self-heating. *Microbial thermogenesis* also has some practical

applications, such as the use of heating manure in hot beds and cold frames and preventing the freezing of newly poured concrete during the winter months.

The production of vinegar by the more rapid methods which employ so-called generators, can result in the formation of sufficient biological heat to destroy the vinegar organisms unless corrective measures can be taken to control the high temperature produced by the microbial action. Tobacco subjected to the "sweating process" is piled in stacks and allowed to heat to temperatures of 55–60° C. before the piles are dismantled and the leaves are hung up to dry. The heat generated in loosely packed silage can be sufficient to interfere with proper fermentation.

Only when heat production by microorganisms is rapid and cumulative is the term THERMOGENESIS usually applied. While it is true that the amount of heat produced by a single bacterial cell is not very great, the combined effect of billions of organisms is considerable. It has even been suggested that in areas where grain is abundant, and cheap in price, homes might be partially heated by microbial thermogenesis in this substrate packed between the walls of the house.

PHOTOGENESIS (BIOLUMINESCENCE)

A curious group of bacteria, found most abundantly in sea water, are endowed with the power of emitting light during their metabolism. Some of these light-producing bacteria grow as parasites or as opportunists on various types of marine life, but no pathogenicity for laboratory animals or for humans has been demonstrated.

If your travels ever take you into a fish market after dark, peering into the barrel where the fish heads are discarded may reveal an eerie glow, particularly if air is blown over the surface of the fish. Should you by chance be carrying a tank of oxygen on your back at the time, a stream of this gas directed into the barrel will activate any bioluminescent bacteria that are present. Caution: Don't smoke during this experiment!

Organisms which are phosphorescent are said to be PHOTOGENIC;

in fact, they can be photographed by their own light. The glow of decaying wood sometimes observed in forests is caused as a rule by fungi, such as certain toadstools and mushrooms. The term "fox-fire" is applied to this luminescence.

Light is emitted during respiration of these organisms under aerobic conditions, rather than being absorbed as occurs in photosynthesis. Part of their waste energy is given off as heat and part as light. Luminescence, apparently, is an unnecessary side-effect of metabolism, since the organisms are capable of growing well without emitting light.

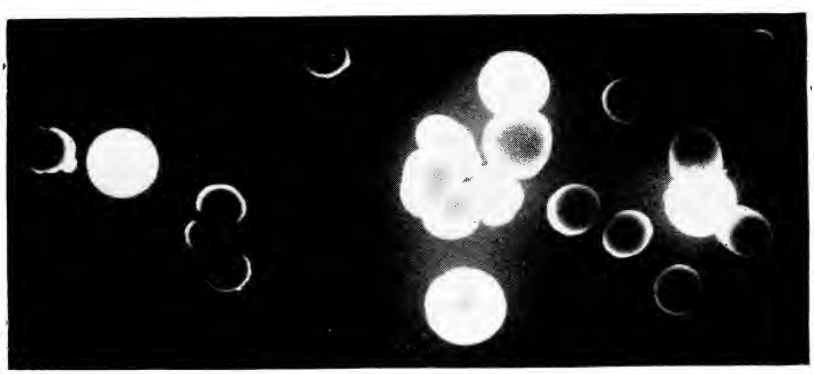


Fig. 30. Luminous bacteria photographed by their own light. (*Courtesy of A. C. Giese, Stanford University.*)

The cold light given off by these microscopic lamps appears to be the result of the action of luciferase enzyme acting upon its substrate luciferin. While a single cell gives off insufficient light to be detected by the usual measuring devices, the cumulative effect of an active culture containing billions of cells is bright enough to permit the reading of a newspaper when a few liters of the culture are actively aerated in a dark room. Those persons who have had an opportunity to be on boats in salt water at night have undoubtedly observed on occasion streaks of light in the water at the stern of the boat as the propeller whipped air into the salt water and activated the bioluminescent bacteria. Some waters are richer in these organisms than are other bodies of water, and

certain seasons of the year, particularly summer, appear to favor the activities of these curious species.

All three morphological types of bacteria—rods, spheres, and spirals—have been found to possess photogenic properties. A great deal of pure research on this interesting phenomenon needs to be done, and for a good review of the topic, interested students are referred to the book, *Living Light*, by Harvey (1940).

CHROMOGENESIS (PIGMENT PRODUCTION)

Most bacterial colonies are white, gray, or nearly transparent, but a few organisms produce pigments with colors extending over the entire range of the visible spectrum. Some of these pigments are confined within the cell (intracellular) and others are secreted through the cell wall into the surrounding medium (extracellular). Chemical analyses have shown that these pigments are similar, if not identical, with those found in flowers and in vegetables. Yellow and shades of yellow, followed in descending numbers by reds, blues, violets, greens, browns, and blacks are the colors found among microbes.

With few exceptions, such as the pigments in the green and purple sulfur bacteria, pigments serve no known useful function for the organisms. In fact, they are generally considered to be waste products of metabolism. Bacteriochlorophyll, while closely related to the chlorophyll of higher plants, is not identical with this vital material which higher green plants depend upon for their survival.

The solubility of bacterial pigments is the basis for schemes of classification, including the following:

1. Those pigments soluble in water. (Including the pigment produced by *Pseudomonas aeruginosa*, the etiological agent in green pus.)
2. Those pigments soluble in alcohol but not in water. (This includes most bacterial pigments, and *Serratia marcescens* is an example of such a chromogenic species.)
3. Those pigments insoluble in either alcohol or water. (*Micro-*

coccus citreus is an example of an organism producing this type of pigment.)

Chromogenesis occurs only under specific conditions of the environment, such as the presence of specific constituents in the medium, *p*H of the substrate, incubation temperature, light intensity, oxygen supply, etc. This last factor is particularly important, and its effect can be demonstrated by examining colonies growing in the depths of a solid medium and comparing them with surface colonies on the same culture plate. Best pigment production occurs when there is an abundant supply of oxygen.

Pigments vary in amount and in intensity of color even within a given species; VARIATIONS (temporary changes) and MUTATIONS (permanent changes) may be demonstrated. The amount of coloring matter within a single cell is too minute to be detected under the microscope, but masses of cells, especially surface colonies on culture plates, provide enough pigment to be seen with the unaided eye. Pigmentation often does not occur until a culture is in the latter stages of growth, which is further evidence to some workers that pigments are accumulations of waste products.

The "bloody bread" of Biblical times was undoubtedly caused by *Serratia marcescens*, the red pigment-producing bacterium, growing on the sacrament wafers. Blue milk, red milk, and other pigments in dairy products are the result of microbial growth on these media, and they can be very troublesome.

A pigment called CYTOCHROME has been found to be present in most, if not all, living cells, and it functions in respiration. Cytochrome, however, is not a pigment in the same sense as the coloring matter in cells described above. Unless cytochrome is concentrated, its color is not detectable. In other words, cytochrome does not cause bacterial colonies to appear pigmented.

The red-purple coloring matter of a select group of bacteria allows the organisms to assimilate carbon dioxide with the aid of sunlight. This pigment is BACTERIOPURPURIN, and it provides a type of metabolism unique among bacteria which ordinarily do not thrive in the presence of sunlight.

TOXINOGENESIS (TOXIN PRODUCTION)

Toxins are poisons produced by living cells—both plant and animal. The reason for their formation is not fully understood, but it has been postulated that bacteria in some cases elicit poisons to destroy surrounding tissues and to create more favorable growth conditions for themselves by neutralizing some of the competition. Are bacterial toxins excretions (waste products) or are they secretions formed as a part of a definite campaign to create more favorable environmental conditions? If we consider the case of a poisonous snake, the lethal bite may be a means for obtaining food, as is the case with spiders. But when a snake bites a man, the act is undoubtedly a protective device. It is questionable that the snake has the ultimate aim of devouring the human being. Certain plants, such as toadstools and castor beans, are extremely poisonous in themselves, but the poison seems to serve no useful end for the plant. Our knowledge relative to RICIN, the poisonous ingredient of the castor bean, is still in need of further elucidation.

Toxins may be given off during the course of bacterial growth, and these are termed EXOTOXINS (soluble poisons). ENDOTOXINS (insoluble poisons) may be produced which are so intimately bound to the cell protoplasm that they are not liberated until after the cell dies and autolyzes (dissolves itself), or until the cells are shattered by physical forces discussed earlier in this book. This is not an arbitrary distinction; it is the basis for important immunological considerations to be taken up later. The symptoms of such diseases as botulism, diphtheria, lockjaw, and scarlet fever are due largely to exotoxins liberated by bacteria. Endotoxins are formed by the typhoid, paratyphoid, dysentery, cholera, and plague organisms.

Exotoxins, which can be separated from the cells which produced them, exhibit the following characteristics: (1) They are formed only by living cells, (2) they are quite specific in the reactions which they produce, (3) they are capable of acting in minute amounts, (4) they can be injected into suitable animals for the production of ANTITOXINS—those important substances em-

ployed in the treatment of diseases caused by bacterial poisons, and (5) they require an incubation period before clinical symptoms are apparent. Fever, malaise, and wasting away of animals left untreated are characteristic of biological toxic reactions, whereas chemical poisons, like strychnine, act almost immediately without a preliminary incubation period.

We think of strychnine as a powerful chemical poison. Yet, the biological toxin produced by *Clostridium tetani* (the lock-jaw organism) is two hundred times as potent as strychnine. A guinea pig may be killed by as little as 1/1,000,000 ml. of a bacteria-free filtrate of a culture of *Clostridium botulinum*—a highly fatal food-poisoning organism. The route by which toxins gain entrance to the body is vital to the ultimate *in vivo* (in the body) response. For example, the toxins of botulinum and of certain micrococci are harmful when swallowed, but the poison generated by the diphtheria organisms can conceivably be ingested with little or no systemic reaction in the individual. When diphtheria toxin is injected into the bloodstream, however, its lethal effect is directed at the nerve tissues and at heart muscles. Cobra venom can be said to be relatively mild in comparison with the toxin produced by a virulent strain of *Corynebacterium diphtheriae*. Exotoxins can be distinguished from each other on the basis of their pharmacological action. Tetanus poison affects motor nerve cells with resultant muscle spasms; botulinum toxin induces early ocular and pharyngeal paralysis; and diphtheria toxin injected into rabbits causes hemorrhages in the adrenal glands.

Whereas the diphtheria microbes may localize in the throat, among other places, the poison given off by these localized bacteria may be carried via the blood stream and the lymph channels to areas of the body directly affected by the poison. The virulence of these exotoxin-producing species is directly dependent upon their ability to form and to liberate their powerful poisons.

Roux and Yersin in 1889 first discovered diphtheria toxin, and they demonstrated that this lethal agent in the filtrate of broth cultures was capable of producing the same symptoms in laboratory animals as an injection of the living microbes. This opened up the

field of toxin investigation, and in 1892 Pfeiffer reported that the cholera organisms produce a poison that is not excreted during the life of the parent cells. It was Pfeiffer who coined the term *endotoxin.*

Repeated injections of sub-fatal doses of exotoxins, or the injection of TOXOIDS—a toxin that has been detoxified by the addition of formalin—will stimulate the production of protective substances called ANTIBODIES. The stimulating agent is called an ANTIGEN, because it causes the injected animals to produce something against the antigen. When an endotoxin-producing organism is introduced into an animal, antibodies will be formed, but they will not be antitoxins; they will be antibodies produced against the entire cell substance, of which the toxin may be but a small fraction.

The comparison of exotoxins and endotoxins below summarizes the principal characteristics of these two types of poisons.

	EXOTOXINS	ENDOTOXINS
Location	Given off through the cell wall into the medium during growth of the cells.	Remain within the cell until the organism is disintegrated by autolysis or by physical means.
Toxicity	Exhibit a high degree of toxicity.	Are less toxic than exotoxins.
Chemistry of Pure Toxin	Are proteins.	May be protein, although some contain other complex substances including carbohydrates.
Action of Formalin	Are converted into toxoids.	Are not converted into toxoids.
Inactivation	Usually inactivated by heat (58–80° C.) in 10 minutes, and by proteolytic enzymes.	Exhibit resistance to heat (80–100° C. for 1 hour) and to proteolytic enzymes.
Antigenicity	Will stimulate the formation of antitoxins in animals.	Are poor antigens for antitoxin production.
Specificity	Specific for antitoxins.	Not specific for antitoxin.

It is not uncommon for an organism to produce several toxins which react in a number of diverse ways when subjected to different tests. Micrococci, for example, produce HEMOTOXIN which dissolves red blood cells; a LEUCOCYTIC TOXIN which destroys white

blood cells; an ENTEROTOXIN which affects the gastrointestinal tract; a LETHAL TOXIN, which kills rabbits; and a skin-destroying poison, called a DERMONECROTIZING TOXIN. Perhaps some, or all, of these reactions are caused by a single toxin manifesting itself in different ways on diverse substances.

ENZYMES

The life of every cell depends upon chemical reactions activated by organic catalysts called ENZYMES. The word enzyme comes from the Greek and means "in leaven or in yeast." Chemists make use of many inorganic catalysts to influence the speed of reactions, but enzymes differ from these catalysts in having their origin only within living cells and in being organic.

Minute amounts of enzymes can catalyze the activity of a great deal of substrate—the name applied to the material upon which the enzyme acts. In fact, the catalyst can act upon substrate one million times heavier than the enzyme without the latter being used up in the reaction. Chemically, enzymes are proteins, or proteins attached to lower molecular weight substances.

Naming of enzymes, with some exceptions, is done by adding the suffix "ase" to the specific substrate. For example, the enzyme which attacks gelatin is termed GELATINASE, and enzymes acting upon protein are designated as PROTEASES, or PROTEINASES. Enzymes may also be designated on the basis of types of chemical reactions which they catalyze—OXIDASES, for example.

The same factors that influence chemical reactions will affect enzyme activity, and these include temperature, *p*H, moisture, concentration of reacting substances, the presence of certain ions which may stimulate or inhibit enzyme activity, etc.

Living cells may produce two general classifications of enzymes: INTRACELLULAR and EXTRACELLULAR. These latter catalysts are secreted, or excreted, through the cell wall, and they prepare food in the surrounding area for passage through the cell membrane. Once the food substance gets within the cell, the intracellular enzymes act upon it and prepare it for metabolism by the cell.

The field of enzymology is a complex study, involving complex

chemical reactions. Suffice it to say here that the metabolism of all cells would grind to a halt were it not for the action of these organic catalysts we term enzymes.

MISCELLANEOUS METABOLIC PRODUCTS

HEMOLYSINS

Hemolysins are agents which destroy red blood cells. They are both filterable and thermolabile (destroyed by heat), and when injected into animals, hemolysins can stimulate the production of antibodies called ANTI-HEMOLYSINS.

When hemolysin-producing bacteria are grown on a suitable culture medium containing whole blood, zones of clearing are distinguishable adjacent to colonies growing on such plates, and these reactions are designated ALPHA HEMOLYSIS and BETA HEMOLYSIS, depending upon the nature of the change in the blood medium. Alpha hemolysis (viridans type produced by pneumococci and so-called green streps) is characterized by a greenish zone on a blood agar plate. Examination of this discolored area under the low power of the microscope will reveal that while a few intact red corpuscles remain, most of the blood cells have disintegrated. Beta hemolysis is characterized by a clear-cut colorless zone, and no intact blood cells can be found in this clear area. Streptococci associated with severe sore throats and pathogenic micrococci are representative of organisms producing beta hemolytic reactions. Any colonies failing to exhibit visible zones of hemolysis are called GAMMA types of growth. There appears to be no strict correlation between hemolysis and pathogenicity.

Hemolysins are similar to bacterial exotoxins in that they are given off in the surrounding medium and can be freed from the cells by filtration. Bacterial hemolysins, however, should not be confused with the type of immune reaction developed in animals injected with the red blood cells of other species of animals. Injected foreign blood cells can stimulate the production of antibodies (also called *amboceptor* or *hemolysins*) which are capable of dissolving the specific blood cells employed as antigen. The

former hemolysins are antigens and are liberated by bacteria, while the latter hemolysins are antibodies produced by the host as a result of antigenic (whole blood cell) stimulation.

COAGULASE

Part of the vital defense mechanism of the body is the deposition of fibrin and the clotting of blood. Bacteria which have gained entrance into the host animal may be kept localized by these reactions. A substance called COAGULASE hastens the clotting of blood plasma. PLASMA is the cell-free liquid of the blood and contains fibrin. Blood serum lacks fibrin and hence is incapable of being clotted by coagulase. Staphylococci tend to remain localized when they set up infections in the body, and the ability of these bacteria to produce coagulase undoubtedly partially explains why these infections remain localized, as in pimples, boils, carbuncles, and the like. When the blood stream is invaded by these bacteria, the formation of blood clots, called *thrombi*, within blood vessels is a characteristic feature of such SEPTICEMIAS (infections within the blood stream). The word BACTEREMIA, strictly speaking, means a transient invasion of the blood stream by bacteria. This is a temporary condition, but in septicemia the organisms are multiplying—a progressive type of blood poisoning.

Rennet, the enzyme which causes sweet-curdling of milk, is another example of a coagulase. After death, the stiffening of muscles in man and in other animals, called *rigor mortis*, is apparently caused by a coagulating enzyme which converts myosinogen of the muscle into firm myosin.

FIBRINOLYSIN

Hemolytic streptococci belonging to Lancefield's Group A (those pathogenic for man) produce a substance called FIBRINOLYSIN which is capable of dissolving blood clots, or plasma clots. Hence, infections with streptococci are less likely to remain localized; they become generalized infections much more readily than do the micrococci which liberate coagulase. Much of the invasiveness of streptococci can be explained on this basis. Fibrinolysin

is specific in that human streptococci can dissolve human blood clots but not clots of blood from other animals.

SPREADING FACTOR

Duran-Reynals reported a SPREADING FACTOR which affects the permeability of invaded tissues to bacteria, toxins, India ink, and other substances. Some bacteria produce spreading factor and it can also be found in the testes of certain animals. The invasiveness of an organism can be correlated with its ability to manufacture spreading factor. Recent studies have shown the similarity of this reaction to that produced with *hyaluronidase,* an enzyme capable of dissolving mucin-like substances. The enzyme destroys hyaluronic acid, a component of the intercellular substance uniting body cells in tissues.

PYROGENS

Unless distilled water is kept free of bacteria, constituents of certain organisms, even though dead, produce fever reactions, called *pyrogenic responses,* upon injection into animals. Exact chemical analyses have revealed pyrogenic fractions which are carbohydrate in character, and when as little as twenty-five to fifty micrograms (millionths of a gram) of this material is injected into a rabbit, the animal of choice, a temperature rise of about 2.5° C. will occur. Pyrogens are not uncommon in the culture medium employed in the manufacture of such substances as penicillin, and great care must be employed to get rid of these undesirable fractions. The Shiga type of the dysentery organism, curiously enough, produces a hypothermic (temperature-lowering) substance in its metabolism.

FERMENTATION

This word comes from the Latin, *ferveo,* meaning to boil, which was the most common observable characteristic of the reaction. As our knowledge increased, fermentation came to signify the breakdown of sugar into alcohol and carbon dioxide (Gay-Lussac's work), and with the studies of Pasteur fermentation became more

closely associated with microorganisms and their ability to cause this breakdown of fermentable substances.

Over one hundred different compounds are produced as the result of microbial fermentation, and these reactions form the basis of some of our large industries today. Yeasts are employed in the manufacture of ethyl alcohol, alcoholic beverages, leavening agents, yeast concentrates, and in the manufacture of glycerol. Bacteria are the biological agents in the preparation of butyl alcohol, acetone and other solvents, lactic acid, and other acids. Molds function in the manufacture of citric, gluconic, and gallic acids, and a host of other miscellaneous products. The chemistry of these reactions is left for discussion in a book designed for more advanced students.

The word *alcohol* is derived from two Arabic words, *al* and *kohl*, which denote a fine powder used by Oriental women to darken their eyebrows. The methods employed in the manufacture of these powders were similar to those used for distilling "spirits." In about the year 1500 the word *alcohol* was adopted to denote a volatile liquid, and today we think in terms of ethyl alcohol when the word alcohol is mentioned.

The quantity of pure alcohol used in the United States alone for industrial purposes and the arts and the sciences was estimated to be six hundred and nine million gallons in 1944, as compared with a little less than nineteen million gallons in 1920. The word *proof* seen on alcohol labels is an old English term meaning strength or quantity of spirit. A one hundred proof spirit contains fifty per cent by volume of alcohol. Such raw materials as molasses, sugar beet, sugar cane, and fruit juices are good saccharine materials for producing alcohol. Such starchy substances as potatoes and cereal grains (corn, barley, rye, rice, etc.) also provide large stocks of raw material for biological alcohol production. Molasses is the most common substance used in the manufacture of industrial alcohol in the United States, except during the World Wars when the molasses supply was materially reduced. Sweden and Norway rely upon the fermentation of wood pulp and waste sulfite liquor from wood pulp mills as their source of alcohol.

PUTREFACTION AND DECAY

Decomposition of protein, including animal and vegetable matter, through the agency of microorganisms in the absence of air is called PUTREFACTION. The end products of such decomposition include solid, liquid, and gaseous matters, some of which have a foul odor. DECAY is a term used to signify aerobic decomposition and the process differs from putrefaction primarily in the state of oxidation in which the products are left. Complete oxidation into stable, non-foul smelling compounds is a characteristic of decay. Both of these processes play important roles in keeping the elements in nature rotating for future generations of plants and animals. We who are living may harbor elements once a part of the living matter of a famous person, an outright scoundrel, or a lovely rose. When our days are over, and our borrowed elements are returned to nature to use as she sees fit, we may become a part of future generations.

Shakespeare's *Hamlet,* Act IV, Scene III, expresses this rotation of elements when Hamlet points out to the King the whereabouts of the dead Polonius in the following passages:

KING. Now, Hamlet, where's Polonius?
HAMLET. At supper.
KING. At supper! Where?
HAMLET. Not where he eats, but where he is eaten; a certain convocation of politic worms are e'en at him. Your worm is your only emperor for diet; we fat all creatures else to fat us; we fat ourselves for maggots. Your fat king and your lean beggar is but variable service, two dishes, but to one table; that's the end.
KING. Alas, alas!
HAMLET. A man may fish with the worm that hath eat of a king; and eat of the fish that hath fed of that worm.
KING. What dost thou mean by this?
HAMLET. Nothing, but to show you how a king may go a progress through the guts of a beggar.

COOPERATIONS AND ANTAGONISMS

Although bacteriologists study organisms as pure cultures of isolated species, that is not the way microbes are found in nature.

Cooperations and antagonisms are very common among microscopic plants and animals just as these relationships exist with higher forms of life. Even in infections, such as a severe sore throat, more than one organism is found in the affected area, but only one species may be attributed as the cause of the severe symptoms.

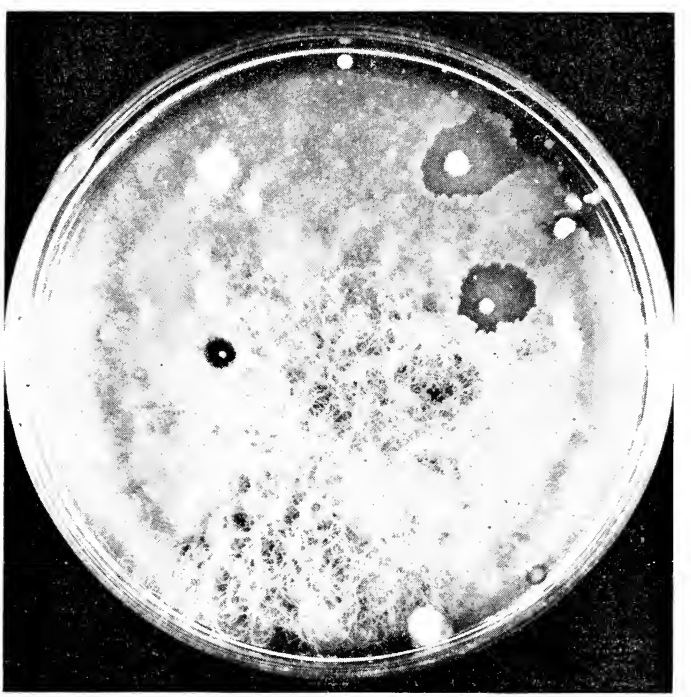


Fig. 31. Microbial antagonisms in an agar plate inoculated with soil. Three of the colonies have produced an inhibitory substance that has prevented the growth of surrounding organisms. (*Courtesy of A. Kelner, S.A.B. No. 143.*)

Blood poisonings, at least in the advanced stages, are usually pure cultures of aggressive organisms. The various relationships existing between microorganisms will ultimately affect their environment. Therefore, a discussion of these reactions seems pertinent in this chapter.

When organisms work together for mutual benefit, we call this

SYMBIOSIS. This relationship can hardly be expected to be a fifty-fifty proposition. In time one or the other of the organisms will predominate; bacteria are no exceptions to the law of competitive spirit.

In nature microbes not only work together with other microbes, but they also cooperate with animal life and with some of the higher plants. One of the better known examples of a symbiotic relationship between bacteria and plants has practical application in the cultivation of some crops that you and I depend upon for food. Legumes (*legere,* Latin, to gather) derive their name from the fact that the peas and beans may be picked without cutting the plants. Nodules develop on the roots of legumes, and in these nodules are formed specialized bacteria which are capable of extracting gaseous nitrogen from the atmosphere which contains about 78% of nitrogen, and converting it into a form available to the plant. Nitrogen in this free state is unavailable to plants unless nitrogen-fixing bacteria are able to convert it into a utilizable form. The leguminous plants, in turn, provide the bacteria with materials and conditions favorable for their growth. In this way both the plant and the bacteria benefit through symbiosis. Nitrogen economy will be discussed in more detail later in this book.

The word COMMENSALISM (literally, eating at the same table—messmates) is applied when an organism lives as a parasite but does not harm or help the host. The bacteria which make up the normal flora of the intestines of warm-blooded animals are representative of this group.

There are some reactions of organisms that depend upon the combined efforts of two or more organisms. Neither organism is capable of causing the reaction by itself, but when combined with another living agent, positive reactions are produced. For example, lactose may not be fermented by bacterium "A" or bacterium "B," but when "A" and "B" are both inoculated into lactose broth, fermentation may occur. Certain multiple microbial infections are examples of this relationship, which is termed SYNERGISM.

METABIOSIS is a condition in which an organism produces in its growth substances or conditions that can be used by another

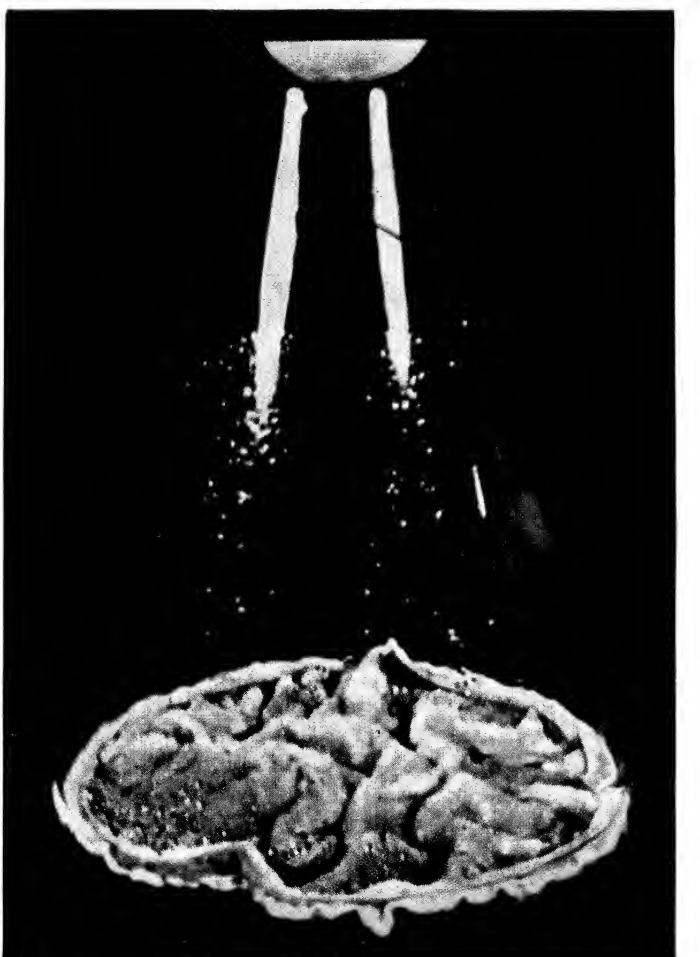


Plate I. Typical mature growth of *Penicillium notatum*. Note the thick, wrinkled growth covered with green spores and the presence of droplets on the surface. These drops are particularly rich in penicillin. (*Courtesy of Merck and Company, Rahway, New Jersey.*)

organism—a favorable effect. For example, when aerobic bacteria grow in the vicinity of anaerobic bacteria, the aerobes reduce the oxygen tension and create conditions favorable for the growth of the anaerobes.

When bacteria exhibit antagonisms—the reverse of symbiosis—the designation of ANTIBIOSIS is applied to the condition. The struggle for supremacy, even among such lowly forms of life as microbes, is vital to our well-being, in fact, to our very survival. Antibiosis partially accounts for the limitations of bacterial growth, which, if left unchecked, might well force all other forms of life from the face of the earth. The old law of survival of the fitter operates even at this reduced level of life.

Man in recent years has put this microbial competitive spirit to work for human good by the development of such medically important antibiotics as PENICILLIN, CHLOROMYCETIN, AUREOMYCIN, BACITRACIN, STREPTOMYCIN, and other substances. The development of these agents represents one of the great milestones in medical science. Many years have been added to the lives of untold thousands of individuals who have been treated with the chemotherapeutic agents, oftentimes called wonder drugs.

Antibiotic means against life, and it refers to an anti-microbial substance produced or derived from living organisms, and which acts adversely on other forms of life, usually microscopic organisms. Students vitally interested in the field of antibiotics are referred to advanced texts and pamphlets in the field, since this discussion will do little more than introduce the topic.

The discovery of penicillin occurred in 1929 when Alexander Fleming, an English bacteriologist, noted that a culture of *Staphylococcus aureus* failed to grow around a mold contamination on one of his culture plates. The mold proved to be *Penicillium notatum.* When this species was grown in nutrient broth, the filtrate of this culture was found to exert a powerful effect against certain bacteria, and when tested against laboratory animals, the filtrate was no more toxic than the nutrient broth alone. Fleming named this active principle *penicillin* and he advocated its possible use in the treatment of infectious diseases. After a lapse of eight years, Florey

and his associates at Oxford University pursued this problem further. They studied ways and means of purifying penicillin and of evaluating its possible usefulness.

From these small beginnings have arisen fabulous industries, and the word fabulous in this case is hardly an exaggeration. Chemical and pharmaceutical houses, under the pressure of war-

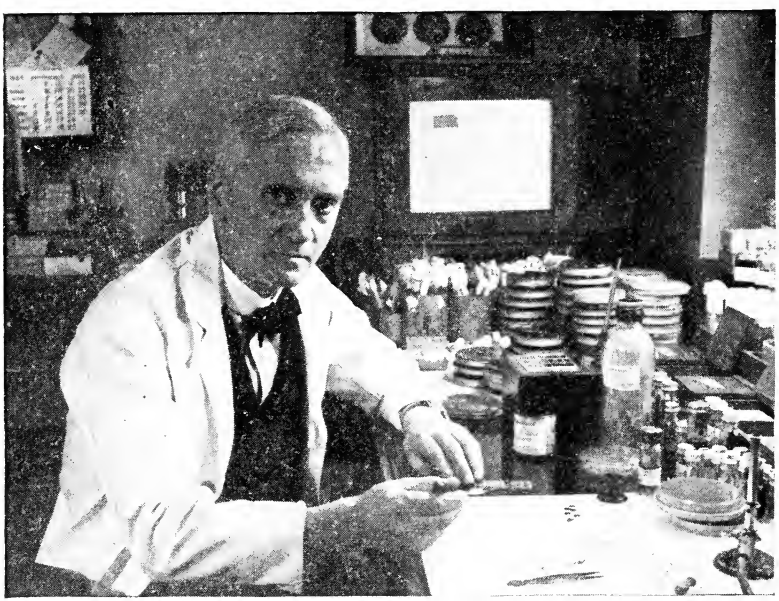


Fig. 32. Alexander Fleming, the discoverer of penicillin. (*From* Medical Microbiology for Nurses *by Erwin Neter, M.D. Copyright 1949, F. A. Davis Company, Philadelphia.*)

time demands, began to produce this antibiotic in quantity, and a better field trial could not have been devised than the opportunities available during World War II. While some of the earlier claims for penicillin were not substantiated—we did not have a panacea—the miracles performed by this drug in the treatment of disease can never be evaluated with any degree of accuracy. It seems a modest enough statement that the contributions of Pasteur and the development of antibiotics have probably saved more human lives

than the number of lives lost during all the wars throughout man's history.

Since penicillin is rather selective in its reaction, organisms found to be resistant to this substance had to await the discovery of additional antibiotics. The search continues today for new and more powerful agents. Unfortunately, with the passage of time,

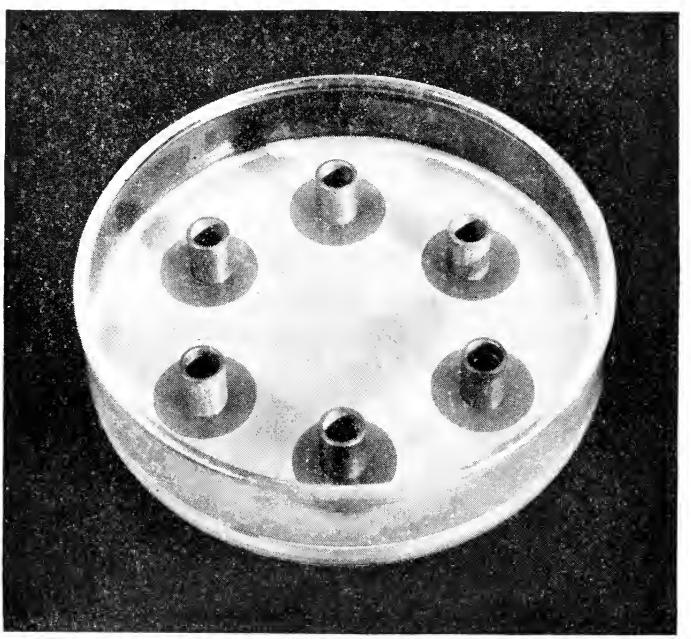


Fig. 33. Bacitracin assay plate showing inhibition zones against *Micrococcus flavus*. (*Courtesy of Commercial Solvents Corporation, New York, New York.*)

medical men have discovered that many bacteria formerly sensitive to the action of particular antibiotics have built up a tolerance which limits the agents it is possible to employ against these infections. Whereas penicillin came about as an accidental discovery, the dozens of antimicrobial agents developed since then have been the result of highly organized searches for new weapons in the antibiotic arsenal. Many organisms produce antibiotics that are

readily demonstrable in routine culture procedures, but too often the active principle is so toxic for the person being treated that the "operation may be a success but the patient will die!"

Soil is the best "melting pot" for microbes that exists in the world; competition for survival is probably no keener anywhere. Hence, thousands of soil samples taken from every corner of the earth are undergoing the searching and screening tests so essential for the discovery of new, usable, microbial, chemotherapeutic agents.

When man toys with the microbial balance of nature, especially in such a culture medium as the intestinal contents of warm-blooded animals, nature is bound to rebel as it has in recent years. Some rather stubborn cases of diarrhea have occurred in selected individuals who have had extensive treatment with antibiotics, and newer agents have had to be perfected to help correct this condition. As bacterial competition diminishes, fungi sometimes take advantage of the new opportunities and are able to flourish. Nobody knows where this chain reaction will end, but in the meantime, looking at the figures with a cold statistical eye, the amount of good done by antibiotics in modern medicine completely overshadows the harm encountered in a relatively small number of patients. As long as the treatment is doing more good for more individuals than was true of pre-antibiotic agents, medicine will continue to be grateful to those who provide new weapons with which to fight microbial diseases. Antibiotics, in one form or another, are here to stay for some time.

CHAPTER 10

The Effect of Chemicals on Microorganisms

DEFINITIONS

Chemical agents are known to affect living organisms in various ways, and this knowledge is the foundation of a multi-million dollar industry—drugs and biologicals. Many products are being sold on the market today in tremendous volume simply because the wording on the labels and in the advertising has sufficient appeal to convince hordes of persons that their lives are incomplete unless

they use the products getting the greatest attention in print and on the air. To protect a gullible public from false and misleading advertising, State and Federal agencies have been established to deal with problems arising from the sale of foods, drugs, and cosmetics.

By adding chemicals to the environment of microbes, it is possible to demonstrate a phenomenon called CHEMOTAXIS—the response of microorganisms to chemicals. If organisms are attracted to chemicals, this is *positive chemotaxis,* and the repelling action of other chemicals results in *negative chemotaxis.* Contrary to earlier beliefs, however, there is no relationship between this migration of organisms and the killing action of the specific chemical. Some agents will lure bacteria to their destruction while other lethal compounds will repel organisms. A completely satisfactory explanation of chemotaxis is still not available.

Many terms employed in discussing chemical effects on bacteria are used with different meanings by various individuals. It seems worth while at this point to define a few words commonly employed in such discussions.

1. ANTISEPTIC: A substance or agency that prevents sepsis, putrefaction, or decay. It is usually employed to control infectious agents on epithelial surfaces, mucous membranes, and superficial wounds.
2. GERMICIDE: Anything that destroys germs (microscopic organisms), particularly pathogenic organisms.
3. BACTERICIDE: Anything that destroys bacteria.
4. BACTERIOSTAT: An agent which inhibits bacteria but does not kill them, at least not for some time.
5. DISINFECTANT: An agent or process that frees from infectious organisms. It is generally thought of in terms of destroying organisms apart from a living animal. The word is generally used to describe the destruction of bacteria which have already initiated an infection, in contrast to an antiseptic which prevents pathogenic organisms from gaining a foothold. Pasteurization, however, may be employed to disinfect milk.

6. FUNGICIDE: An agent which destroys fungi.
7. STERILIZATION: The act or process of freeing something of all living cells—plant and animal, microscopic and macroscopic.
8. CHEMOTHERAPY: The treatment of infectious diseases with substances which kill or inhibit the growth of pathogenic organisms in the host's body without causing serious injury to the host. This term dates to the early 1870's when Weigert applied aniline dyes for tissue staining, and Ehrlich began considering the use of dyes *in vivo* (in the body) as bactericidal substances. Ehrlich introduced the term *chemotherapy* when he discovered "606," the synthetic, arsenic-containing aniline compound used for the treatment of syphilis.

Some chemicals that exert powerful destructive action on microorganisms may, in lower concentrations, exhibit a stimulatory effect on these same organisms. Disinfectants may act as antiseptics or as bacteriostats at different concentrations, and in very dilute amounts these same compounds may actually stimulate microbial growth.

CHARACTERISTICS OF AN IDEAL DISINFECTANT

There is no such thing as an ideal disinfectant on the market today. If you were given the task of trying to design such a compound, the following minimum considerations would have to be fulfilled:

1. *High Germicidal Power.* The word germicide is used here rather than bactericide because an ideal disinfectant should have a wide killing range with respect to microorganisms. The chances of finding a pure microbial culture in the area to be disinfected are rather remote. Therefore, any chemical aimed at freeing an area of infectious agents should be sufficiently broad in its killing power to give reasonable assurance of success.

2. *Stability.* If the chemical has strong affinity for all kinds of organic matter, the disinfectant may be dissipated before it has had an opportunity to combine with and to destroy microorganisms. Organic matter must be anticipated in the locations where disin-

fectants are to be applied. The chemical should not spontaneously break down upon standing, or the decomposition products may have little or no lethal effect, and the user may be left with a false sense of security.

3. *Homogeneity.* Every drop of the disinfectant should be just as effective in its killing capacity as every other drop in the container. Whenever the label calls for shaking the compound well before using, the human element is introduced and interpretations of "shake well before using" vary widely from person to person.

4. *Ready Solubility in the Strength Required for Disinfection.* If a compound is to be employed as a disinfectant, it must, in many cases, ionize (dissociate into ions). To do this the chemical must be soluble in the concentration that is toxic for microorganisms.

5. *Non-Poisonous to Higher Animals and Man.* The narrow margin existing between the concentration of medication required to kill microorganisms and the strength of the chemical that destroys healthy tissue cells of the host limits the usefulness of some compounds that have a high germicidal power. Not only must the bacteria be killed when the disinfectant is applied, but healthy tissues should be kept intact, if possible, to speed subsequent healing of the wound. If the chemical is readily absorbed into the animal system and has lethal effects on internal organs, the application of the disinfectant must be confined to use on inanimate objects.

6. *Noncorrosive.* This is mainly a storage problem, but if the disinfectant is to be used on metals, such as in operating rooms, etc., any corrosive action may so damage the equipment that it becomes impractical to use an otherwise effective agent.

7. *Penetrative Power.* Liquids exhibiting low surface tension have greater penetrating power than high surface tension substances. In order to reach bacteria that are deeply entrenched in a wound, penetrating power of the agent is an important factor. It is for this reason, among others, that tinctures (alcoholic solu-

tions) are employed in preference to aqueous (water) solutions when treating cuts and wounds with disinfectants. Alcohol has a much lower surface tension than water, and it can thus be considered to be wetter than water. So-called wetting agents, including Lauryl Sulfate, Tween 80, and Tergitol 7, can be incorporated into liquids to improve their wetting power and penetrating ability.

8. *Moderate Cost.* If a compound is priced excessively high, it will be restricted in its usefulness. While price is a relative thing, there are limits if the product is to be put on the market for common use.

Other considerations may be added to this list characterizing an ideal disinfectant, but they would probably not be nearly as important as the factors already discussed. Some compounds approach being ideal as disinfectants, but none of them fulfills all of the desired characteristics.

FACTORS INFLUENCING DISINFECTION

Disinfection is in part a chemical reaction, and factors influencing chemical reactions will, in general, affect the disinfection process. It is agreed that temperature, time, moisture, concentration of reacting substances, presence of extraneous matter, and surface tension have a direct bearing on the outcome of this chemical change. Undoubtedly there are other factors which are involved, but a brief statement about each of the above six considerations seems warranted.

1. *Temperature.* The speed of a chemical reaction will increase with the temperature up to a certain point. Many chemical reactions will go to completion in a matter of a few minutes under the influence of heat, but these same reactions at room temperature or lower, might require days, weeks, months, or even years, if they took place at all. Heat may be considered to act like a catalyst when it accelerates the speed of a reaction.

2. *Time.* The most rapid chemical reactions known to man require time to occur. Disinfection is not instantaneous, although this is a common misconception. Many persons firmly believe that

the very second a disinfectant is applied to microbes, the organisms are killed. This is not true. Time and temperature are very difficult to separate, as was previously pointed out with respect to thermal death time and thermal death point.

3. *Moisture.* Moisture plays an important role in the coagulation of protein, and since coagulation at least partially explains some disinfection reactions, available moisture influences the ultimate disinfection reaction. Moisture also serves as a carrier for heat and for transporting chemicals into the cells. We know that 100% alcohol is practically worthless as far as killing bacteria is concerned, but when the alcoholic strength is cut to between 50 and 70% by the addition of water, the added moisture aids in the coagulation of protoplasm and in the eventual destruction of microbes.

4. *Concentration of Reacting Substances.* With chemical reactions in general, the higher the concentration of reacting substances—within limits—the more chemical end product will be formed. We do know, however, that the most concentrated solutions of disinfectants are not always the most effective for killing bacteria. There is an optimum strength, above and below which there is a diminution of activity.

5. *Presence of Extraneous Matter.* Chemicals vary in their disinfecting ability in the presence of organic matter. Some chemicals, like chlorine, are rapidly neutralized if extraneous matter is present. Other chemicals have less affinity for organic matter and kill microorganisms quite promptly even when "dirt" is present.

6. *Surface Tension.* This consideration was touched upon in the discussion of penetrating power as a characteristic of an ideal disinfectant. It has been demonstrated that marked changes in some organisms, especially surface changes of the cells, can be induced by lowering the surface tension. Cells are easier to "get at" when the chemical agent is in a solution of low surface tension.

It goes almost without saying that the type of organism, its age and history, and many other factors will influence the disinfection process.

SPECIFIC COMPOUNDS EMPLOYED AS DISINFECTANTS

HEAVY METALS

When metals were first considered as possible agents in disinfection, their activity was referred to as an OLIGODYNAMIC ACTION (*oligo,* small; *dynamic,* powerful), because a small amount of metal appeared to exert a highly lethal effect on microorganisms. The salts of silver and of mercury represent two of the better known

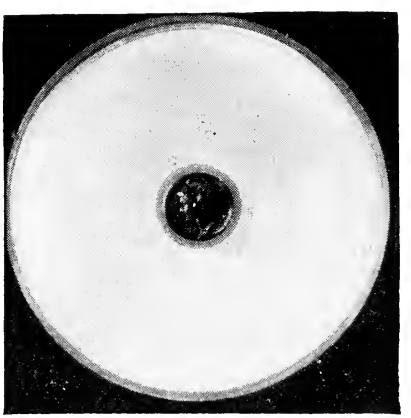


Fig. 34. Oligodynamic action of silver. Note the absence of growth in the area immediately surrounding the dime in a pour plate of *Escherichia coli.* (*By permission from* Introduction to the Bacteria *by C. E. Clifton. Copyright, 1950. McGraw-Hill Book Company, Inc.*)

compounds employed as disinfectants. Recent evidence, however, points to the fact that these salts act more as bacteriostats than as bactericides.

1. *Bichloride of Mercury* ($HgCl_2$) is commonly used in a concentration of 1:500 or 1:1000 as a disinfectant, with higher dilutions acting as bacteriostats. As effective as this compound appears to be, it has several disadvantages which limit its usefulness. It is highly corrosive; in fact, it is commonly referred to as corrosive sublimate. The compound is poisonous to man and to animals, and it does cause coagulation of proteins. This latter feature explains why, when someone swallows bichloride of mercury,

either accidentally or with suicidal intent, egg white is administered as an antidote. The mercury combines with the egg protein, and by inducing vomiting, much of the mercury can be expelled before it works its way into the body, if the antidote is given promptly.

By converting mercury into complex organic compounds, much of the toxicity, corrosiveness, and protein-coagulating properties are reduced. Such compounds as merthiolate, metaphen, mercurochrome, and phenyl mercuric nitrate are representative of these organic combinations, and they include some of our best skin disinfectants. Merthiolate in particular enjoys an excellent reputation as a pre-surgical applicant for the skin. Many home medicine chests and first aid kits, which formerly contained iodine, now have tincture of merthiolate as the disinfectant of choice for superficial wounds. Laboratory tests confirm the high killing power of merthiolate. An aqueous solution of mercurochrome has relatively little bactericidal power, but a tincture of this same compound has more value.

2. *Silver Salts* are rather effective in killing bacteria, due to the oligodynamic action of the silver resulting from the reduction of silver ions. Silver nitrate ($AgNO_3$) is a well-known silver compound, and it is found to be bactericidal in a concentration of 1:1000 and bacteriostatic up to 1:10,000. Argyrol and protargol contain colloidal silver.

Until Credé introduced silver nitrate as a prophylactic, gonorrhea of the eyes of young babies was one of the leading causes of blindness in children attending schools for such handicapped persons.

3. *Zinc Salts* are weak antiseptics, at best. Not all heavy metals have the same bacteria-killing capacity, and zinc salts are mentioned merely to note why they are used at all. Zinc sulphate is commonly employed as an eye wash because it does exert a soothing action on the delicate eye membranes. The chloride of zinc is a constituent of some dentifrices and mouth washes, but the germicidal claims sometimes attributed to the action of this compound do not stand up under close scrutiny.

4. *Copper Salts* are much better algicides than bactericides. Suggestions have been made that copper sulphate be added to water supplies to serve the double purpose of inhibiting the growth of algae and of killing bacteria. Since we know little about the cumulative effects of ingested copper and other metals in small amounts over a long period of time, it seems wise to adhere to the tested technics, especially chlorination, for treating public supplies of drinking water.

THE HALOGEN COMPOUNDS

The electro-negative substances, chlorine, bromine, fluorine, and iodine, are not found in a free state in nature; they are much too active and readily combine with many other substances.

1. *Chlorine.* Phenol is often considered by the layman to be a rather powerful disinfectant, probably because it has "that hospital smell." But free chlorine has a bactericidal power up to two hundred times that of phenol, because the halogen is a strong oxidizing agent and its ions are quite toxic for protoplasm. The firmness with which the chlorine is bound to other substances or elements determines how effective the chlorine is going to be in killing germs. Sodium chloride (table salt) is a tight combination of sodium and chlorine (NaCl), and no measurable germicidal effect of this compound of chlorine can be registered. Loosely-bound chlorine, such as that found in calcium hypochlorite, can be active in killing organisms. Upon prolonged storage, however, these loosely bound compounds lose their active principle, and a false sense of germicidal activity may result from their use. Chlorine can combine directly with protein in a process called *chlorination*, or it may act by *oxidation*. In either case, the living cell's normal protoplasmic balance is disrupted and the cell eventually dies. Liquid chlorine has largely replaced hypochlorite in treating water supplies, and this purified liquid chlorine adds no inert matter to the water supply. But for smaller operations, it is safer to handle the hypochlorite.

Calcium hypochlorite, also known by the names of chloride of lime, hypo, bleaching powder, or bleach, is widely used in sanita-

tion on a small scale, and the effectiveness of this compound is measured in terms of "available chlorine." Since the principal action depends upon oxygen, available chlorine is probably not the best way of expressing this oxidation reaction, according to some sanitarians. When you purchase Chlorox, B-K, HTH-15, and Diversol, you are buying hypochlorite compounds.

During World War I a popular substance for treating wounds was Dakin's solution (0.5% available chlorine), which was prepared from sodium carbonate, chlorinated lime, and boric acid. It was designed to control infections without harming the underlying healthy tissue, which is essential for optimum healing of the wound. When the sulfa drugs were added to our list of available compounds just before World War II, Dakin's solution was relegated to history.

2. *Bromine.* Not enough work has been done with bromine to make very specific statements about its possibilities as a disinfectant, but it has been shown to exhibit some disinfecting action. This substance needs further investigation.

3. *Fluorine* is the most active, chemically, of the four halogens, and this high activity practically eliminates it as a disinfectant. However, another application of this chemical in recent years has been the fluoridation of public water supplies as a means of reducing tooth decay. This topic will be discussed at more length under the heading of dentifrices later in this chapter.

4. *Iodine.* Compounds of iodine, as well as iodine alone, have been used extensively through the years as disinfectants. Of all the iodine compounds employed, however, standard tincture of iodine is one of the most commonly used in the treatment of wounds and for preparing the skin prior to surgery. A concentration of 0.2 parts of iodine per million is quite effective in the treatment of small quantities of water for drinking purposes, provided at least a thirty minute contact time is allowed for disinfection to occur. Iodoform (CHI_3) has antiseptic properties, and its slow decomposition in the presence of organic matter, with the resultant liberation of free iodine, probably accounts for its effectiveness.

Before the introduction of merthiolate and other organic mercurials, iodine as a tincture was without question one of the most extensively used skin disinfectants. While iodine is still employed by many hospitals, some of the newer compounds of mercury are ascending the ladder of popularity.

OXIDIZING AGENTS

One of the better-known oxidizing agents used for disinfection purposes is 3% hydrogen peroxide. The assuring sight of active bubbling and frothing observed when this compound is put on a cut is visual evidence to the layman that the process is effective. Too much faith, however, has been placed in hydrogen peroxide, particularly in deep, dirty wounds. Unless the compound is stored in a cold place and is tightly stoppered, it may lose some of its effectiveness. It does kill some bacteria, but its lack of penetrating power limits its effectiveness to topical application on superficial abrasions.

ACIDS AND ALKALIES

Many acids and alkalies are capable of destroying bacteria, but their use is confined to locations other than in or on the body of man and other animals because of their destructive action on tissues.

ALCOHOL

Many persons, especially physicians and nurses, have much greater faith in the germicidal power of alcohol than bacteriological tests indicate is warranted. That magic bottle of 70% ethyl alcohol is highly over-rated as a skin disinfectant prior to hypodermic injections. Soap and water are probably just as effective, or are better than alcohol, for mechanically removing skin organisms. The time factor does not allow sufficient contact between the alcohol and the bacteria to do much, if any, killing. The fact that alcohol has a low surface tension improves the cleansing properties of the agent on skin that is normally oily, but soap and water are still considered to be reliable substitutes for alcohol.

The reason people do not contract more infections following hypodermic injections after a "lick and a promise" with alcohol

can probably be attributed to the efficient defense mechanism that the body has at its command to ward off infections. How else can we explain the "relatively few infections" experienced by drug addicts whose practice of asepsis must be questioned? Since chemicals applied as disinfectants to the skin prior to hypodermic injections do not have an opportunity to act in the short contact period, some physicians prefer to use acetone as a cleansing agent in preference to either alcohol or soap and water.

At best, ethyl alcohol in a concentration of between 50 and 70% is a mild disinfectant, and its use should be confined to such technics as soaking thermometers which have been previously wiped with cotton to remove the visible organic matter. While a 30-minute contact period with 70% ethyl alcohol may disinfect thermometers, other more reliable compounds are available for this and similar procedures.

FUMIGANTS

A few decades ago the custom of fumigation of homes in which individuals had been suffering from communicable diseases was a common procedure, but today this practice is largely directed against insects and rodents which might be carriers of disease. Formaldehyde gas, chlorine, and sulfur dioxide were extensively used for terminal disinfection after a patient had recovered from a communicable disease. Strict attention to sanitary practices, coupled with adequate washing of bedding and extensive ventilation, are the usual modern treatments for the sickroom. Hydrocyanic acid gas is a deadly poison for insects as well as for man and lower animals. Military barracks are usually fumigated with this substance to rid the premises of roaches, bedbugs, and vermin. Because the fumes of hydrogen cyanide are so toxic to humans, extreme care must be exercised by the workers engaged in extermination operations. Special canisters must be used with the gas masks to protect workers from the lethal fumes.

Aerosol mists and vapors, whose active ingredients include pyrethrum, DDT, or one of the glycols, are popular for treatment of air in closed places. Propylene glycol and the superior triethylene glycol have come into wide use in an attempt to minimize the

spread of upper respiratory diseases via the air. It is surprising to note the many ideal characteristics displayed by these glycols which make them suited for disinfecting air. They have a low toxicity for man and other animals, are odorless, tasteless, reasonable in price, and are non-irritating.

Triethylene glycol has been found to be effective for use in isolation wards of hospitals and in military barracks where people are living in close proximity to one another. Some large industrial concerns have found that the incorporation of these vapors and mists in their air-conditioning systems has reduced the number of man-days lost as a result of common colds, particularly in large offices where large numbers of typists and clerks must work in the same room.

There is no conclusive evidence available that the continued exposure of humans to glycol vapors has any detectable deleterious effect on man. Prolonged exposure of selected laboratory animals to high concentrations of these vapors has resulted in no ill effects. Portable vaporizers may be purchased for use in small rooms. A special glycol-impregnated paper is mechanically drawn over the surface of heated rollers at controlled speeds, and the vapors may disinfect the air in a moderate-sized room in an hour or less.

CHEMICAL REACTIONS INVOLVED IN DISINFECTION

It is not the intent of this book to go into the elaborate chemical explanations involved in disinfection, but even with a limited background in chemistry, students should be able to comprehend the general nature of what probably occurs when an organism is subjected to chemical action. It should be borne in mind that a multiple reaction often occurs, and no single response can be attributed as the complete explanation for the death of microorganisms.

OXIDATION

If oxygen is liberated in the chemical reaction, destruction of organisms may take place. This is at least part of the explanation of the killing power of chlorine and of hydrogen peroxide, to mention but two common substances.

HYDROLYSIS

When water is added to a substance, followed by a splitting of the molecule, the reaction is called HYDROLYSIS. Concentrated acids and alkalies, and hot water exert some of their disinfecting power through this means.

FORMATION OF SALTS WITH PROTEINS

Certain heavy metals, including mercury and silver, act as good disinfectants when their salts come in contact with microorganisms. The salts combine directly with the protein of the organisms, throwing the protoplasm out of balance, and causing the eventual death of the organism. The halogens kill bacteria by a direct combination with the protein, although other chemical reactions also play a part in the disinfecting process.

COAGULATION OF CELLULAR PROTEIN

Treatment of organisms with disinfectants often results in the coagulation of the colloidal protoplasm. This severe physical and chemical shock kills microbes.

While other chemical responses may be offered to explain disinfection, the four reactions briefly touched upon above probably include the more important changes involved in this lethal process.

METHODS OF EVALUATING AND STANDARDIZING DISINFECTANTS

The general public is usually impressed by "scientific claims" in support of product "A" in preference to product "B." If this were not so, advertising would not base its success in selling campaigns on these laboratory findings. To one who has never had the advantage of an exposure to courses in science, the half-truths of some advertising have a strong appeal. There may be nothing wrong in the claims as far as they go, but an informed person should take time out to ask himself a few questions about what has not been said in the advertisements.

The importance of conducting uniform tests for evaluating

various materials cannot be over-emphasized. This is particularly important in the case of disinfectants. Qualitative and quantitative tests have been standardized to permit laboratories to duplicate results obtained by other testing laboratories when products are subjected to scientific scrutiny. The ramifications of the tests employed for disinfectants are too great to dwell upon, but the underlying principles of some of the accepted procedures might be of interest.

MARBLE CUP TECHNIC

One of the earlier methods for evaluating the effectiveness of chemical disinfectants was to inoculate rather heavily a tube of nutrient agar with a test organism such as *Micrococcus pyogenes* variety *aureus*. Since this organism is a frequent cause of localized skin infections, it is commonly employed in disinfectant testing.

After the seeded medium has been poured into a culture dish and before the agar has had an opportunity to solidify, a sterile marble is placed in the center of the agar plate and is left in position until the medium solidifies. By carefully removing this marble with a pair of sterile forceps, a cup-like depression is left in the medium. The test chemical, whether it be a liquid, a cream, or an ointment, is placed in this depression and the plate is incubated at body temperature. If the test material has any bactericidal or bacteriostatic properties, a zone of clearing will appear adjacent to the depression in the agar. The test organism should grow heavily in all parts of the plate except where the chemical has had an adverse affect on the organisms. In general, the wider the zone of clearing around the cup, the more effective is the chemical being tested. This might also be considered to be a semi-quantitative measurement of the penetrating power of the test chemical.

If one were interested in determining whether the clear zone represented killing (bactericidal effect) or mere inhibition (bacteriostatic effect) of the organisms, a small piece can be scooped out of the clear area of the agar and can be subcultured into a tube of nutrient broth medium. If the organisms have only been inhibited, the dilution effect of the broth on the test chemical in

the agar will permit the dormant cells to grow and produce a turbidity in the tube of broth. However, failure of the organisms to grow in the broth would indicate a bactericidal action of the test chemical on the specific microorganisms.

PENICYLINDERS

There are definite objections and limitations to the marble cup technic of measuring the effectiveness of disinfectants. The tendency of the agar to split when removing the marble is one of the more serious of these drawbacks. Cracks in the agar allow seepage of the test chemical and a false measurement of penetrating power may result.

An outgrowth of this marble method has been the development of what are termed penicylinders—cylinders of glass, porcelain, or metal about $^6/_{16}$ of an inch long with an inside diameter of ½ inch and open at both ends. By having one end beveled the penicylinders can be imbedded in the agar which has been previously seeded with the test organisms. A measured amount of the chemical is placed in the open end of the penicylinders that are left in the agar. As is true with the marble technic, zones of clearing adjacent to the penicylinders are measured after the plates have undergone prescribed incubation. This is a routine method employed for determining the potency of antibiotics.

FILTER PAPER DISC METHOD

Another technic for testing sensitivities of organisms to various disinfectants and antibiotics is the use of standardized circles of special filter paper which have been impregnated with the material to be tested. The surface of an agar plate is heavily seeded with specific organisms, and the paper discs are aseptically transferred to the agar surface. Zones of clearing are interpreted in the usual way.

Clinical laboratories employ such discs as an aid to physicians who are interested in knowing which antibiotic has the best chance of combatting a given infection. A half-dozen or more antibiotics may be tested on a series of plates, and with rapidly growing or-

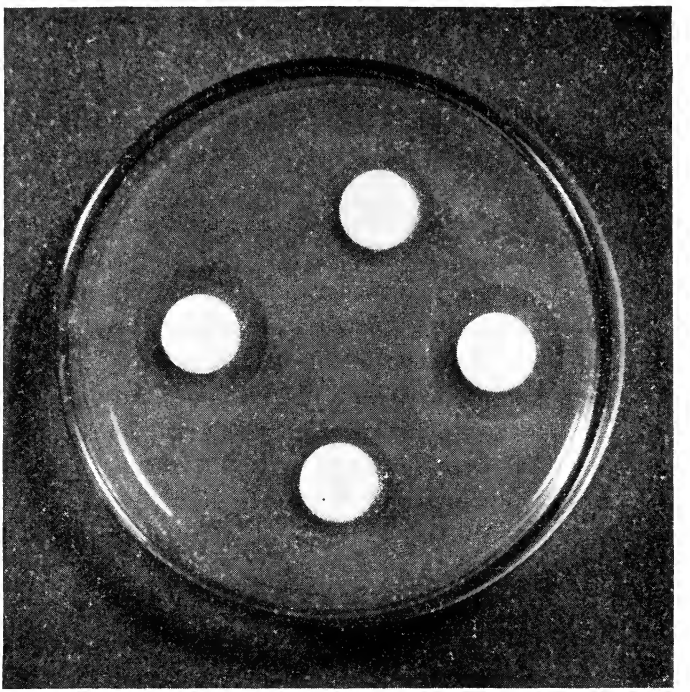


Fig. 35. Paper disc method for the determination of penicillin concentration in a solution. The size of the inhibition zone is related to the amount of penicillin in the paper discs. (*Courtesy of Eli Lilly and Company, Indianapolis, Indiana.*)

ganisms decisions can be arrived at within a normal working day. Impregnated discs may be purchased in graded strengths, and the relative merits of each antibiotic can be determined by comparing the width of the zones of clearing.

THE PHENOL COEFFICIENT TEST

An official method for evaluating the usefulness of disinfectants is that prescribed by the Food and Drug Administration (F.D.A. method). In brief, the procedure is a comparison of the relative effectiveness of liquid disinfectants with that of pure phenol under one set of highly standardized laboratory conditions. If the ulti-

mate use of the chemical is directed toward killing pathogens in excreta and wastes, the test organism is customarily the typhoid organism—*Salmonella typhosa*. On the other hand, if the chemical is designed for use on the skin and in wounds, the more appropriate skin organism, *Micrococcus pyogenes* var. *aureus*, is employed in the test.

The bactericidal strength of the chemical is expressed as a number. With phenol, the standard, being assigned the number 1.0, a chemical having a coefficient higher than 1.0 can be said to have greater killing power than phenol *under the standard conditions of this test*, while coefficients less than 1.0 indicate that the test material is less effective than phenol under these specific conditions. The phenol coefficient is derived by determining how high substance "X" can be diluted and still exhibit a lethal effect equal to phenol. For example, if a 1:500 dilution of "X" produces killing comparable to that of a 1:100 dilution of phenol, the coefficient would be 500 divided by 100, or 5.0.

There are many exact details which must be borne in mind when conducting a phenol coefficient test. Interested students are referred to advanced texts in the field for additional information.

SOAPS AND DETERGENTS

The importance of soap and water in sanitary practices for preventing the dissemination of pathogenic microorganisms cannot be over-emphasized. While soaps as a group possess little or no disinfecting power, they are capable of mechanically loosening and removing "dirt" and organisms trapped in this material. The term "sanitizing" has come into popular use in recent years, and the expression has considerable merit. Too often people have the false notion that dishes are sterilized when they are washed in hot soapy water. The temperature of the water must be well above that tolerated by human hands before disinfection can occur, to say nothing of sterilization. When dishes and equipment are sanitized, the visible organic matter is removed and pathogenic bacteria find conditions unfavorable for their multiplication or even their residence. It is unnecessary to sterilize eating utensils to

make them safe for human use, but public eating establishments should make certain that utensils are sanitized.

When surgeons and their assistants subject hands to the usual surgical scrub with soap and a stiff brush, they are attempting to reduce the microbial population of their skin by mechanical action. You cannot sterilize the skin with any known compound developed to date without destroying or severely injuring this tissue. Skin is composed of many layers of cells, and while the outer cells may be freed of organisms by a combination of mechanical and chemical procedures, the underlying cells may still harbor potentially dangerous bacteria, especially deep in the pore spaces.

So-called medicated soaps, while they have value, do not disinfect the hands with the ease the advertisements would lead you to believe. If such medicated soaps are used to the exclusion of other types of washing preparations, there is evidence that the thin film of soap and its active medication can reduce the bacterial flora to a small fraction of that normally found on the skin. Some surgeons have found that they can materially shorten the length of a surgical scrub if they use selected medicated soaps and no other cleansing preparations on their hands at home, in the office, and in the hospital. The usual minute or less that is devoted to a single washing of the hands by most persons is not going to drastically reduce the bacterial population, whether medicated or non-medicated soaps are employed. Only prolonged exposure to the active germicidal ingredient will effectively do the job that the "ads" would have you believe.

Wetting and cleaning agents, classified as synthetic detergents, have come into prominence in recent years, and the claims that these compounds can clean as well as disinfect in one operation are true only under designated conditions. These soapless-soaps are advocated for use in dishwashing and in clothes-washing machines because of their relative stability in both acid and alkaline solutions and because of their failure to form precipitates in hard water. The low surface tension of detergents gives them the advantage of being able to penetrate and to wet surfaces better than either water or ordinary soaps.

BACTERIOSTATIC AND BACTERICIDAL ACTION OF DYES

Many dyes employed in industry today contain aniline, a substance which can be treated in various ways to create stable pigments. Some of these dyes adversely affect microbial protoplasm, and they can be incorporated into selective media for retarding the growth of specific organisms. Often a given concentration of a specific dye will inhibit bacterial growth, while a higher concentration of that same dye may kill the organisms. There is a relationship between the gram staining reaction and the effect of the dyes. Medicine takes advantage of the action of gentian violet in the treatment of wounds, burns, and some skin infections.

DENTIFRICES

The published claims and the true merits of dentifrices are an interesting subject for extensive investigation, but here only the highlights of the topic are discussed.

If we knew with finality the cause of tooth decay, undoubtedly drastic changes in our way of living might be warranted. But since all available evidence points to a multiplicity of causes for this human affliction, man attempts to treat the condition from a number of standpoints. Lower animals do not experience the extensive tooth troubles affecting man. What do these lower animals do that is different?

Nutritionists have demonstrated that at least part of tooth decay can be attributed to diet. Perhaps poor nutrition is the primary cause of dental caries and other factors stem from this original weakness in tooth structure. Since calcium and phosphorus, among other elements, make up the chemical composition of our teeth, it is logical to assume that if these vital elements are lacking in our diet, their shortage will be reflected in poor tooth structure and in ultimate decay.

Millions, even billions, of viable bacteria can be found in a human mouth, and the varied species that can be isolated present an interesting field for study. We know that among other end products of bacterial metabolism, acids are formed, and the amount

of acid produced by some organisms is considerable. In fact, some investigators claim that the enamel of teeth can be visibly etched by the acids produced by bacteria lodged in the crevices between the teeth. Not one of the fancy-designed toothbrushes is able to remove effectively these entrenched organisms, advertising claims to the contrary. The use of dental floss is one means of dislodging these hidden microbial cultures. Since much decay of the teeth occurs in the tight, hidden crevices, there is some support for the claims that microbial end products are capable of attacking the relatively resistant enamel on our teeth.

Some years ago considerable research was directed toward studies of the lactobacilli, those high acid-producing bacteria found in varying numbers in the mouth. Claims were presented that persons harboring an abundant flora of lactobacilli appeared to have an abnormally high number of dental caries. The correlation between these two factors needs further clarification.

If acids in the mouth are the cause of the breakdown of tooth enamel, it appeared logical to some persons to pursue a practice recorded in ancient writings. The Chinese reported that some factor in urine appeared to reduce tooth decay. Ammoniated dentifrices have been an outgrowth of this revived information. By incorporating into dentifrices chemicals capable of neutralizing acids, tooth pastes and powders can not only aid in the mechanical removal of organic matter and bacteria, but the alkaline ions can conceivably help to neutralize acids and thus aid in reducing tooth decay. The lasting effect of one such treatment is rather short-lived, but it is probably fair to state that the ammoniated dentifrices may do *some* good.

The deodorizing power of chlorophyll—that green-pigmented constituent of higher plants—has been capitalized upon by manufacturers in recent years. As is true for so many new products put on the market, the claims for chlorophyll as an ingredient of toothpastes, soaps, dog biscuits, etc., have been rather sweeping. As some doubting wit has phrased it:

> Why reeks the goat on yonder hill
> While he feeds all day on chlorophyll?

Under controlled conditions, chlorophyll does exhibit deodorizing properties, but the substance has limitations. The public has been greatly impressed with the claims made for this material, and as long as advertising makes clever appeals the average person will continue to purchase these products.

While on the subject of dentifrices, it might be of interest to inject a note about how toothbrushes might serve as fomites in the transmission of upper respiratory infections, particularly the common cold. Many household bathrooms still have a porcelain container for holding the array of family toothbrushes. Even though each person may have his own brush, much of this sanitary care can be nullified when the wet brushes are allowed to rest on the rack surface where pools of water can collect and permit migration of organisms from one brush to another. Toothbrushes should hang with the bristle end down, with sufficient space between the brushes to prevent direct contact, and the location should allow rapid drying of the bristles. Qualitative as well as quantitative studies might well be undertaken on this phase of disease dissemination by fomites.

FLUORIDATION OF PUBLIC WATER SUPPLIES

A number of years ago it was observed that persons who drank water containing fluorides appeared to have harder teeth and experienced less tooth decay than is normally found in the average population. Certain sections of the United States, parts of Colorado in particular, have water supplies in which the fluoride content is above the optimum for the prevention of dental caries, and mottling (spotting) of the teeth is common in these areas. Research has revealed that the ideal concentration of this chemical appears to lie in the range of one part of fluorine per million parts of water if mottling is to be prevented and if sound teeth are to be expected.

Since the natural fluoride content of most drinking water is too minute to be of much value from the standpoint of dental health, the practice of adding fluorine in the form of its salts to municipal

water supplies has been gaining favor in recent years. Such treatment is known as *fluoridation.*

A small, but active, minority of opponents to this procedure have made fluoridation a lively topic in state legislatures, and the uphill battle on the part of public health authorities is a repeat performance of the struggle experienced in instituting pasteurization of milk and chlorination of public water supplies. Meddling with nature, according to the opponents, is a dangerous practice. Enough research has been conducted, however, to indicate the strong desirability of fluoridation, and in time the practice will undoubtedly be just as common as chlorination. Published claims indicate that up to 60% reduction in tooth decay can be accomplished with controlled fluoridation of water supplies.

In localities where the practice has not been introduced, young children can avail themselves of topical application (by their dentists or dental hygienists) of a 2% solution of sodium fluoride. The procedure involves painting the teeth with this solution for four successive weeks at the ages of three, seven, and ten. There are indications that pronounced reduction in dental caries results from such applications of the chemical. Just what the mechanism is that makes topical application effective is still not clearly understood. Some workers in the field have suggested that a direct combination occurs with the tooth enamel. Others feel that a prolonged bactericidal effect may account for the success of the practice. Whatever the mechanism, decided reduction in tooth decay is reported from widely scattered areas throughout the United States.

There appears to be a relationship between consumption of refined sugar and tooth decay. The bacteria in the mouth are able to ferment these sugars and destruction of tooth enamel is a direct result, according to some oral hygienists. Thorough brushing of the teeth immediately after each meal appears to reduce the opportunity for bacteria to produce destructive acids from food residues, especially in the hidden crevices of the teeth. The use of dental floss is an important phase of oral hygiene, particularly

for individuals whose teeth are very close together and are difficult to keep clean with a toothbrush or with the normal flushing action of saliva.

If this discussion has done nothing more, it should have raised questions in the mind of the reader relative to the effectiveness of some of the chemical compounds sold on the market. It has not been the intent to discredit all of the claims made for these products, but rather to stimulate thinking individuals to discriminate between products. The reader should learn to weigh the claims of the manufacturer against possible things that have been left unsaid. What is stated on the labels may be true up to a certain point, but half-truths can be misleading.

CHAPTER 11

Polluted Water Can Kill You

An examination of maps will disclose that most large cities are located where they are because an abundant water supply, among other things, was available. When sanitary engineers are confronted with the problem of determining how large a reservoir should be to accommodate the projected growth of a community, the figure of one hundred gallons of water per capita is a usual consideration. This does not mean that each individual needs this volume of

water for his personal drinking, bathing, and cooking needs, but industries which grow up with a community draw heavily on water supplies. To provide the benefits of these services, the one-hundred-gallon figure is useful in the calculation.

DEFINITIONS

Whenever sudden, widespread disease breaks out, the water supply is one of the first suspicions by the public. With the careful attention devoted to providing a clean, safe, public water supply, such fears are usually unfounded. This has not always been so, however, and many serious epidemics have been traced directly to polluted water. But before pursuing this topic further, a few definitions of accepted terms seems warranted.

1. Pure Water. To a chemist, pure water means a liquid with a combination of two parts of hydrogen and one part of oxygen, but to a microbiologist it is understood to be water which contains no disease-producing bacteria or chemicals harmful to man or animals. *Safe water* is understood to be pure water, since a laboratory examination confirms its potability.

2. Impure Water. This is considered to be the reverse of our definition for pure water. Impure water contains bacteria or chemicals known to be harmful to man or animals.

3. Polluted Water. People formerly called this contaminated water, and it indicates that sewage has found its way into the supply. Since human or animal wastes may contain pathogenic organisms, polluted water is *potentially* dangerous.

4. Dangerous Water. When one wishes to specify that water contains known disease-producing microbes, it is necessary to be more specific than merely to say that the water is polluted or contaminated. Polluted water contains sewage which may or may not cause disease, and contaminated water may have a high bacterial count due to saprophytic organisms. The term "infected" water is sometimes used, but this is probably a misuse of the word. Tissues may become infected but water, strictly speaking, may not. Perhaps "dangerous" is a better designation for water that is potentially harmful because it contains known pathogenic organisms.

Water-borne diseases include typhoid, paratyphoid, bacillary dysentery, amoebic dysentery, cholera, and hemorrhagic jaundice, but the potential pathogens found in water vary with the geographic location.

SOURCES OF WATER

As moisture particles condense into droplets in the clouds, sheer weight will eventually compel the water to fall toward the ground. On the way to the earth's surface the rain picks up dirt and microorganisms suspended in the atmosphere. Toward the end of a prolonged shower, however, the air may be free of microorganisms, and this moisture reaches the earth devoid of living things. As soon as the water strikes the ground, all kinds of microbes are picked up and carried along in the water. Since open reservoirs are exposed to contamination carried through the air and through ground washings, very few natural bodies of water can be relied upon to yield a supply safe for human consumption. The magnitude of the problem increases with the population to be served.

DUG WELLS

Securing water in rural areas involves the construction of wells of one type or another, with a simple dug well being the most common. The depth of such wells varies with the locality, some shallow ones being hardly more than ten or fifteen feet in depth. Unless a person is aware of some of the pitfalls of shallow wells, such a water supply can be the cause of serious family illnesses. The surface organisms that might find their way into the water supply are legion in both number and types. Nevertheless, a carefully constructed well with proper walls and tight seals around the top to prevent the entrance of soil-surface washings, can serve as a reliable supply of safe water for a limited number of individuals. During prolonged periods of drought, however, the water table may recede and shallow wells are subject to running dry. It pays to dig deeper than one originally figures might normally be required in order to provide a reserve water supply.

After arriving at the maximum anticipated needs, a well can be dug wide enough and deep enough to provide the desired

volume of water. The following table will be found helpful in these calculations.

DIAMETER OF THE WELL IN FEET	APPROXIMATE NUMBER OF GALLONS FOR EACH FOOT OF WATER
3	50
4	95
5	150
6	200
7	290
8	375

In locating wells it is important to bear in mind that as much distance as possible should be allowed between the sewage outlet and the location of the well. While each installation is a separate problem, a minimum distance of fifty feet is generally considered sufficient to allow proper filtration of sewage through the soil to eliminate pollution of water supplies. This may not be sufficient, however, if the drainage slopes steeply toward the well, or if the type of soil does not allow efficient filtration. If possible, the water supply should be located on ground higher than the sewage outlet with surface drainage directed away from the well. Unless the top of the well is properly constructed, surface washings from barnyards, etc., may gain access to the water supply, particularly during heavy rainstorms. The extra time and expense devoted to the details of construction will more than pay for themselves over a period of years.

DRIVEN WELLS

By drilling deep into the earth, often through a layer impervious to water, a more abundant and usually safe water supply can be expected. Just because water comes from a driven well, however, is not reason enough to insure its potability without subjecting it to bacteriological examination. In general the water from such wells has come from a distance, and has undergone natural filtration as it seeped through the soil. But if, through improper construction, surface water is allowed to gain entrance to the supply, pollution problems might well arise.

The depth of driven wells varies with the location, but in some areas they average between fifty and one hundred feet. Many

persons who are faced with seasonal water shortages find it expedient, even if expensive, to drill a well and insure themselves of an abundant year-round supply of potable water.

SPRINGS

There are few sights more refreshing to a hot, tired person than cool water flowing from a spring in a shady glen on a warm summer day, but opinions vary as to the safety of such water for human consumption. Spring water is an underground supply that finds its way to the surface of the ground. Theoretically, the filtration this water has undergone should make it potable, but unless the sanitary conditions around the outlet of a spring are known, pollution is always a possibility. Surface pollution from man or animal wastes must never be minimized. Should there be any doubt about the potability, there are simple ways of treating water to make it safe for drinking. A few of these technics will be discussed later in this chapter.

CISTERNS

In some sections of the United States a cistern is an important means of providing soft water for residents of the community. Where rainfall is limited, or when chemical elements in the soil tend to make water "hard," it is customary to gather rainwater from the roofs of the houses and to collect it in storage tanks after a preliminary filtration to remove visible dirt and at least part of the trapped organisms.

RESERVOIRS

Cities and municipalities are faced with the challenge of supplying an adequate water supply to the inhabitants at a reasonable price, and the reservoir is a common means of storage. The water is collected in open areas, usually behind a dam in a process called *impounding*. As the name reservoir indicates, the collecting area provides a reserve supply to be drawn upon as needed.

Any attempt to cover these huge areas to prevent outside contamination is impractical. While it is true that upon standing, water tends to improve itself from bacteriological standpoints, some

form of treatment—filtration or chlorination—is usually necessary to insure the public a safe product.

LAKES

Naturally occurring lakes provide water to some communities and eliminate the necessity for constructing reservoirs. Since lakes are also subject to pollution, the water must be treated before it can be consumed with safety.

RIVERS AND STREAMS

Many large cities are located beside rivers, both large and small, and such bodies of water may serve at least a two-fold function. The upstream end may be used as a source of water for the city, and sewage may be pumped back into the river at a downstream point. Major problems relative to river and stream pollution have arisen through the years and represent some of the knottier considerations facing public health authorities. Overloading streams with sewage ruins the fish supply and has already wiped out fishing areas that man used to rely upon for food. Like reservoir and lake water, the supply pumped from rivers must undergo some form of treatment before it is safe for human use.

There is a popular notion that running water purifies itself, but in the light of newer knowledge this statement is not strictly correct. Running water improves itself, but to say that the water becomes purified is stretching the truth unless qualifications are stated. The rate of flow, the degree of pollution, the temperature, and other factors must all be considered before it is possible to state how far a body of water must travel before it can be considered free of pathogenic organisms. We know that disease-producing bacteria come out second best in competition with saprophytic organisms in polluted water, but the time factor cannot be stated without reservations.

Sanitarians have made great strides in the last fifty years with respect to improvement of water and milk supplies. Our morbidity (sick rate) and mortality statistics reflect this change especially in

enteric diseases. The typhoid, paratyphoid, and dysentery rates have dropped off sharply since measures have been instituted to improve water supplies on both small and large scales. Widespread water-borne outbreaks in the United States can be a thing of the past as long as constant vigilance is the watchword. While typhoid vaccination may attenuate the symptoms of the disease, it is a false notion that a person vaccinated against typhoid is completely protected from the disease. Vaccination is designed to protect the individual from a chance contact with a few of these organisms. The ingestion of large numbers of *Salmonella typhosa* may still cause typhoid fever.

TREATMENT OF DRINKING WATER

The treatment of water to make it potable involves the physical removal of enteric pathogens or their chemical destruction. The use of disinfectants goes back to at least 1897 when Jewell added bleaching powder to the water supply of Adrian, Michigan. Since this date the treatment of public water supplies to make them safe has become widespread. Chlorine gas, liquid chlorine, and various chlorine compounds have been employed since Jewell's early work in the field, and sharp declines in the typhoid rate were recorded upon the introduction of this practice. Rates of twenty deaths or more per hundred thousand population have dropped to less than one death per hundred thousand at the present time.

The concentration required to satisfactorily disinfect water is usually not over one part of chlorine per million parts of water (abbreviated P.P.M.), with a residual of one tenth to three tenths P.P.M. at distant points throughout the distribution system. Properly chlorinated water ordinarily cannot be detected organoleptically (by taste or smell) by the average consumer. It should be mentioned that occasional gastro-intestinal outbreaks of non-bacterial origin might arise from filtered and chlorinated water. These outbreaks are probably caused by viruses which are unharmed by the physical and chemical treatment. Amoebic dysentery might conceivably be disseminated through chlorinated water,

but only if such water has not been filtered. The amoebae cannot pass a sand filter, but they can withstand the usual concentrations of chlorine employed in water purification.

SMALL VOLUMES OF WATER

Campers, unless they carry their own drinking water, are faced with the problem of finding water near the camp site and treating this water to make it fit for consumption. Other methods being unavailable, water can be vigorously boiled for ten minutes and the usual enteric pathogens will be inactivated. The higher the altitude, the lower will be the boiling point of water in an open container. But even on the highest peak in the United States, the boiling point will be sufficiently high to insure killing undesirable organisms within the prescribed ten minute period.

During World War I the Army Medical Corps advocated the use of two drops of tincture of iodine for each quart of water to make it safe for human consumption. A thirty-minute waiting period, however, is required for insuring this disinfection. The use of tablets containing available chlorine was a recommended practice in the army during the second World War. Two such tablets for each canteen (about one quart) of water will disinfect this volume in thirty minutes, but if the water is very muddy, four tablets are required to allow for the affinity the chlorine has for organic matter.

WELLS IN RURAL AREAS

Water from newly constructed wells will normally have a very high bacterial count which will not "settle down" for a matter of weeks or even months unless the supply is treated to reduce the bacterial population that found its way into the water during construction operations. A common treatment is to dissolve the contents of a freshly opened one pound can of chloride of lime in two quarts of water. After the insoluble lime has settled, introduce into the well two ounces of the clear supernatant solution for every estimated one hundred gallons of water. This concentration of chlorine is high enough to be detected by smell. Allow the

chemical to remain in the well overnight, and then pump out the water until the odor of chlorine disappears. A bacteriological examination will usually show that such treated water is now ready to be used for drinking purposes.

Persons engaged in testing small water supplies are frequently confronted with requests for information as to what should be done when small animals are found in the well. Had the supply been properly covered, such animals as rabbits, skunks, cats, mice, etc., would not be able to get into the well. The presence of these animals is often not detected until advanced decomposition has set in and strong tastes and odors are noticed in the water. The usual advice for such conditions is to remove the animal, or its remains, and to chlorinate the water as described above. Such chemical treatment will leave the water with a good taste and the bulk of the bacteria generated during the decomposition are usually eliminated.

RESERVOIRS AND OTHER LARGE BODIES OF WATER

Large bodies of water contain appreciable quantities of organic debris, and upon standing for long periods of time, much of the undesirable material will settle out. Microorganisms are carried down with these particles, and bacterial numbers are then materially reduced through natural antagonisms. To speed up this process, various chemicals can be added to the water as it is stored in settling tanks. These chemicals (including iron and aluminum sulfates) form a flaky precipitate and carry a great deal of suspended matter with them to the bottom of the tanks.

While a high percentage of organisms may be removed from water in these chambers, it has been found desirable to follow sedimentation with filtration through sand. Filtration dates to 1829 when the river water of London was passed through sand to remove undesirable matter. Even though the germ theory of disease had not yet been established, records reveal that filtration appeared to reduce the incidence of intestinal upsets among those who drank the treated water.

One of the classics recorded in microbiology is the famous case

in Germany of the cholera epidemic of 1892. Hamburg, using the raw water from the Elbe River, reported hundreds of new cases of cholera each day, while Altona, adjacent to Hamburg, drew its water from the same source but subjected it to filtration prior to use. Altona reported few or no cases of cholera each day.

SLOW SAND FILTER

Small communities may resort to slow sand filtration in purifying their water supply. These filters may be several acres in size.

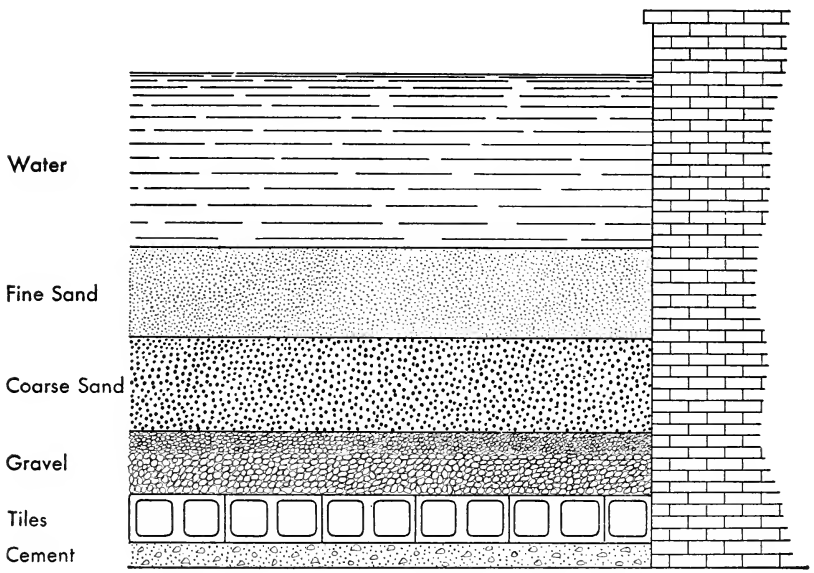


Fig. 36. Cross section of a sand filter. (*C. E. Turner, Elizabeth McHose,* Effective Living, *Copyright 1941, 1945, 1950 by Prentice-Hall, Inc., New York, p. 346. Reprinted by permission of the publisher.*)

After laying a concrete base, perforated drainage tile is placed on the floor and covered with coarse gravel, on top of which is added fine gravel. Finally, from two to four feet of graded sand is placed on top of this. Without previous treatment, the water is allowed to flow through the filter bed by natural gravity flow. The sand becomes covered with a colloidal flocculent mass called a *Schmutzdecke* which aids in the filtration process. As the layer

of debris becomes thicker, filtration slows down to the point where the filter must be cleaned. A slow sand filter can be expected to treat between three and six million gallons of water per acre of filter surface per day. The combination of mechanical trapping of the organisms, electrical charges, and the natural eating habits of protozoa and other organisms accounts for the removal of up to 98% of the bacteria in the water. All pathogens are generally removed or die during this operation.

RAPID SAND FILTER

Until the colloidal layer becomes well established on a slow sand filter, the operation is not too efficient. And when the filter

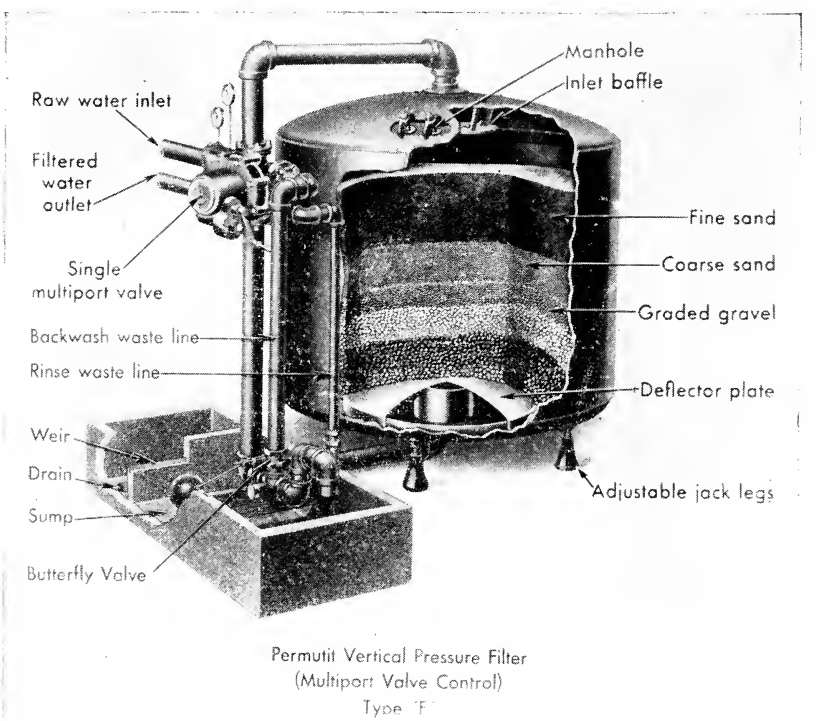


Fig. 37. A vertical pressure filter. Water is forced under pressure through successive layers of fine sand, coarse sand, and graded gravel. (*Courtesy of the Permutit Company, New York, New York.*)

become covered with a thick zoogloeal material, the filtration speed is reduced to an uneconomical level. By treating water with a coagulating chemical first, it is possible to establish an effective *Schmutzdecke* more quickly than is true of slow sand filtration. But clogging of the filters also takes place more rapidly. To compensate for this, water flow is reversed through the filter bed with the aid of mechanical devices. Such rapid filters can treat up to one hundred and twenty-five million gallons of water per acre of filter surface per day.

Rapid sand filters are almost as effective bacteriologically as slow sand filters and they occupy a good deal less space, but because of the added mechanical devices, rapid filters are more expensive to operate than are slow sand filters. No matter which system is employed, however, chlorination is a wise practice as an added precaution.

As one looks over the progress that has resulted from the combined efforts of engineers, chemists, and biologists in providing an abundant, safe water supply to our centers of dense population, it is truly a remarkable achievement in the history of preventive medicine.

SWIMMING POOLS

Persons engaged in performing clinical bacteriological analyses can usually tell when late spring or early summer has arrived on the basis of the number of cultures submitted from persons suffering from ear infections. The call of the "ole swimmin' hole" prompts individuals to bathe in ponds which may at times be grossly polluted. Rats commonly live on the banks of such bodies of water, and they may spread pathogenic organisms. According to careful surveys, between 50 and 90% of wild rats harbor *Leptospira,* which may cause fatal jaundice, and these bacteria are excreted in the urine of rats. Weil's disease, which has a fatality rate of about 25%, is caused by *Leptospira icterohemorrhagiae.*

Sinus trouble for some persons may be traced to swimming holes that were grossly polluted, and fungus infections are frequently suspected of having a similar origin.

Artificial pools constructed indoors and outdoors can be the

source of many unnecessary infections unless strict attention is directed to cleanliness, both of the individual bathers and of the pool itself. Chlorination under controlled supervision is an important step in maintaining sanitary conditions for the swimmers, and a residual concentration of at least six-tenths of a part of chlorine per million parts of water should be maintained. Eye irritation may be caused by stronger chlorine residuals.

Persons suffering from open skin infections should not be permitted to swim in these pools, and special medicinal foot baths between the pool and the locker rooms can help to minimize such skin diseases as "athlete's foot."

ICE AS A POTENTIAL AGENCY IN DISEASE DISSEMINATION

Until a relatively few years ago ice could be harvested from any pond, pool, lake, or reservoir, and it could be sold on the market irrespective of the quality of the water before it was frozen.

Before the widespread use of refrigerators many a city-bred child looked forward to the arrival in the neighborhood of the ice man so he could "appropriate" a sliver of ice when the man was busy making a delivery. In looking back upon that era, public health authorities might well wonder if any cases of typhoid fever might have originated from the eating of "infected" ice.

This so-called natural ice is customarily harvested during the latter part of winter and is stored in ice-houses until the summer demand moves the cakes from storage. Authorities differ as to the relative danger involved in eating ice made from polluted water. The one group points out that as ice forms on the surface of open bodies of water, the crystals tend to come down in a pure state and impurities are excluded. It is interesting to note that these same persons frequently summarize their opinions by stating that, if possible, ice should not be harvested from bodies of water known to be polluted. Jordan remarked that ice from highly polluted water could not be expected to contain many, if any, disease-producing organisms after three or four months of storage. After six months of storage ice can be considered to be safe, and this

period is commonly accepted by health authorities who have control of the sale of ice harvested from open bodies of water.

Artificial, or manufactured, ice presents a little different aspect to the problem. Since the entire volume of water in the container is to be frozen, the bacteria cannot escape the way they might on an open body of water where they can be squeezed out, so to speak, as they are pushed downward by the forming ice crystals. For this reason it is of the utmost importance that all water used in manufacturing artificial ice be of the highest purity, and the containers in which freezing is to take place must be scrupulously clean to avoid the spread of disease. It goes almost without saying that the persons employed in such ice plants should be known to be typhoid-free, and they should be made to understand and to practice the accepted rules of sanitation. Hilliard found relatively high bacterial counts in artificial ice, traceable to a cloth filter used by the manufacturer to strain out coarse dirt and sediment from the water prior to freezing it.

It should be emphasized, however, that there is little, if any, authenticated proof that ice has been instrumental in the dissemination of disease. The topic is discussed merely to point out that ice may be a potential source of certain pathogenic bacteria, and more work needs to be done to prove the point one way or another.

COMMON DRINKING CUPS AND PUBLIC DRINKING FOUNTAINS

It was not until a few years ago that the common drinking cup was eliminated from the faucets on the public square of many towns. The very thought of the types of diseases one might contract from these fomites makes the hair stand up on the back of the neck of anyone who understands bacteriology. It is impossible to calculate the number of persons who might have contracted such diseases as septic sore throat, diphtheria, tuberculosis, and possibly syphilis from the common drinking cup.

Due to the perseverance of public health authorities, the common drinking cup is a thing of the past except in "uninformed" communities. Public drinking fountains have replaced these cups,

and in general these fountains are of sanitary design. Some unsanitary bubblers are still being used, however, and they should be replaced. Proper construction allows the water to leave the spout at an angle, and no water that touches an individual's lips has an opportunity to fall back on the spout. Too many fountains have fancy metal guards that are very artistic, but they defeat the purpose of a so-called sanitary fountain. When the stream of water is so reduced that persons must come in intimate contact with the guard to get close enough to obtain some of the water coming out of the faucet, the guard has lost its usefulness and has reverted to a health hazard.

BOTTLED WATER

Another popular notion exists that all bottled waters are bacteriologically pure, but unfortunately this is not the case. We have made progress in the control of bottled waters, but careful laboratory examination has found some of them to be below the accepted bacteriological standards. Control by public health authorities has brought about improvement in this regard, but vigilance must never be relaxed.

STANDARD BACTERIOLOGICAL TESTING OF WATER

Before too much reliance is placed upon the results of a single bacteriological analysis of a water sample, the bacteriologist should be made aware of all conditions in the area from which the sample originated, especially as these conditions bear on present or future pollution possibilities. This *sanitary survey* aids in the interpretation of the results of the test and will influence the final recommendations of the analyst making out the laboratory report. Such considerations as the slope of the land, the distance from the nearest sewage outlet, the precautions taken to reduce and eliminate the entrance of surface washings, etc., must be carefully interpreted.

It is revealing to many students embarking on their first course in bacteriology to discover that when water is subjected to laboratory analysis, no attempt is made to find or to isolate pathogenic bacteria. If typhoid, paratyphoid, and dysentery are the

common diseases we are trying to avoid, why don't we look for these specific organisms when we analyze water bacteriologically? The answer is that these pathogenic bacteria are relatively difficult to isolate from water, even from badly polluted water. The procedure available for the cultivation and identification of these species are too involved and time-consuming to be very practical. If a quart of water harbored one typhoid organism, it would be extremely difficult to isolate that one cell. Yet, that apparently low concentration of organisms in a water supply might well be the cause of a serious typhoid outbreak.

However, by using the index organism, *Escherichia coli*, we can determine with a relatively high degree of accuracy whether a water supply is polluted. *Escherichia coli* is found in extremely high numbers in the intestinal wastes (feces) of warm-blooded animals, and apparently in few other places. If we can detect this organism in water, feces can be said to have found their way into the supply, and such water is *potentially dangerous.* Since a small percentage of humans who have had typhoid fever remain for months or years as healthy carriers of these organisms which they excrete intermittently, there is always the potential possibility that the *Escherichia coli* found in the water came from the intestines of such an infected individual. Not all polluted water contains dangerous pathogens, but in order to be ultra-safe, sanitarians prefer to condemn all polluted water to protect the public from possible infection from sewage originating from carriers. It is a much wiser policy to condemn more water than necessary than to allow one sample to be called safe when it may not be free from pathogenic bacteria.

Persons interested in the finer details of bacteriological water testing are referred to *Standard Methods for the Examination of Water and Sewage* prepared through the joint efforts of the American Public Health Association and the American Water Works Association. It is not the intent of this chapter to repeat all of the laboratory procedures, but general comments on the underlying principles will be discussed.

The physical appearance of water is not a reliable index of the

safety of that water for human consumption. Water may be undesirable because it looks "dirty," but the water is unsafe only when it has been proven to be so by scientific analysis. The chance that water will be non-potable from a purely chemical point of view is rare, and for this reason chemical analyses are not routinely carried out unless the individual is interested in determining what harmful effects the water might have on boilers, pipes, and manufacturing equipment. Certain chemical tests, chiefly those concerned with nitrogen in the combined form, will give useful information to sanitarians who are interested in determining how recently a supply of water has been polluted. As the sewage undergoes decomposition, the bacteria convert the nitrogenous compounds into ammonia, nitrites, and nitrates. The stage of decomposition yields valuable information to a trained interpreter.

From the standpoint of human health the most important test conducted on water is the bacteriological analysis. While we are interested in knowing the numbers of living organisms present in water to be used for drinking, we are more interested in the source of these bacteria.

The standard technic calls for the determination of the presence of pollution, and if sewage organisms are found, the water is condemned. One of the easiest ways to detect sewage is to look for *Escherichia coli* (the genus is named in honor of Escherich, the discoverer, and the species denotes the origin of the bacteria—the colon). This organism is found in the excreta of man and other warm-blooded animals. Hence, if *Escherichia coli* is present, there exists the possibility that enteric pathogens might also be expected.

Patients suffering from enteric diseases, together with healthy carriers of these pathogens, represent potential sources of the pollution. Some persons feel that unless water is grossly polluted, the chance of infecting humans is remote. While it may be true that much polluted water could be consumed with no harmful effects, would you knowingly have a member of your family drink potentially-harmful water? Statistically, the number of persons who might contract typhoid or some other enteric disease from slightly polluted water may not be significant. But if that number

in the statistical table happens to represent you, or someone near and dear to you, the figure has tremendous significance. A single case of disease caused by poor water is one case too many.

Escherichia coli is a short, gram negative, non spore-forming rod that ferments lactose with both acid and gas production. This is a unique set of reactions reserved for a limited number of organisms, and its very uniqueness provides a simple screening technic for determining the presence of sewage. Bacteriological water analysis involves three major steps.

THE PRESUMPTIVE TEST

When tubes of lactose broth are inoculated with water and the tubes are incubated at body temperature, the production of acid and gas through the breakdown of lactose leads the analyst to

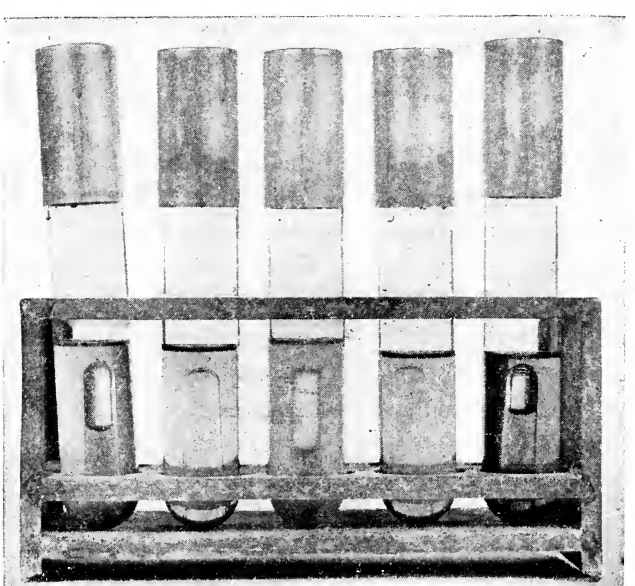


Fig. 38. Presumptive test for the coliform group of organisms in water. The fermentation tubes of lactose broth show gas production in three out of five tubes. The aluminum caps shown here are preferred to cotton plugs by some workers. (*From* General Bacteriology, *D. B. Swingle and W. G. Walter, 2nd ed., Copyright 1947, D. Van Nostrand Company, Inc.*)

presume that *coliform* bacteria are present in the water. The term *coliform* refers to all species of the genera *Escherichia* and *Aerobacter*. The *Aerobacter* is generally of soil or plant origin, but as high as 10% of fecal samples may be found to harbor these organisms. *Escherichia coli* is considered to be primarily of fecal origin. The interaction of two or more species of bacteria, however, may yield acid and gas from lactose due to synergism, discussed in an earlier chapter. For this reason, it is important that positive presumptive tests be carried at least one step further before the water can be said to contain sewage.

THE CONFIRMED TEST

By employing selective media containing substances bacteriostatic for gram positive bacteria, the gram negative species present in the presumptive lactose broth tubes will have a better opportunity to develop when they are streaked on the selective agar media. Endo agar, eosine methylene blue agar, and others may be used to confirm the presence of coliforms as the cause of the positive presumptive test. Typical coliforms appear as colored colonies with or without a metallic-like lustre on the surface when examined in reflected light. A newer medium, Tergitol 7, appears to be better than either Endo or eosine methylene blue agar as a confirmatory medium, and after more research has been conducted, Tergitol 7 may be preferred by more laboratories.

COMPLETED TEST

Having isolated suspicious-looking colonies on the selective medium, the next step is to select a typical colony and inoculate a fresh tube of lactose broth and a standard nutrient agar slant with growth from such a colony. If this pure culture ferments the sugar with acid and gas production, and if a gram stain of the agar slant growth reveals short, gram negative rods, it has been confirmed that the water contains coliforms.

Should the analyst desire to determine the species of the coliform with which he is dealing, a series of additional tests may be run to pinpoint the organism as to genus and species. These tests were discussed in Chapter 6.

CHAPTER 12

Biological Sewage Disposal

GENERAL CONSIDERATIONS

As important as the problem is of obtaining an adequate safe supply of water for a community, a greater challenge, at least in some areas, presents itself when sanitary engineers are faced with the disposal of used water, which becomes sewage. We can define sewage in a number of ways, but the term is usually accepted to mean the used water supply of a household or of a community. It is a complicated mixture, both biologically and chemically. If large volumes of chemical industrial wastes are involved, fewer bacteria and more chemicals would be present than if the sewage were composed principally of household wastes. But

such wastes as those derived from a packing house, for example, might add nutrients which would encourage microbial multiplication in sewage.

When human excreta contain enteric pathogens, these organisms survive for varying periods of time, depending upon the competition with saprophytic organisms. Enteric pathogens usually come out second best when saprophytic competition is keen.

The problem of safe disposal of potentially harmful organisms commonly revolves around dumping the sewage into large bodies of water or processing the material, usually biologically, to make it odorless and harmless. Sanitary engineers have determined that sewage can usually be adequately handled without creating a public nuisance if one part of sewage is diluted in not less than fifty parts of water, but some form of pre-treatment is required by certain state and federal laws, before sewage can be dumped into such bodies of water. This ratio usually allows proper biological decomposition without overloading the water.

The term *sewerage* is applied to the pipes, mains, tanks, etc., which comprise a disposal system. The designation does not include the liquid wastes themselves. Sewage may contain matter in solution, substances in colloidal suspension, and large and small gross particles which must undergo decomposition to make them soluble.

TYPES OF SEWAGE DISPOSAL SYSTEMS

It is estimated that more than half of the people in the United States must dispose of their own sewage since no central system is available to them. The procedures employed for the disposal of human wastes in rural areas include sanitary pit privies, cesspools, or septic tanks. The latter two are generally used where running water and flush toilets are involved.

SANITARY PIT PRIVIES

The "outhouse," so common in rural areas, has served, and is serving, a useful function in the disposal of human wastes. If properly constructed to prevent a fly problem, these pit privies

are meeting the challenge of waste disposal quite satisfactorily. It is important that such privies be provided with self-closing lids to minimize the entrance and the multiplication of flies and other insects capable of disseminating enteric pathogens. The filthy habits of flies, which lay their eggs in human and other animal wastes and then fly to places where food is being prepared for human consumption, create a potential health hazard. More will be said in a later chapter relative to insects and their possible role in the transmission of disease. Suffice it to say at this point that flies should be denied access to human wastes, which might contain enteric pathogens.

When sanitary privies fill up to within a few feet of the soil surface, the wastes should be properly covered with dirt after first pouring oil on the material to minimize opportunities for insects to breed in the excrement. As was mentioned in the previous chapter on water bacteriology, sewage outlets should never be placed nearer than fifty feet from water supplies, and drainage of sewage should be in the opposite direction from that of water sources. If the soil conditions do not favor adequate decomposition of sewage, fifty feet may not be sufficient distance between the sewage outlet and the water supply. Each case is an individual problem.

CESSPOOLS

Disposal of sewage on a small scale, such as from a private home, is oftentimes accomplished by means of a cesspool, which can be described as a hole in the ground with walls constructed of stone or bricks piled up without the use of mortar. Such construction allows seepage of sewage into the surrounding soil. Solids drop to the bottom of the cesspool where they undergo decomposition, principally anaerobic, and pathogens are eventually destroyed through the antagonistic action of saprophytes. Unless the ground around the cesspool is rather porous to permit proper drainage, however, cesspools may fill up and overflow, with unpleasant results. Many properly constructed cesspools have been in operation for years requiring little or no attention.

SEPTIC TANKS

These are closed, underground, metal or concrete tanks in which sewage is subjected to anaerobic decomposition for periods varying from twenty-four hours upwards. This principle of utilizing bacteria to liquefy sewage solids was first proposed by Donald Cameron in England. The process involves a breakdown of sewage solids similar to that found when organic matter is attacked in the soil. A minimum capacity of thirty gallons of sewage per person per day may be used in calculating septic tank requirements, but forty or even fifty gallons is more realistic. Temperature, among other factors, has a direct bearing upon the speed with which digestion takes place, and when the temperature drops too low, the rate of decomposition of sewage may slow down to a point where solids might build up more quickly than biological digestion is able to liquefy them.

After the preliminary anaerobic decomposition in the septic tank, the sewage is carried out into the soil by means of tile piping embedded in gravel or coarse sand. By leaving a space between the sections of the tile or by employing porous tile, the partially decomposed sewage can seep into the sand or gravel and undergo aerobic breakdown. Another common practice is to empty the sewage into cesspools after the material has undergone decomposition in a septic tank. This leaves a relatively stable, odorless end product.

Some septic tanks are reported to have been in operation for over fifty years with no cleaning, but in general some non-digestible sludge accumulates in the tank and needs to be removed periodically. It is helpful, however, after cleaning a septic tank to put back some of the active material (sludge) to serve as a culture "starter." Unless this is done, the proper bacterial flora may require an extended period to become established, and the efficiency of the tank is thereby reduced. When septic tanks demand frequent attention, the difficulty can usually be attributed to one or more of the following:

1. Overloading the tank because of inadequate tank capacity.
2. Insufficient or improperly prepared drainage field to accommodate the effluent from the septic tank.
3. Lack of a grease trap between the kitchen and the tank. It is just as undesirable to have a grease trap that is not regularly cleaned as it is to have no grease trap at all.
4. Introducing materials into the sewage which are unable to undergo biological digestion.

While persons engaged in building their own home naturally are interested in keeping costs to a minimum, economizing by purchasing and installing a septic tank of insufficient capacity is, in the main, false economy. They should anticipate any eventuality and provide a tank large enough to allow for future increased volumes of sewage. If precautions are taken to obviate the difficulties listed above, septic tanks will generally provide an efficient and reliable means of disposing of sewage on a relatively small scale.

IMHOFF TANKS

For capacities up to 300,000 gallons of sewage per day, a two-story septic tank called an *Imhoff Tank* is frequently employed.

As the sewage passes through a v-shaped trough, the solids drop to the bottom of the tank where anaerobic action occurs. Since undigested material accumulates in time, a sludge-removal vent is provided. Gas being liberated in the process is sometimes collected and used to burn garbage, or it may be used to warm the building in which the Imhoff tanks are kept. Thus a more favorable temperature can be maintained to encourage sewage decomposition.

CONTACT BEDS

Disposal of the effluent from large sewage digestion tanks is handled in a number of ways. The contact bed, while not as popular today as it once was, may be employed in this process. A contact bed is prepared by filling water-tight basins with stones, slate, slag, broken crockery, etc. The basin is filled with sewage

and allowed to remain for several hours to permit anaerobic action to become established. Then the liquid is drained off through the bottom of the bed, and the solids clinging to the stones and other rough materials in the contact bed are permitted to under-

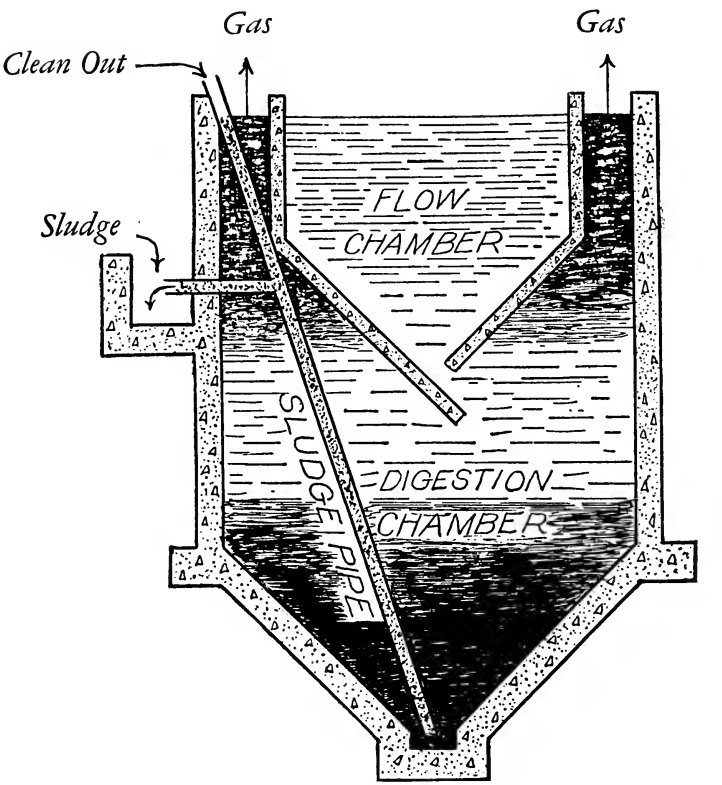


Fig. 39. Section through an Imhoff tank. A typical tank may be about 30 feet by 80 feet in size. (*From* General Bacteriology, *D. B. Swingle and W. G. Walter. Copyright 1947, D. Van Nostrand Company, Inc., New York.*)

go aerobic action in which oxidizing bacteria play an important role. The complete cycle of filling, standing, and emptying usually entails about eight hours for an acre-sized bed processing up to 800,000 gallons of sewage a day.

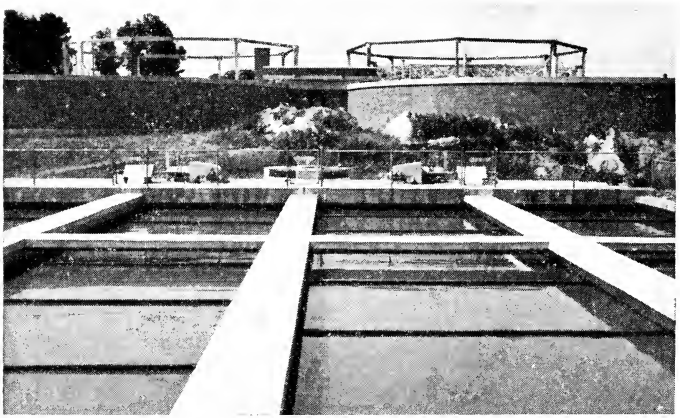


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Fig. 40. (A) Primary settling tanks. Solids settle out as the sewage flows slowly through these tanks. (B) Trickling filter. The effluent from the settling tanks is sprayed intermittently onto the bed of rocks where aerobic microorganisms oxidize the organic matter in the liquid. (*From* Microbiology, *W. B. Sarles, W. C. Frazier, J. B. Wilson, and S. D. Knight. Copyright 1951, Harper and Brothers, New York.*)

TRICKLING FILTERS

These filters are composed of beds about ten feet deep filled with rough materials similar to those found in contact beds. After preliminary anaerobic digestion, the sewage is sprayed on the surface of the bed where aerobic decomposition continues the breakdown process. It may require as long as three months to build up a suitable zoogloeal mass within the bed, but once this is established, sewage decomposition is very efficient. Too high a concentration of industrial wastes, particularly those wastes from chemical plants, may destroy large numbers of bacteria on the beds, and the efficiency of the operation will suffer accordingly.

SAND BEDS

A top layer of about eighteen inches of fine sand resting on three feet of coarse sand and an additional three feet of coarse gravel serves as an excellent bed upon which partially digested sewage can be sprayed. After standing for a period lasting up to several days, depending upon the temperature, another layer of sewage can be applied to the sand bed where it undergoes aerobic breakdown. When the organic matter builds up to an appreciable thickness, it can be scraped off and the cycle repeated with a fresh application of sewage.

ACTIVATED SLUDGE

A common method for the disposal of sewage is an aerobic process in which air is forced through a large tank containing raw sewage, resulting in the formation of activated sludge. The bubbling air encourages the growth of aerobic bacteria and it also keeps the solids in motion, which hastens decomposition. From 85 to 95% of the solids may be oxidized in such an aeration tank in about five hours, if the oxygen content is maintained at one part, or higher, per million parts of sewage.

Since not all of the solids are decomposed in the aeration tanks, it is customary to pump the treated sewage into settling tanks where the solids are removed. Dried activated sludge contains up

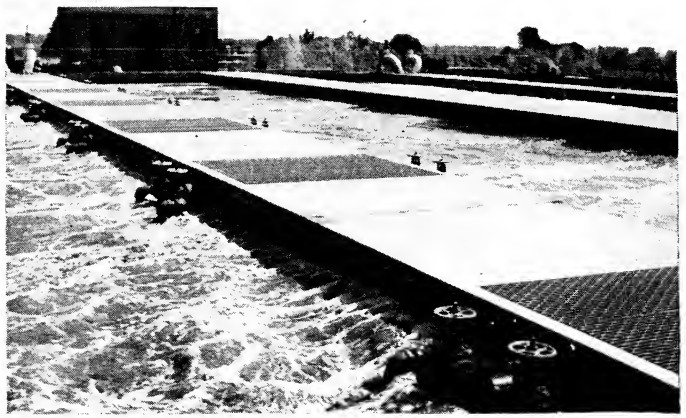

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Fig. 41. (A) Aeration tanks in activated sludge system. Air is pumped rapidly into the sewage that has been inoculated with activated sludge, and rapid oxidation of organic matter takes place. (B) An empty aeration tank. Many small bubbles are formed by forcing air through the long porous plates visible at the left on the bottom of the tank. (*From* Microbiology, *W. B. Sarles, W. C. Frazier, J. B. Wilson, and S. D. Knight. Copyright 1951, Harper and Brothers, New York.*)

to 15% of nitrates, is practically odorless, and harbors large numbers of nitrogen-fixing bacteria. Such sludge, therefore, serves as a valuable fertilizer. When starting a new tank of activated sludge, it is customary to inoculate the tank with "ripe" sludge from a previous batch. This inoculum provides desirable organisms required to decompose sewage into carbon dioxide and water, and thus cuts down on the length of time required for breakdown of the organic matter.

If the aerated sludge is not to be used as fertilizer, anaerobic decomposition is permitted to take place, and sewer gas (methane) is liberated during this reaction. Since methane is combustible, it can be collected and employed to warm the sludge tanks and speed up microbial activity. Solids remaining after anaerobic decomposition of sludge have little or no value as fertilizer, but they can be burned, be used as fill, or even be employed as soil conditioners in sandy regions.

DISEASES POTENTIALLY TRANSMISSIBLE THROUGH SEWAGE

Statements are frequently found in the literature to the effect that pathogenic bacteria do not multiply in sewage. Careful determinations probably have not been made to support this contention, but it is reasonable to assume that the high biological competition in sewage does not favor the development of pathogens. It has been reported, however, that *Salmonella typhosa* can be isolated from sludge after several weeks of storage. Perhaps these microbes have remained in a dormant state, even though active multiplication of their numbers may not have taken place.

In some European countries, and particularly in the Far East, a process called SEWAGE FARMING is commonly practiced. Raw sewage is spread out on the land and crops are grown in these areas, but it is not advisable to raise vegetables on these farms, especially the types of vegetables that are normally eaten raw. Serious outbreaks of enteric diseases have been traced to the consumption of small crops harvested from sewage farms and consumed without previous cooking. Grain and hay are frequently

grown on such farms. In China and in other countries where fertilizers are scarce, it is routine practice to save human excrement and use it for fertilizer. Such material is termed NIGHT SOIL. Undoubtedly a great number of pathogenic microorganisms are transmitted to humans by this practice.

Overloading bodies of water with excessive quantities of organic matter lowers the concentration of dissolved oxygen, and this endangers fish life. The effluent from sewage disposal plants should be treated to remove organic matter before dumping the wastes into streams, rivers, or lakes. State Water Commissions are constantly faced with the problem of controlling pollution of water, and their efforts are beginning to bear fruit.

Pollution of water near public bathing beaches has become increasingly serious in recent years, with pollution reaching such proportions that health authorities in some localities have been forced to close specified areas for human bathing. Shellfish gathered in these locations have spread typhoid and other enteric diseases when the food has been consumed without proper cooking. While sewage has been shown to contain the virus of poliomyelitis, no one has conclusively demonstrated that this disease is transmitted through bathing in such polluted water. Tuberculosis organisms can also be isolated from polluted water on occasion, but this disease is not normally considered to be contracted by persons bathing in such water. When authorities quarantine beaches at the height of polio epidemics, the restriction is usually enforced with the object of minimizing close contact of large numbers of individuals. In general, this restriction is not enforced as a result of fear that persons will ingest the virus from the contaminated water.

GARBAGE AND OTHER WASTES

Edible food becomes garbage in one sweep of the arm when plates are removed from the table. Garbage may be defined as refuse animal or vegetable matter. Since this material is capable of undergoing decomposition with accompanying foul odors, to say nothing of its unsightly appearance, proper garbage disposal is of extreme importance from the standpoints of human health,

economics, convenience, and general cleanliness. Uncovered garbage attracts flies and serves as a breeding ground for rats. While insects and rodents probably pick up relatively few pathogenic organisms from the garbage itself, large numbers of insects and small animals can become an acute nuisance and a definite potential health hazard, since they are natural vectors of many microbes.

Under the same heading as garbage, we must think of other solid waste material from human habitations not carried through sewage systems. The general term "refuse" is applied to these wastes, and includes ashes, rubbish, street sweepings, and dead animals. In rural areas the disposal of refuse is a relatively simple problem handled by individual families, but in large communities the problem becomes magnified. The choice of available systems depends upon local conditions, with economical sanitation an important consideration. A few common practices are listed below.

DUMPING

Towing garbage out to sea has been a routine procedure for a number of years in coastal areas, but since tides are capable of carrying the unsightly material back to the shore, the practice has been abandoned in many communities. There is little doubt that dumping garbage out to sea is an economical means of disposal, but other factors outweigh the advantages in many instances.

FILLING

Dry refuse can be used as fill for lowlands, but the dust problem on windy days can create very undesirable conditions, particularly if the prevailing wind carries the material back over the city. Combustible trash is always a potential fire hazard, and the number of times that the fire department is called to fight dump fires during dry weather is sufficient evidence of the undesirability of this disposal technic.

BURIAL

Deep trenches can be dug and covered with soil after organic refuse has been dumped into these pits. Proper coverage to prevent a rat and fly problem is necessary.

INCINERATION

This is the destruction of wastes by fire, and the ashes left after such treatment can be saved and used for fill in lowlands. When garbage is burned at a high temperature, the odor is not offensive.

FEEDING

Cooked garbage may be fed to pigs if non-digestible materials are kept separate from the edible garbage. Raw garbage has been the cause of a great many cases of trichinosis, and health authorities are seeking legislation to curb the practice of feeding raw garbage to pigs. The great amount of food provided by garbage, however, has prompted some communities to pass laws allowing these wastes to be fed to pigs if the garbage is cooked according to prescribed specifications.

CHAPTER 13

The Air We Breathe

GENERAL CONSIDERATIONS

Without delving too deeply into the field of physiology, it might be of interest to quote a few figures relative to the bulk of air inhaled each day by human beings. Since air carries microorganisms, the volume of air inspired will directly influence the number of organisms inhaled.

A quiet inspiration is usually found to average about 500 c.c. (30 cubic inches), but lung capacities vary tremendously among

persons. Adult females have approximately one-fifth less lung capacity than adult males. The respiration rate falls within the range of 13 to 18 per minute in adults, and with an average volume of approximately one-half liter per inspiration, a person inhales better than 10,000 liters of air each day. Inspired air consists of about 21% oxygen, 78% nitrogen, 1% argon, and 0.04% carbon dioxide; expired air consists of 16% oxygen, 4% carbon dioxide, and other gas percentages remain about the same as that found in the inspired air.

When the air is drawn through the nose, the hairs lining this passage serve as a filter, and as the air passes through the tortuous nose passages it is warmed and moistened. Many bacteria are trapped in the fluid bathing the mucous membranes lining the nose. Mouth breathers do not have as efficient a mechanism for filtering out microorganisms with the result that such persons appear to be more prone to respiratory infections than is found true in normal nose-breathers.

Air microbiology has attracted the attention of biologists since the time of Pasteur's famous experiments designed to disprove abiogenesis. Pasteur felt that there was a direct relationship between dust count and bacterial count. Miquel reported that one cubic foot of air in Paris contained one hundred and fifty bacteria. After a rain this same source of air yielded but six bacteria. A gram of house dust was reported to contain 2,100,000 organisms.

Many persons today think of Charles A. Lindbergh only as the "Lone Eagle" who flew the Atlantic Ocean non-stop in a Ryan monoplane from New York to Paris in May, 1927, but Lindbergh is also recognized as a biological scientist. During the summer of 1933 when he flew over the Arctic Seas, he collected data about the microbiology of the atmosphere. These findings were reported by Meier in the January 1935 issue of *The Scientific Monthly,* and they point out that air currents can be instrumental in the dissemination of spores which might cause plant diseases at points some distance from the origin of the spores.

Subsequent investigations have shown that the higher the altitude, the fewer microorganisms, in general, one might expect to

find. Powerful updrafts of air currents heavily laden with street dust, however, can carry organisms to unbelievable altitudes.

In addition to being transported on particles of dust, bacteria can find their way into the air via moisture droplets expelled by man during talking, coughing, and sneezing. Even the bark of a dog adds microbes to the air. The number and the size of droplets varies with the intensity of the expulsion from the nose or mouth. Larger particles settle out relatively quickly, while smaller moisture droplets remain in suspension in the air for periods of time depending upon the temperature, humidity, speed of air currents, and other factors. The potential possibility for the dissemination of upper respiratory diseases is graphically shown in high-speed photographs of an unstifled sneeze. It does not require too much imagination to realize how simple it would be for a heavily infected individual to spread his misery to large numbers of susceptible persons with whom he might have close contact in crowded, poorly ventilated rooms.

Air in itself will not support growth of microorganisms; it is not suitable as a bacteriological medium. Air is merely a passive transfer agent for organisms being borne on dust particles or in moisture droplets. The types of organisms one isolates from the atmosphere depend upon the source of the samples.

AIR POLLUTION AND HEALTH

Besides carrying "ordinary" dust, air can be the vehicle for the spread of silica dust in stone-cutting and grinding operations. Unless the operator wears a protective mask, he might inhale dangerous amounts of sharp-edged particles of silica which are capable of setting up a condition in the lungs known as SILICOSIS. The shearing action of silica can predispose an individual to subsequent microbial infection of the lungs.

Since house dust is notorious for its high bacterial count, manufacturers of vacuum cleaners have capitalized on this point in their sales promotion schemes. There is no doubt that cleaning the floor, rugs, and furniture is more efficient when the operation is performed with a vacuum cleaner than it would be with "old-

fashioned" broom and dust cloth technics. Housewives probably inhale fewer bacteria when these vacuum devices are employed. But manufacturers have, at times, used misleading advertising in this regard when they call their machines "air purifiers." The bags in which the dirt is collected vary in their efficiency, and it has been demonstrated that some bacteria are capable of passing through certain bags very rapidly and returning to the air of the room at the exhaust end of the vacuum cleaner.

Qualitative determinations have revealed that potentially dangerous respiratory organisms, particularly streptococci, are quite numerous in the air and dust of military barracks, dormitories, and hospital wards. By oiling the floors and by treating the bedding with oil-containing compounds, such as mineral oil in special emulsifying agents, the air can be protected against high dust counts and therefore against high bacterial counts. This oiling technic has proven satisfactory enough to warrant adoption by both the British and the American armies. When properly applied to bedding, these compounds leave no greasy feeling, no odor or fire hazard.

In regions where considerable soft coal is used as fuel, the smoke belching from the chimneys can fill the surrounding air with high concentrations of dust and chemical vapors which cannot be considered as conducive to good health. While the number of microorganisms in the air of such localities may not be very high, the non-living entities can inflict potential harm over a prolonged period. Especially affected are asthmatics whose condition is aggravated by smog. Various mechanical devices are available for installation in chimneys to precipitate these smoke particles before they are expelled into the atmosphere, and in communities where anti-smoke campaigns have been instituted, such devices are playing an important part in attaining the desired results.

QUALITATIVE TECHNICS FOR MICROBIOLOGICAL AIR ANALYSIS

One of the simplest approaches to the problem of determining the microbial content of the air is to expose the agar surface of

petri dishes to the atmosphere for varying periods of time. While this is not a quantitative method, the flora can be evaluated qualitatively by employing media designed to encourage growth of specific organisms. A blood agar medium, for example, will favor the growth of fastidious organisms, including certain streptococci. Zones of clearing adjacent to colonies growing on a blood agar medium represent a reaction known as HEMOLYSIS (destruction of blood cells). The type and the degree of blood change has some diagnostic significance to a trained observer. If the zone appears green, this reaction is called ALPHA HEMOLYSIS. So-called green streps fall into this category. If a colony exhibits a clear zone with a punched-out appearance, this is BETA HEMOLYSIS, and if no visible change in the blood can be seen around a colony growing on a blood agar plate, the reaction is called GAMMA.

Tomato agar will encourage the growth of yeasts and molds, while such selective media as Endo agar and eosine methylene blue agar can be employed to evaluate the gram negative flora of the atmosphere. By exposing several different types of media to the atmosphere, it is possible to accumulate considerable data pertinent to aerobiology.

QUANTITATIVE AIR ANALYSIS

A complete study of the microbiology of the atmosphere should include quantitative determinations, and since the pioneer work of Pasteur a number of published reports have advocated the use of simple and ingenious devices to accomplish these ends. It is desirable for surgeons to perform their operations in an atmosphere where bacterial numbers are reduced to a minimum. Ultra-modern hospitals provide their operating rooms with washed or filtered air pumped into the room through air-conditioning systems. It is amazing, however, what fine surgery can be accomplished with relatively little post-operative infection in military hospitals set up in tents out in open fields. While it may be true that the air in these installations is not as clean as one would expect to find in permanent hospitals, the standard aseptic practices employed can do a great deal to minimize infections.

Devices for filtering known quantities of air for microbiological analysis have been devised by Ruehle and Kulp, by Rettger, and others. These technics are based upon siphoning water from a graduated carboy, and as the water leaves the container, an

Fig. 42. Electrostatic air sampler showing exposed petri dishes. It employs a high voltage electrostatic field to precipitate organisms from a given volume of air into two specially prepared petri dishes. (*Courtesy of the Lamp Division of General Electric Company, Schenectady, New York.*)

equivalent volume of air passes through measured amounts of sterile sand or sterile physiological saline. Plate counts are then made of these trapped organisms using media designed to encourage the growth of specific organisms. Another device sucks known volumes of air into a special centrifuge which spins the air

against the walls of the apparatus where agar media are able to trap the organisms.

It was estimated by one investigator that a person living in London breathes in about 300,000 microbes each day. Some of these organisms are undoubtedly potentially pathogenic, but the efficient filtering mechanisms of the human nose and throat, coupled with other internal defense mechanisms to be discussed later in this book, help to explain how man is able to remain healthy much of the time.

Persons working in air-conditioned offices probably breathe in much fewer than the number of organisms reported in the London survey, while other individuals engaged in "dusty occupations" may inhale millions or billions of organisms during a twenty-four-hour period. The number of bacteria in the air has no deep sanitary significance, but the kinds of organisms may be important. Inhaling moisture droplets expelled from persons suffering from respiratory infections is considered to be almost a direct contact, and the public health significance is greater than the mere inhalation of large numbers of saprophytic bacteria.

AIR AS A VEHICLE FOR DISEASE TRANSMISSION

The air exhaled through the nose during ordinary respiration contains no organisms. But talking, even in low tones, requires forceful expulsion of air. Hundreds, thousands, or even millions of droplets are expelled as we increase the explosive force in loud talking, coughing, and sneezing.

The use of face masks is an attempt to minimize the spread of organisms by individuals working in such places as operating rooms, maternity wards, and the bottle-filling and capping sections of biological supply houses. Unless a mask is properly prepared, however, unwarranted reliance may be placed upon the effectiveness of the technic. A mask made of six layers of special gauze is considered effective if it is laundered before use and snugly fitted over the nose and mouth of the wearer. Since only dry shields are efficient, masks which become moist with perspiration should be replaced. It must be remembered that forced ex-

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Fig. 43. (A) An unstifled sneeze with clouds of bacteria-laden droplets being expelled. (B) A sneeze stifled with the bare hand. Some droplets get past the hand into the air. (C) A sneeze stifled with a handkerchief. Still fewer droplets are expelled into the air. (*Courtesy of M. W. Jennison, S.A.B. Nos. 5, 9, 10.*)

halations, such as sneezing and coughing, may allow bacteria to pass through these masks, but the organisms are reduced in number. The value of masks worn by personnel during influenza epidemics has been questioned by some who feel that viruses are not held back by gauze traps placed over the mouth and nose. Some good is undoubtedly accomplished, but the limitations should be recognized.

Fig. 44. Atomizing of droplets into the air by blowing out the last drop from a pipette. (*From Johansson, K. R., and Ferris, D. H.* Journal of Infectious Diseases, *1946*, **78**, *241*.)

Recent studies conducted by the United States Public Health Service and other agencies have graphically shown by means of high-speed cameras the occupational hazards involved in pipetting pathogenic organisms. The colored films available on a rental basis will be a revelation to persons engaged in bacteriological work. The aerosol mists that are created by shaking and pipetting are capable of contaminating the air with large numbers of organisms unless precautions are taken to reduce the volume of these

aerosols by modifying some of the standard technics commonly employed in bacteriology laboratories.

PHYSICAL AND CHEMICAL FACTORS AFFECTING AIR AND HUMAN HEALTH

There is nothing wrong with being microbe-conscious, but like anything else, the practice can be overdone. This is not a plea to cast aside common sanitary practices which have been shown to be important in reducing the spread of infection, but there are sensible approaches to this business of learning to live in the presence of hordes of parasites awaiting an opportunity to attack and destroy us.

Since the nose and mouth are commonly the portal of entry for many organisms, and since these areas are sources of large numbers of microorganisms, it behooves us to adhere to the accepted practice in modern sanitation of using a handkerchief or sanitary tissue to stifle coughs and sneezes. All of us harbor potential pathogens in our nose and throat; we may serve as healthy carriers of these microbes. But these same organisms may be highly virulent to other susceptible persons, while their normal bacterial flora, in turn, may prove to be highly virulent for us. The carrier is the best explanation we have for the fact that diseases do not die out.

Those individuals unfortunate enough to be allergic to dust or to pollen may lead a very uncomfortable existence. Various filters have been devised to be worn in the nose of such afflicted persons, but the results are variable and questionable.

ULTRA-VIOLET LAMPS

Light having wavelengths from about 2000 to 3000 Ångstrom units is highly lethal to microorganisms, and this band is known as the ultra-violet range. An Ångstrom unit, the measurement employed to express wavelengths, has a value of 0.1 millimicron (0.0001 micron) and is abbreviated Å. A wavelength of 2536 Å is particularly lethal to bacteria. Sunlight contains a relatively low concentration of ultra-violet rays which are reduced still further by dust, fog, moisture, etc., in the air, but artificial sources of

these radiations are available from quartz mercury vapor lamps or from carbon arcs.

Ultra-violet lamps enjoy a wide use in biological supply houses and in hospital nurseries as a means of reducing microbial populations. Some hospitals have these lamps installed in their operating

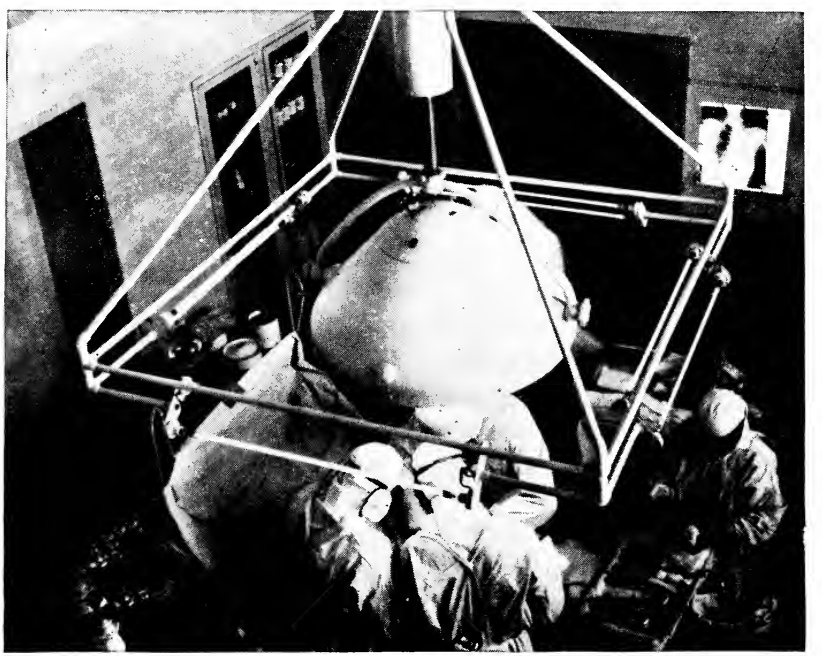


Fig. 45. Suspended above the operating table are four ultra-violet Sterilamps which emit ultra-violet light in the bactericidal range and kill air-borne bacteria. (*Courtesy of the Westinghouse Electric Corporation, Pittsburgh, Penna.*)

rooms to minimize post-operative infections. An unusual use of ultra-violet radiation has been attempted in certain banks where these light waves form a "protective shield" for tellers who daily deal with hundreds of individuals at a relatively close range. Unless the exposure is prolonged, however, microorganisms may survive direct contact with these wavelengths of light. The value of this practice in banks is highly questionable.

AIR CONDITIONING

Temperature, humidity, and movement of air are intimately related in any consideration of human comfort. Even when the temperature and the humidity are high, the mere circulation of air by use of a fan can make a person more comfortable. Improper air conditioning, such as maintaining too low a temperature, too low a humidity, or too rapid circulation of air, can be detrimental to health by chilling persons, and some believe this induces the onset of common colds.

AIR TREATMENT DEVICES

We might consider Lister's work in introducing carbolic acid sprays into the air surrounding patients during surgery to be the earliest attempt to "purify" the air. Little progress was made in expanding research in this direction until quite recently when aerosol mists were introduced. Glycol vapors, particularly propylene and triethylene glycol, are employed as air disinfectants, and they exhibit many characteristics ideal for the killing of microorganisms. As little as 0.5 mg. of propylene glycol vapor per liter of air can virtually sterilize heavily contaminated air in fifteen seconds, according to some investigators, and triethylene glycol is apparently even more effective than propylene glycol. Chemical treatment of the air with aerosols, however, bears a relationship to temperature and humidity.

While most studies on the treatment of the atmosphere with aerosols have been aimed at reducing the count of viable organisms, a great deal more information is needed before it can be stated conclusively that these mists have a direct effect on reducing disease.

ODORS AND HEALTH

The role played by bad odors in human health has been a matter of pure speculation for decades. It was commonly believed that bad air was the direct cause of certain fevers. A disease still masquerading under a false name is *malaria*, which literally means bad air. Since persons engaged in work around sewers

contracted this fever, the notion arose that it was the foul-smelling air in the sewers that caused the malady. It was not until 1880 that Laveran revealed the mosquito transmission of malaria, but the old misnomer, malaria, has persisted through the years.

The sense of smell varies widely between individuals. Odors can be perceived more readily in a moist air than they can be detected in a dry atmosphere. Many poisonous gases, including carbon monoxide, are practically without odor. It should be remembered that the foul smell of exhaust fumes from a motor vehicle is not due to the lethal carbon monoxide; other products of combustion overshadow the barely detectable monoxide fumes.

Rosenau has stated that science has demonstrated that our sense of smell is a poor sanitary guide. While disagreeable odors may not be harmful, they should be eliminated for esthetic and psychological reasons, as well as for decency and cleanliness.

The more recent fundamental investigations of Winslow and his colleagues relative to the effect of odors on health have added a great deal of knowledge to this phase of science. Odors, for instance, affect the appetite, just as clean and pleasant surroundings are conducive to better appetites. Winslow summarizes the information by stating that we may conclude that while air does not as a rule bear the microorganisms causing disease and does not carry with it mysterious and specific organic poisons, an atmosphere laden with offensive odors is not only objectionable on esthetic and economic grounds, but may under certain circumstances constitute a real menace to public health.

There are differences of opinion about such scientific matters, and that is what makes research such a challenge.

RAISING MICROBE-FREE ANIMALS

The tremendous number of bacteria found in the intestines of warm-blooded animals, particularly in the colon, raised a number of questions within the minds of early investigators. One of these questions concerned the ability of rearing animals in the complete absence of living microbes. Some workers expressed the opinion that proper digestion of food might not take place in the absence

of bacteria. To answer this question, as well as many others in biology and medicine, Reyniers and his colleagues at Notre Dame have set up elaborate equipment to study the problems. This research has been under way since 1928 and it is an interesting chapter in the development of microbiology. Needless to say, animals have been successfully raised microbe-free and they serve the same function in science as a sterile tube of media, or as a chemically pure reagent in chemistry, according to the investigators at Notre Dame.

CHAPTER 14

The Soil and They That Dwell Therein

PHYSICAL AND CHEMICAL COMPOSITION

The upper layer of the earth's surface, varying in thickness from six to eight inches in the case of humid soils and up to ten or twenty feet in certain arid soils, possesses characteristic properties which distinguish it from the underlying rocks and rock ingredients. This relatively thin layer of the earth's crust is called SOIL. A dictionary defines soil as "finely divided rock material mixed with decayed vegetable or animal matter, constituting that portion of the surface of the earth in which plants grow, or may grow." The principal difference between soil and the sub-soil is the presence of living organisms—plant and animal. It is a combination of climate and living organisms which determines to a great extent the type of earth in a given locality.

Soil is such a complex substance, both chemically and biologi-

cally, that it would be impossible to go into adequate detail in order to give the student a complete and clear idea of what transpires in this medium hour after hour, year on end. A few of the fundamental concepts relative to soil microbiology can be presented, however.

The very food we eat comes directly or indirectly from the soil. We may grow crops to be consumed directly, or we may feed these crops to lower animals which in turn furnish us with meat, hides, wool, and many other useful items.

Too often the layman looks upon the soil as an inert aggregation of minerals and organic matter, but in reality soil is dynamic —changing every second as microscopic and macroscopic organisms act upon available food materials and upon each other. The better medium that the soil provides, the higher will be the bacterial count, and there is a direct relationship between bacterial count and soil fertility. It is just as important for the soil to fulfill the prerequisites of a good medium as it is for a bacteriologist to compound foods which meet the needs of the organisms he is attempting to cultivate in the laboratory. Unless temperature and oxygen supply are properly controlled, however, an otherwise satisfactory medium may not support the growth of the desired organisms. When a farmer drains, plows, limes, and fertilizes a soil, he may not realize all of the technical reasons involved, but he is, in effect, trying to create a favorable environment for the cultivation of organisms.

Because of the diversity of life found in rich soils, no single diet can be expected to supply all of the requirements of so many different appetites. Most soil bacteria prefer complex food materials which they can attack (analyze) with the aid of enzymes; such bacteria are known as HETEROTROPHS. Another group of organisms, AUTOTROPHS, demand simple food elements which they can build up (synthesize) to suit their peculiar requirements. Waste products excreted by one organism may be sought as food by still other bacteria. Little or nothing is wasted in the soil.

When bacteria attack complex organic matter, the presence of oxygen is an important prerequisite for insuring oxidation of or-

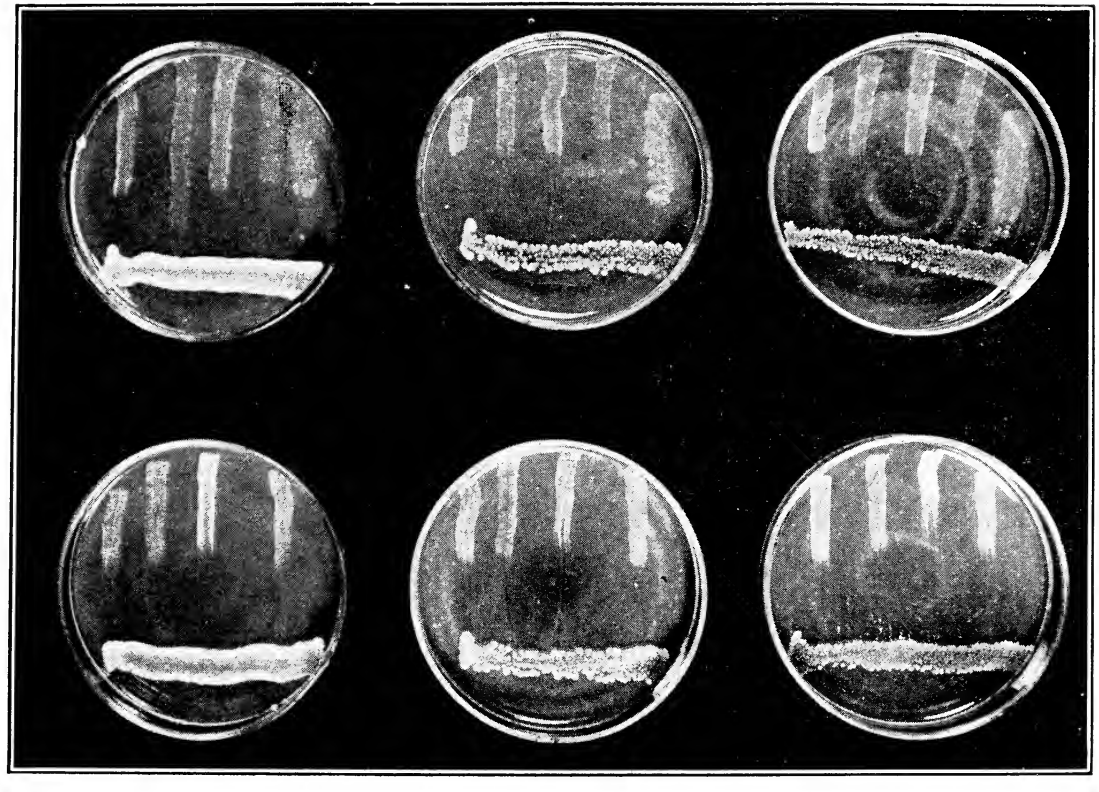


Fig. 46. Cross-streak method for testing production of antibiotic substances. (*From* Soil Microbiology, *S. A. Waksman. Copyright 1953, John Wiley and Sons, Inc., New York.*)

ganic substances. A loose-textured soil will permit the entrance of atmospheric gases which contain about 20% oxygen, but as active metabolism continues in an organic-rich soil, carbon dioxide and other gases produced during microbial metabolism tend to displace the dissolved oxygen, sometimes reducing the latter to a surprisingly low concentration. Organic matter helps the soil to retain moisture, and it aids soil fertility in many direct ways. The inter-dependence of these various chemical and physical factors makes the soil far from a static medium; in fact, the biological activities in the soil put a three ring circus to shame. Out of this cauldron emerge the foods we depend upon for our survival.

What is meant by the terms clay, silt, sand, and gravel? These are arbitrary designations based upon the particle size of the material, and they may be tabulated as follows:

Clay	0.005 mm. and smaller
Silt	0.005 to 0.05 mm.
Very fine sand	0.05 to 0.10 mm.
Fine sand	0.10 to 0.25 mm.
Medium sand	0.25 to 0.50 mm.
Coarse sand	0.50 to 1.00 mm.
Fine gravel	1.00 to 2.00 mm.

Surrounding these particles is a moisture layer, appearing as a film, which transports in solution the minerals which are dissolved from the inorganic soil constituents and the carbon dioxide and other substances produced from the decomposition of organic matter.

Growing plants obtain their required nutrients by absorbing them from this solution via the root hairs which penetrate between the soil particles. Some of the soil elements are only slightly soluble, and as the plants absorb these dissolved elements, more of the chemical goes into solution and becomes available to higher plants.

SOIL ORGANISMS

The microbial population of the soil is made up essentially of bacteria, yeasts, molds, algae, and protozoa, and these organisms are found principally in the upper layers of the earth's crust grow-

ing as saprophytes, with the possible exception of protozoa which consume other microorganisms. Millions, or even billions, of organisms may be found per gram of soil, the numbers depending upon how good a medium the particular soil happens to be at the moment. In a previous chapter the various technics available for determining the total and the viable bacterial counts of liquids were discussed. Some of these methods, with modifications, are available to soil microbiologists, but the procedures will not be discussed here.

While most soil organisms are generally considered to be nonpathogenic, there are bacteria which can initiate serious disease in man. When a person cuts himself, the soil microbes that gain entrance to the wound are usually not as dangerous as is the introduction into the flesh of the individual's own skin bacteria—the pyogenic (pus-producing) cocci—which can cause wound infections and septicemia (blood poisoning).

Soil that has been fertilized with manure, particularly horse manure, may contain unusually high numbers of *Clostridium tetani,* the etiological agent in lockjaw. This is important information for persons engaged in directing sports and physical education. Football fields and baseball diamonds should not be fertilized with horse manure, or persons sustaining injuries of the flesh may have to be administered tetanus antitoxin to prevent the development of lockjaw. It is much more desirable and it is less hazardous to employ commercial preparations for maintaining good grass in these areas.

Another disease that can be contracted when deep wounds are contaminated with heavily fertilized soil is gas gangrene, caused by *Clostridium perfringens.* From the generic name of this organism and the one causing lockjaw, it is known that these two species are anaerobes; they grow in the absence of free atmospheric oxygen. When an individual sustains a deep, dirty flesh wound, both aerobes and anaerobes are usually forced into the injury together with the soil particles, and after the aerobes have depleted the supply of free oxygen in the depths of the wound, the anaerobes find conditions suitable for their growth and they flourish.

THE NITROGEN CYCLE

Nitrogen, as an element of protein, is a constituent of every living cell. Unless there were some means for releasing nitrogen and other elements from dead plant and animal cells, the world's supply of available elements would in time be bound up and un-

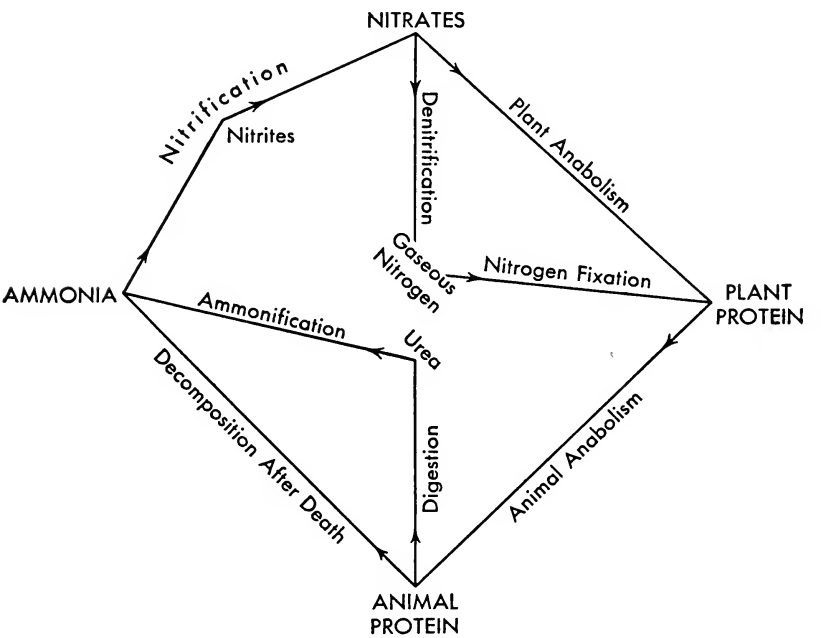


Fig. 47. The nitrogen cycle.

available for future generations of living things. Through the action of microbes, bacteria in particular, the elements are kept rotating in a so-called cycle.

Commencing at the bottom of the diagram depicting the nitrogen cycle, labeled *animal protein,* it can be seen that at least two paths are available as the cycle is followed clockwise. During the metabolism of higher animals one of the waste products, excreted principally through the urine, is *urea,* which has the chemical formula $CO(NH_2)_2$. When urea is acted upon by microorganisms

in the soil, it is converted into *ammonia* (NH_3). Animal protein can also be changed into ammonia during the decomposition of the dead animals.

Ammonia is rapidly oxidized in a well-ventilated soil to a substance called *nitrous acid* (HNO_2) through the action of two bacterial genera, the *Nitrosomonas* and the *Nitrosococcus,* but the acid is soon converted into *nitrites* (NO_2, KNO_2, or $NaNO_2$). Another group of bacteria pick up the ball, so to speak, and oxidize the nitrites into *nitrates* (NO_3). This entire process of converting ammonia into nitrates is termed NITRIFICATION; the organisms involved are called *nitrifying bacteria.*

Plants are capable of assimilating nitrogen when it is supplied in the form of nitrates, and this is indicated by labeling the next step in the cycle *plant protein.* There is evidence, however, that some plants may assimilate ammonia as a source of nitrogen. Unfortunately, when soil is not sufficiently aerated, anaerobes and facultative anaerobes are capable of reducing the nitrates back into the nitrite form—a highly undesirable process from the standpoint of soil fertility. Still other bacteria are able to *denitrify* nitrates and liberate gaseous nitrogen (N_2) which constitutes about 78% of the atmosphere and is largely unavailable to plants. This DENITRIFICATION results in a loss of soil nitrogen, and hence the process is to be avoided if possible.

A few genera of bacteria have the capacity to bind atmospheric gaseous nitrogen and to utilize it in their metabolism. These organisms are called *nitrogen fixing* bacteria. Members of the *Rhizobium* genus can live in symbiosis with the root systems of legumes (clover, peas, beans, alfalfa, vetch, peanuts, etc.), causing the formation of root nodules. These organisms furnish available nitrogen to the plants, and the legumes reciprocate by supplying necessary nutrients and proper living conditions for the rhizobia. It has been calculated that this symbiotic relationship between legumes and root nodule bacteria results in fixation of as much as 240 pounds of nitrogen per acre during a single growing season. When growing independently of the legumes, however, these bacteria are apparently incapable of fixing gaseous nitrogen.

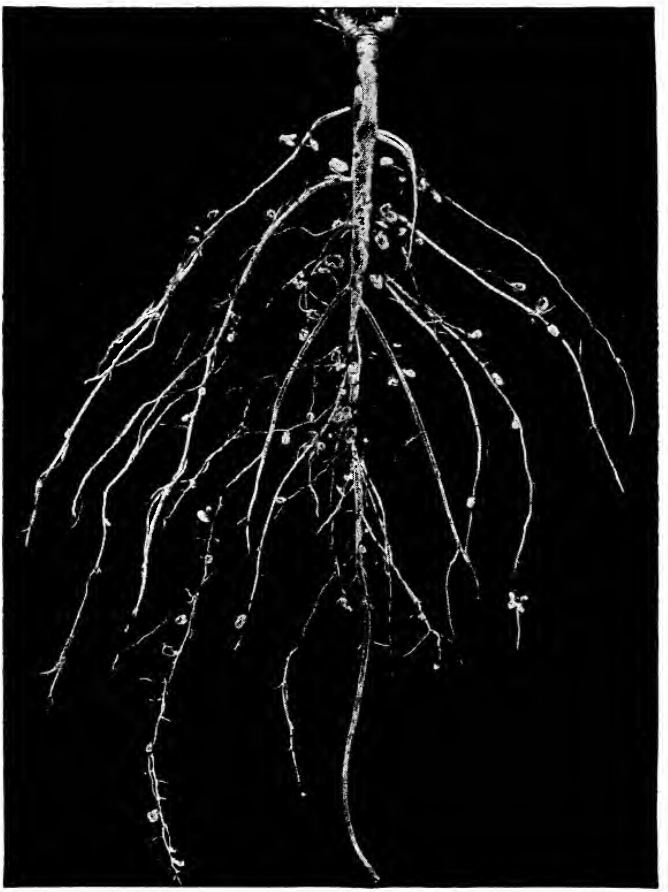


Fig. 48. Root nodules on Alsike clover. (*From* Plant Life. A Textbook of Botany, *D. B. Swingle, 2nd ed. Copyright 1942, D. Van Nostrand Company, Inc., New York.*)

The genus *Azotobacter* includes organisms which live independently in the soil (non-symbiotically) and which can fix atmospheric gaseous nitrogen. When the *p*H falls below about 6.0, however, *Azotobacter* cease fixing nitrogen until conditions become more favorable for their development.

The tremendous amount of nitrogen suspended over every acre of ground throughout the world is largely wasted as far as plants

are concerned, and man must purchase costly fertilizers to replenish the nitrogen lost through the harvesting of crops. Manufacturing processes have been devised, however, for the extraction of atmospheric nitrogen. The Haber Process, for example, forces the combination of nitrogen and hydrogen at about 200° C. under

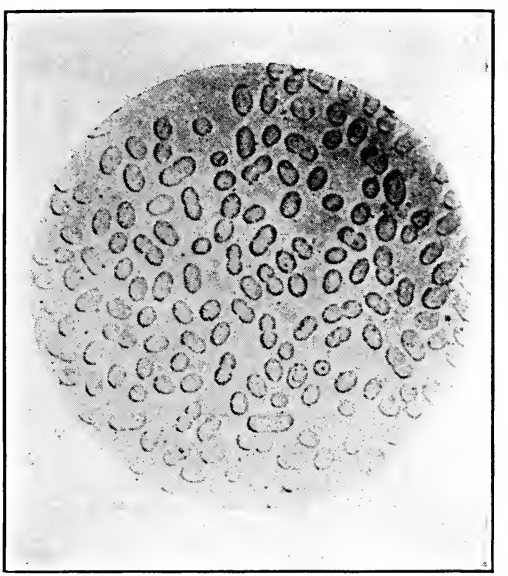


Fig. 49. *Azotobacter chroococcum.* (*From* Soil Microbiology, *S. A. Waksman. Copyright 1953, John Wiley and Sons, Inc., New York.*)

a pressure of 200 atmospheres (an atmosphere is about 14.7 pounds per square inch at sea level) in the presence of iron particles which serve as a catalyst. Ammonia is formed by this chemical union, and the ammonia in turn is converted into ammonium salts, several millions tons of which are produced annually in the United States.

THE CARBON CYCLE

Carbon constitutes another primary element in all protoplasm, and thus carbon is an essential substance in nature. Although the

atmosphere contains only three parts of carbon dioxide (CO_2) in ten thousand (0.03%), it is difficult to conceive of plants and animals existing without this material.

The equilibrium created by plants and animals with respect to carbon economy is one of those orderly processes we see so frequently when nature takes a hand in things. Higher plants re-

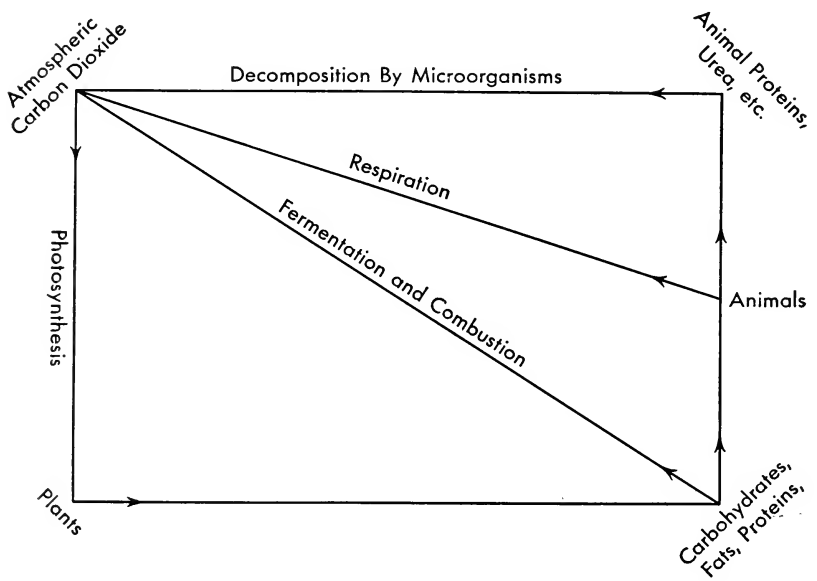


Fig. 50. The carbon cycle.

quire carbon dioxide in order that they might synthesize sugar from this gas with the aid of sunlight. This building up of energy substances from water and carbon dioxide is catalyzed by the green pigment, *chlorophyll*, found in higher plants, and the chemical process is called PHOTOSYNTHESIS. While it is generally stated that plants take in carbon dioxide and give off oxygen in their metabolism, and that animals utilize oxygen and liberate carbon dioxide through respiration, plants also return some carbon dioxide to the atmosphere during their life processes.

Combustion of fuel—coal, oil, gasoline, kerosene, etc.—releases

some carbon dioxide to the air, but in the main the over-all atmospheric concentration of this gas is not materially raised by such combustion except in the immediate locality.

CYCLES OF OTHER ELEMENTS

A scheme similar to those outlined for nitrogen and for carbon can be diagramed for each element in nature, but space will not be utilized in this book to present these cycles. Other textbooks can be consulted by those having a further interest in this phase of science.

Death is the fore-runner of life. Were it not for the death of plants and animals, available elements would eventually become depleted. Man borrows chemicals for his lifetime, and then after his death these elements are released for future generations when he keeps his rendezvous with the soil.

MICROBIOLOGY OF PETROLEUM

A relatively new phase of microbiology is that study concerned with the formation and the destruction of petroleum and its products. It appears possible that the temperature and the pressure required for the formation of petroleum are within the range compatible with bacterial life. There is further evidence that the types of materials, including microorganisms, found on the bottom of seas, resemble those substances found in deep oil wells. Even a pressure of 150,000 pounds per square inch, which may be the pressure in deep oil deposits, will permit microbial activity to occur.

Spoilage of gasoline may take place when certain bacteria begin to utilize this hydrocarbon as a source of food, and a list of such organisms includes some of the common soil genera—*Pseudomonas, Sarcina, Achromobacter,* and *Alcaligenes.* Even molds and yeasts are capable of attacking petroleum products as a source of carbon. This knowledge is made use of by investigators searching for new petroleum deposits. By inoculating a medium containing all elements necessary for growth except carbon with a petroleum-

utilizing organism, flasks of such cultures can be lowered into wells suspected of containing oil-bearing deposits. If petroleum vapors are present in the wells, the organisms will utilize the carbon in these vapors and the test culture will grow. So the bacteriologist joins hands with the geologist and with other scientists in this fascinating detective work for the petroleum industry.

CHAPTER 15

Food Poisoning and Food Infection

THE NATURE OF THE PROBLEM

This should prove to be one of the more practical chapters in the book. When students are asked for their comments after completing an introductory course in microbiology, they frequently reply that the information they have derived from the lectures and the readings on food poisoning seems to stay with them as long as any other topic covered in the course.

Those who have been duly impressed with what they have been taught will more than likely spread their new-found knowl-

edge—somewhat distorted at times, to be sure—to members of their family and to their acquaintances. Education can initiate a chain reaction whose limits are almost without bounds.

It is probable that at least 100,000 persons are victims of food poisoning each year in the United States. Since public health records usually contain only the statistics concerned with mass food poisoning contracted at public functions, it is impossible to state how many outbreaks occur in family units and are never recorded. The great tragedy is the fact that such poisonings are easily avoided by the application of a few fundamental principles of sanitation and common sense.

Whenever a newspaper prints an account of mass illness from food and attributes the cause to "ptomaine poisoning," you can be certain that the designation is a false one. Ptomaines are products resulting from putrefaction of proteins, and there is evidence that these end products are nontoxic when taken by mouth. Selmi, an Italian toxicologist, introduced the word PTOMAINE in 1870. It is derived from the Greek word *ptoma,* meaning corpse. Jordan stated it well when he claimed that the term "ptomaine poisoning" is a convenient refuge for etiologic uncertainty. The unfortunate feature of most food-borne outbreaks is the lack of visible evidence that the food is contaminated or harbors toxins. Putrefaction is rarely associated with the condition.

Food poisoning is a general expression which includes both poisoning due to pre-formed toxins in the ingested food, and infection of the gastro-intestinal tract by living organisms. Strictly speaking, these two conditions are more correctly designated as food intoxication and food infection, respectively.

Whether a given food is likely to cause food poisoning depends upon how suitable a medium it happens to be. If all of the prerequisites for a good microbiological medium have been fulfilled with the exception of one, microbial activity will be slowed down or will actually be prevented. Dry cereals, for example, lack sufficient moisture to allow active bacterial growth. Concentrated sugar syrups are not likely to support active microbial growth because of their high osmotic pressure. Fats do not contain the

desirable balance of available food substances, and hence they are not commonly implicated in food-borne outbreaks. Any food, however, under a given set of conditions may become contaminated by carriers with living organisms capable of causing severe food-borne infections.

Food poisoning is characterized by a sudden onset with one or more of the following symptoms: abdominal pain, headache, nausea, vomiting, and diarrhea, usually within two to twenty-four hours after ingestion of the food, although longer incubation periods may be true for some of the bacterial infections.

CANNED GOODS

When considering the potential danger of canned goods in food poisoning, it is necessary to think in terms of commercially canned and home-canned foods. Because of the high temperature and the long processing time employed by commercial canners, the amount of spoilage found in the millions of cans of food marketed annually is virtually infinitesimal. On the other hand, home-canned foods are probably responsible for proportionately higher numbers of gastric upsets, sometimes with fatal results.

The tin can has revolutionized home life, and even though it is frequently remarked that many families would starve should the housewife lose the can opener, our diets are better balanced and more interesting because of the wider selection of foods available to us in cans and in frozen form all year round, rather than just during the growing season.

An old belief still persists that once a tin can of food has been opened, the contents should be removed immediately to prevent tin poisoning. While it is true that flavors may develop in time when foods are stored in open cans, the danger from food poisoning is no greater than it is for foods stored in bowls or dishes. In fact, a tin can will probably be cleaner than many dishes. It is the types and the numbers of organisms present in the foods, not the tin container itself, that determine whether the food will cause poisoning when ingested. Some cans are lacquered or enameled on the inside, and preference is shown for this type of

lining to help retain the natural color of foods during processing. There is not sufficient evidence to warrant the statement that illness is more likely to occur from foods preserved in cans with a thin coating of tin.

Because foods may contain heat-resistant, spore-forming bacteria prior to processing, some spoilage must be expected in the finished product, since spores frequently survive this heat treatment. A leaky can or one having bulged ends due to gas pressures built up within the container obviously should be discarded. Any cans with distinct off-odors should also be thrown away. Oftentimes an off-odor does not become apparent until heat is applied to the food.

MILK AND DAIRY PRODUCTS

Because milk is one of nature's most nearly perfect foods, microorganisms thrive in it when the temperature is raised much above that found in a properly functioning refrigerator. Serious milk-borne outbreaks have occurred in the past due to pathogenic organisms which have gained entrance to the milk either directly from infected persons and from healthy carriers, or indirectly from milk-producing animals which have served as carriers when man has contaminated their udders. There are still other diseases which can be transmitted to man directly from infected animals, and tuberculosis and undulant fever fall into this group.

TUBERCULOSIS

Pulmonary (lung) tuberculosis is usually transmitted from one infected person to another through the agency of discharges containing the bacteria *Mycobacterium tuberculosis.* The protective capsule surrounding these organisms allows them to withstand considerable periods of drying and a relatively long contact with sunlight and chemical agents before the cell succumbs. Viable tuberculosis organisms have been isolated from house dust many months after a patient has left the area.

Once the leading cause of death in the United States, tuberculosis—formerly called the white plague—has dropped far down the list of diseases affecting man. This reduction is largely the result

of notable advances in preventive medicine and in therapeutic discoveries. Lest the reader get the impression that tuberculosis is no longer an important disease, consider the fact that more than 30,000 persons die each year from this cause in the United States alone. Nearly a half-million individuals are afflicted at any one time. During periods of war and in times of famine this disease is one of the first to show an increase, and there are parts of the world today where tuberculosis is still a leading cause of death.

UNDULANT FEVER

The etiological agent of this disease has the generic name of *Brucella,* in honor of Bruce who isolated the organism in 1882 and studied its mode of transmission. Technically, this disease is called BRUCELLOSIS, rather than the more popular designation of undulant fever.

Brucellosis is not transmitted from person to person except, perhaps, through blood transfusions. It may be contracted by drinking raw milk from infected animals, by eating infected meat that has been improperly cooked, and by working with infected animals or their carcasses. There is some evidence to support the idea that these small gram negative rods can penetrate the unbroken skin or enter the body through minute defects in the skin. Veterinarians and slaughter house workers should wear rubber gloves to protect themselves from this disease when handling carcasses. It is not clear whether inhalation of the organisms is a mode of transmission.

There are three recognized species of *Brucella: abortus, melitensis,* and *suis. Brucella suis,* from pigs, causes a more severe attack in humans than *Brucella abortus* from cows or *Brucella melitensis* from goats.

The wavy nature of both the temperature curve of the affected person and the course of the clinical symptoms gives the disease its designation of undulant fever. It is the chronic, debilitating nature of the malady which leaves the sufferers in such a depressed state. Many infected individuals find themselves unable to hold down a full-time position because of the clinical symptoms of the

disease. Before the advent of antibiotics, treatment for brucellosis was decidedly limited, but good results have been reported with the administration of selected antibiotics.

While notable progress has been made in the control and in the eradication of brucellosis in animals through vaccination and slaughtering programs, the fact remains that the danger of human beings contracting undulant fever, tuberculosis, and other diseases through the agency of raw milk, is a constant threat. Proper pasteurization, however, can destroy these pathogens and make milk safe for human consumption. This heat treatment originated with Louis Pasteur, after whom the process is named, when he employed the technic with French wines in an effort to prevent spoilage. Present-day pasteurization, as applied to the dairy industry, is aimed primarily at making the food safe for human consumption, with improving the keeping quality a secondary consideration.

Two general heating technics are employed in pasteurization of milk today. The first is the HOLDING METHOD, in which the milk is heated to between 142° and 145° F. (61.7° and 62.8° C.), held at that temperature for at least thirty minutes, followed by rapid cooling to a temperature below 50° F. (10° C.). A more recent process is the SHORT TIME HIGH TEMPERATURE PASTEURIZATION in which the milk is heated to a temperature of 160° F. (71.1° C.) for a period of from fifteen to seventeen seconds. Both of these methods are based upon the time and temperature relationship necessary to insure the killing of the tuberculosis organism, which is the most resistant pathogen one might expect to encounter in milk.

Pasteurization ranks with chlorination of water supplies as far as reduction of the spread of disease is concerned, but educating the public to accept pasteurization has not been, and still is not, a simple matter. A small but vociferous minority objects to man tampering with nature's food. But if we consider that man has raised and developed animals for milk production beyond the normal requirements of the young calves for whom the milk was intended, considerations other than those of nature's intent must

be reckoned with. The steps through which the milk must pass before it is poured into a glass on your dinner table necessitate that proper safe-guards be instituted to correct for any mistakes made in sanitary practices along the line. When a human being draws milk from the animal, whether it be by hand or by milking machine, there is always the possibility that pathogenic bacteria from the person or organisms from an improperly disinfected milking machine might find their way into the milk with eventual harm being done to the unsuspecting consumer. Even if we did drink milk directly from the cow's udder the way the calf does, there is always the possibility that the cow may be suffering from tuberculosis or from brucellosis in the early preclinical stages not readily detected from day to day by casual observation.

A frequent argument heard in opposition to pasteurization is that "boiling the milk imparts a cooked flavor to it and ruins the food value of the product." A temperature of 145° F. is a long way from 212° F., the boiling point at sea level. Properly pasteurized milk cannot normally be detected when the blind-fold test is run on a large number of subjects. As for the food value being ruined, the greatest harm that pasteurization does to raw milk is to affect vitamin C, in which raw milk is deficient to begin with. Even though a child is fed raw milk, some vitamin C and iron are added to supplement the diet. If it is necessary to feed orange juice, cod-liver oil, and vegetables to make up for these nutritional deficiencies in raw milk, why not feed a little more of these supplements, pasteurize the milk, and be assured that the child is getting a safe product?

The evidence that pasteurization is a desirable health measure is too overwhelming to be refuted. Some large progressive cities have passed ordinances requiring that only pasteurized milk be sold within their city limits. Such measures will pay rich dividends in public health, but you can't expect to convince every resident that pasteurization is a good thing. After all, "Grandfather drank raw milk all his life, and he lived to be 90, etc." Wasn't grandfather fortunate? Should anyone need convincing as to the wiseness of pasteurization, let him observe just one small child

crippled for life because of tuberculosis of the bones contracted from drinking raw milk. Suppose this cripple were you, or your brother, or your sister!

STAPHYLOCOCCUS FOOD POISONING

The human skin harbors large numbers of staphylococci (micrococci) as part of the normal microbial flora. As long as these organisms remain on the outside of the unbroken skin, they do no harm to the host. But as soon as a break appears in the skin—whether it be a pin-prick size or larger—these staphylococci are ready, willing, and able to establish themselves in the opening where the food supply and the general living conditions are agreeable to the invaders. When the internal defense mechanisms of the host are unable to cope with the microorganisms, an infection becomes established at the site. Pus cells (leucocytes) are rushed to the area by the body, and these white blood cells attempt to engulf and destroy the microbes in a process called PHAGOCYTOSIS. When this condition occurs, a pimple, boil, or carbuncle is formed.

Under normal conditions the invading cocci remain localized and do not spread through the blood stream causing blood poisoning. At times, however, when individuals break down nature's protective barrier by tampering with the infection, a generalized bacteremia occurs and prompt medical attention is needed to prevent progressive blood poisoning and death. Some pathologists believe that skin infections above the line of the nose should not be squeezed or irritated by non-medical persons. The combination of a rich blood supply and the proximity to the brain may set up conditions favorable for the development of a brain infection. However, any skin infection which "gets loose" can find its way into the circulation and may be carried to the brain as well as to other organs of the body.

Since human skin is such a good source of staphylococci, it should not be too surprising to learn that food poisoning caused by these organisms is so prevalent. When grown in a favorable medium, staphylococci can generate a powerful toxin which has a strong effect on the gastro-intestinal tract. Not all strains of

staphylococci, however, are equally toxigenic. *Micrococcus pyogenes* variety *aureus* (formerly called *Staphylococcus aureus*) is the most frequent coccus implicated in food poisoning outbreaks, but the *albus* variety has also been isolated from some foods which have caused severe illness when ingested. Because this poison affects the intestinal tract, it is called an ENTEROTOXIN. Unfortunately, this poison is thermo-stable and heating foods containing this toxin will not inactivate it.

It is surprising the number of staphylococci that it is possible for us to ingest without displaying symptoms of poisoning, but relatively small amounts of their pre-formed toxin can cause serious gastric upsets. The incubation period between ingestion of the toxic food and the appearance of clinical symptoms is generally less than twelve hours, with three to six hours being the most common in staphylococcus poisoning. The severity of the symptoms will depend upon the amount of poison ingested, but the fatality rate is extremely low. Persons who have experienced a frank case of food poisoning usually sum up their reactions by saying that as the symptoms intensify they are afraid that they are going to die. Then when their misery continues, they are afraid that they are not going to die!

The unfortunate part about food poisoning is that it is so unnecessary. If persons who handle food were careful to wash their hands thoroughly—not just give them a lick and a promise—and were aware of the importance of proper refrigeration of foods, a great deal of disease of this type could be eliminated. The role played by insects and rodents must not be minimized, however, since their bodies can passively transfer pathogenic organisms to food left uncovered or improperly protected.

Since improper refrigeration is the ultimate cause of most staphylococcus poisoning, a few words on this topic are warranted. To be efficient in preventing the spoilage of food, a refrigerator should be maintained at about 40° F. (about 5° C.). Frequent defrosting of some refrigerators may be necessary to keep the temperature this low. But another very important consideration, especially when attempting to chill foods like potato salad, is to

store the food in shallow layers not over three inches thick to permit rapid cooling when the salad is placed in the refrigerator. Failure to do this has been the cause of many food poisoning outbreaks. A huge bowl or bucket of potato salad, as prepared for large groups, may be such an efficient insulator, that the center of the container may not become sufficiently cold to stop microbial activity for several hours after the food is transferred to the cooling chamber. Once the salad dressing has been added to the potatoes, which should be thoroughly chilled beforehand, waste no time in chilling the mixture in shallow layers. It is not a wise policy to serve left-over potato salad a day or two later, especially if it stood at warm temperatures for any period of time during the first serving. Staphylococci thrive on salad dressings, and in a warm room it does not require very many hours before considerable toxin can be generated in this favorable medium.

Cream-filled bakery products—pies, eclairs, cream-puffs, etc.—meat products, especially left-overs, gravy that is kept luke warm for extended periods of time, dressings used in stuffing poultry, and dairy products made from milk drawn from infected animals are the usual products involved in staphylococcus food poisoning. Avoid these foods in warm weather, especially in public eating establishments where what goes on in the back room is unknown to the patron out in front.

One of the characteristics of food-borne intoxications is that not all persons who partake of the poisoned food are necessarily affected. Each individual has his own level of tolerance for poisons before he will manifest clinical symptoms, and the range of tolerance is rather broad. Secondly, not all portions of the food are necessarily equally poisoned. In the case of a large container of potato salad, for example, those persons eating the portion from the outer layers which are cooled more quickly than the center core will not get nearly as much, if any, toxin as those persons who are unfortunate enough to be served that portion from the depths where microbial activity might have continued for hours after the container was placed in the refrigerator.

Public health authorities can usually find the cause of an

outbreak by submitting samples of all foods served, if available, to a bacteriology laboratory for analysis. By questioning all affected individuals, one or more foods will be found to have been eaten by all, and bacteriological examination will usually confirm the investigator's suspicions. It should be borne in mind, however, that some who partook of the food will not show clinical symptoms, for reasons already discussed. On the other hand, some who become ill might not have ingested any of the food which the investigators conclude caused the outbreak. Psychic vomiting can sometimes account for these irregularities.

If a single individual can be spared the misery of food poisoning by being informed of a few fundamentals and precautions, all of the lectures and writings on the subject will have been worth while. Unless you have experienced the poisoning yourself, or unless you have seen firsthand how acute the symptoms are in a real outbreak, it is difficult to fully appreciate the problem. As one observer remarked, "It is a moving experience."

We take a great deal for granted when we walk into a strange restaurant and order a meal that has been prepared behind closed doors out of the vision of the customer. Standards of cleanliness vary widely between individuals. What may be to one food handler a perfectly satisfactory technic for handling food, may be revolting to another person who is fastidious. One measure of sanitation, although by no means the only index, is the cleanliness of the food-handler's hands, especially his fingernails, which should be kept very short at all times to prevent the accumulation of bacteria-laden dirt. A dirty apron showing signs of long use is another criterion of cleanliness. A person who pays attention to small details of sanitation will usually devote proper attention to larger matters as well.

SALMONELLA FOOD INFECTION

This type of disease differs from staphylococcal food poisoning in that the living *Salmonella* germs are ingested, and the typical clinical symptoms are delayed until the organisms have had an opportunity to become established in the gastro-intestinal tract.

This is a food infection rather than a food poisoning. The incubation period with *Salmonella* usually lasts longer than twelve hours with a sudden onset of chills, headache, abdominal cramps, vomiting, diarrhea, fever, and eventual prostration.

Food may be infected by human carriers and by rodents and insects which may serve as either passive or active carriers. Improperly cooked meat from animals infected with *Salmonella* may also cause this type of food infection. The microbes are gram negative rods, non-sporulating, and they usually exhibit active motility in young broth cultures. Since these organisms are excreted in the feces of man, the importance of excluding carriers as food handlers should not be minimized. If a carrier happens to be a housewife who prepares food for her own family, sound sanitary practices with respect to adequate hand washing are imperative.

Salmonella typhimurium, the cause of mouse typhoid, has been the cause of some serious food infections, especially in military camps. Insects and vermin must be excluded from areas where food is being prepared and being served if infection of individuals is to be avoided.

Since salmonellae are non spore-forming, they are not particularly resistant to heating; boiling temperature will kill them in a few minutes. The importance of proper refrigeration should be mentioned again as an important link in helping to minimize the chain of infection.

BOTULISM

The name botulism is derived from the Latin and means sausage. It had its origin many years ago in Europe where sausages were found to be the cause of serious food-borne poisonings, and the etiological agent is *Clostridium botulinum,* an anaerobic spore-forming rod. Because of the marked resistance of these spores to heat, the canning industry sets the time and temperature of processing foods with the destruction of these spores as the basis.

During growth of *Clostridium botulinum* it excretes a true exotoxin, which is one of the most powerful biological poisons known to man. The toxin has a marked affinity for nerve tissue

with the paralysis of the respiratory muscles as the eventual cause of the death of persons unfortunate enough to ingest a lethal dose. Since the organism is an anaerobe, sealing cans or jars with the exclusion of oxygen sets up ideal conditions for the bacteria to multiply and to produce their deadly potion. Home canning of neutral protein foods such as peas, beans, and corn cannot be safely carried out unless the foods are processed with steam under pressure. Ordinary canning methods in boiling water baths are not sufficient to insure destruction of these resistant spores which have their origin in the soil. The concentration of *Clostridium botulinum* in soil varies with different locations, with our western states having greater numbers of these spores than are usually found in eastern soils.

There are relatively few cases of botulism in the United States with the result that the average person is not even aware that such a disease exists. With the fine work being carried out by home demonstration agents hired by our State Colleges and Universities, education of home-makers with respect to the importance of the use of pressure cookers in canning certain types of foods has proven valuable.

Botulism is the most fatal of the food poisonings and infections generally encountered by man, with death rates as high as 65%. When the exotoxin begins to take effect, double vision and swelling of the tongue are characteristic symptoms. Relatively little pain is associated with the disease until just before death.

An off-odor or an off-color in a freshly opened can of food may furnish the clue that all is not well with the canned product. Under no circumstances should such foods be tasted until they have been boiled for at least ten minutes. Such heating inactivates the toxin, although it will not kill the spores of *Clostridium botulinum*. There are cases on record where only minute bits of canned food were swallowed before the food had been cooked, and death of the individual ensued within a few days.

The question is frequently posed by students as to whether frozen foods are potentially dangerous from the standpoint of botulism. The answer is that they normally are not dangerous.

In the first place, anaerobic conditions are lacking, and *Clostridium botulinum* grows only in the absence of free atmospheric oxygen. Secondly, the low temperature at which the food is stored allows little microbial activity to occur, and the toxin must be preformed in the food before clinical symptoms will occur in persons who consume the food. Frozen foods may cause poisoning, however, if large numbers of specific organisms were present in the food which was held too long at warm temperatures prior to freezing, or if the thawed product becomes contaminated and is allowed to remain too long at warm temperatures before being consumed. Freezing generally does not destroy bacteria; it merely preserves them.

Because true exotoxins are produced by the botulinum organisms, antitoxins are available and are indicated for use if the disease can be diagnosed early enough. The more time that elapses before the administration of antitoxin, however, the less promising results can be expected. Five specific toxins are produced by different strains of *Clostridium botulinum,* and five specific antitoxins are available to counteract these poisons. Since antitoxins are specific only for the particular toxin against which they are produced, it is necessary to administer a mixture of antitoxins, called a bivalent or polyvalent antitoxin, to meet any eventuality. Time cannot be taken to determine the specific type of poison involved in a given case, or the injection of antitoxin may be delayed too long to do the patient any good.

STREPTOCOCCUS AND OTHER BACTERIAL POISONINGS

While streptococci are not found as commonly in food-borne outbreaks as staphylococci or *Salmonella* species, they can cause severe illness. The alpha (green) streptococci are usually involved if this organism is the cause of the poisoning. Since these bacteria are normal inhabitants of our nasopharynx, persons who cough and sneeze over food may spray enough streptococci to serve as an inoculum which can grow in food under proper temperature conditions. Following an incubation period of from

six to twelve hours after ingestion, the enterotoxin produced by these organisms can cause typical food poisoning symptoms.

Reports are occasionally encountered that such bacteria as *Proteus* and *Escherichia* species appear to be implicated in food poisoning. The evidence is not too conclusive to warrant classifying these organisms in the same category as others mentioned as the usual causes of food-borne outbreaks.

CHEMICAL FOOD POISONING

When compared with microbial food poisoning, chemical poisoning comes in a poor second as far as the number of cases is concerned. In spite of warnings on labels and precautionary measures advocated by public health authorities, there are thousands of cases of chemical poisonings in the United States every year. Volumes can be written on the subject, but the presentation of a few examples might serve to call the attention of students to the problem. Since the clinical symptoms are frequently mistaken for microbial food poisoning, a brief discussion here seems warranted.

As ridiculous as it may seem, sodium fluoride, a constituent of many rat poisons, is commonly mistaken for baking powder, starch, and similar appearing materials. Why people insist on storing rat poison in the same area with foodstuffs is difficult to explain, but they do. Housewives have been known to prepare biscuits and pancakes from sodium fluoride which has been taken for baking powder or baking soda. Either the label on the container was lost or it was not read before the powder was carefully added to the mixture of other ingredients. When a physician prescribes aspirin for a sick child, it is hardly sporting to feed the child arsenic just because the two words happen to begin with the letter *a* and have four other letters in common. This may sound facetious, but the example is grimly true.

Cheap enamelware has been the cause of severe illness when foods, particularly acid foods, are cooked in these utensils. The underlying antimony may be dissolved in the food in high enough

concentration to result in vomiting within a few minutes after ingestion.

Arsenate of lead, a common spray employed to kill pests on fruit trees, may cause severe illness to individuals who purchase fresh fruits, and eat them without proper washing. Persons engaged in industries dealing with paints, batteries, gasoline, glazes for pottery, and insecticides are exposed to unhealthy concentrations of lead unless suitable precautions are observed.

Many chemicals, including formaldehyde, boric acid, potassium permanganate, hydrogen peroxide, and others, have been discontinued as preservatives in foods since passage of the Food and Drug Act of 1906 and its revision more than twenty years later. Each year tighter controls on foods, drugs, and cosmetics are necessary in the interests of public health and welfare. State and national agencies charged with this watch-dog responsibility are doing creditable work in assuring the public that they are getting what they are paying for, and that dangerous products are not being sold on the open market without proper labeling.

POISONOUS PLANTS AND ANIMALS

Poisonous mushrooms, snakeroot, water hemlock, and rhubarb leaves may produce gastro-intestinal symptoms similar to those associated with microbial food poisoning. Water hemlock resembles parsnips or carrots as it grows in swamps or in wet meadows. When food was scarce during the first World War, the use of rhubarb leaves was recommended for human consumption to supplement the diet with leafy vegetables. Time proved that chemical constituents of the rhubarb leaf were toxic to humans and increased the clotting time of blood to dangerously high levels.

PREVENTION OF FOOD POISONING AND INFECTION

To summarize briefly, most suffering from food poisoning can be avoided by the application of the following precautions:

1. No person should be permitted to handle food for public consumption unless he understands and practices the essential rules of sanitation, both with respect to the cleanliness of his

own person and the utensils and equipment employed in the preparation of food.

2. No person known to be a carrier of enteric pathogens should be permitted to prepare food for others.
3. No person should be allowed to handle food when he has any draining infection, whether it be on his hands or on other parts of his body. During periods when he is suffering from an upper respiratory infection, he should be kept away from places where food is being prepared for others.
4. Foods should not be allowed to stand at warm temperatures for extended period of time before use if they are the types of foods in which rapid bacterial multiplication can occur.
5. Foods should be adequately refrigerated, preferably at a temperature near 40° F.
6. If foods like potato salad must be stored for any period of time before being served, distribute them in shallow layers (two or three inches deep) to allow rapid chilling of the entire mass.
7. Protect all foods from insects and vermin.
8. Avoid cream-filled pastries and custards in public eating establishments during the warm months of the year.
9. Do not attempt to home-can protein foods, especially non-acid types, unless the product is subjected to processing with steam under pressure.
10. Never taste canned foods that have an "off odor" unless they are first boiled for at least ten minutes. Food that is obviously spoiled should not be eaten even after cooking, because thermostable toxins may withstand the cooking and when ingested may cause severe gastro-intestinal disturbances.
11. Do not cook foods in cheap enamelware that is chipped.
12. Wash all fresh fruits before eating.

If a detailed list of all precautions were to be tabulated, there are undoubtedly other warnings that might be mentioned. But if persons would follow the twelve rules above, a great deal of human suffering could be avoided.

CHAPTER 16

Disease Transmission and Man's Resistance

DEFINITION AND THEORIES OF DISEASE
MODES OF TRANSMISSION AND PORTALS OF ENTRY
TYPES OF DISEASE
MECHANICAL AND PHYSIOLOGICAL BARRIERS OF MAN
FACTORS AFFECTING RESISTANCE TO DISEASE
IMMUNITY
ANTIGENS AND ANTIBODIES
PREVENTIVE MEASURES IN DISEASE

DEFINITION AND THEORIES OF DISEASE

It is difficult to define DISEASE. When freely translated the word disease means a lack of being at ease—a state of discomfort. Discomfort is not always caused by disease, but disease always results in some malfunction or discomfort, whether it be to a cell, to a tissue, or to an entire body. Disease may be considered as a departure from normal (whatever normal is), or perhaps a harmful departure from what might be considered the well-being of an individual. When a microorganism is the cause of this discomfort, we designate it as a pathogenic (*pathos* means sadness, and *genic*

means producing) organism. The condition the microorganism produces may be an INFECTION. In some diseases products of microbial metabolism, either in the presence or in the absence of the cells which produced them, can initiate changes in the host which cause symptoms of disease. Food poisoning exotoxins fall into this category. Many microbes are non-pathogenic and do not cause disease.

Health and disease are relative terms. To a person who is deathly ill, someone who "only has a sore throat or a common cold" may be extremely healthy by comparison. But the cold sufferer may feel that he is miserable when compared with someone else who, at the moment, exhibits no signs of illness. Disease is rarely stationary. It may end in recovery, in permanent injury, or in death. HEALTH is defined by some as complete harmony between the organism and its internal and external environment. VIRULENCE is the capacity of an organism to produce disease. Not all disease is caused by microorganisms. There are a multitude of malfunctions of an organic nature that have an etiology other than microorganisms.

Theories as to the cause of disease date to the beginning of man's existence, and since the germ theory was not established until about seventy-five years ago, how did man explain these mysterious maladies which picked out an individual here, and cut short the life of an individual there? Out of the multitude of theories put forth through the ages, a few explanations had greater support than other proposals that had been advanced and rejected.

THE DEMONIC THEORY OF DISEASE

The concept that a body becomes possessed by evil spirits has been one of the more popular explanations of that intangible change that comes over persons who were in good health and spirits such a short time previously. Some remote tribes still employ witch doctors to drive out the demons or evil spirits with incantations and ceremonies. Superstition has a powerful hold on many "civilized" persons, as can be observed almost every day. Bad luck (evil spirits?) will pursue that individual who chances to

walk under a ladder, or that person whose path is crossed by a black cat. Biblical writings give references to "those who are possessed." While the methods employed for removing evil spirits make interesting reading, there is little that is scientific in the procedure.

THE MIASMIC THEORY OF DISEASE

The observation was made in years gone by that persons who lived in areas abounding with swamps and bogs appeared to have a disproportionate amount of illness. Polluting agents in the vapors, or *miasmas*, were believed to be the cause of the sickness. Miasma is a word of Greek origin and means defilement, and such emanations from swamplands defiled the atmosphere, according to popular notion. A direct carry-over from this era is the word *malaria*, which means bad air. What more proof did anyone need than the observation that those who worked in sewers contracted severe chills and fever which might last for prolonged periods? The misnomer, malaria, has persisted through the years, even though we know that even in the finest air-conditioned atmosphere, a person may contract malaria if the right species of an infected female anopheline mosquito bites a susceptible individual. It just so happened that the sewers provided the breeding ground for such mosquitoes, with the foulness of the air being an unrelated concomitant factor. It is so easy to draw hasty conclusions in science!

THE HUMORAL THEORY OF DISEASE

Hippocrates (460–395 B.C.) believed that a harmonious relationship between the four humors of the body—phlegm, blood, yellow bile, and black bile—was essential if a person was to remain in a healthy condition. When any one or more of these humors got out of balance with the others, disease was the expected alternative. Blood-letting as a means of restoring the proper balance between the humors was commonly practiced in the United States at the time of George Washington. A carry-over of this theory of disease is found in such common non-medical expressions as *sanguine*, denoting too much blood and a confident, and hopeful attitude. *Melancholy* is derived from black bile, indicating a state of low

spirits due to an over-abundance of this humor. *Phlegmatic*, which today means a sluggish, indifferent reaction, originally indicated a disproportionate amount of phlegm.

PYTHOGENIC THEORY OF DISEASE

Dirt and filth, according to this theory, was the cause of disease, especially typhoid fever. Decaying vegetation and animal matter provided an excellent breeding ground for disease. This was pure speculation with no scientific evidence to back it up when it was originally proposed.

THE GERM THEORY OF DISEASE

The belief that sub-visible organisms were the cause of much of man's misery was postulated early in the nineteenth century, when a few poorly conducted experiments were carried out with no conclusive findings. Jacob Henle, a pathologist, advocated in 1840 that more careful investigations be conducted to prove or to disprove this germ theory. If germs really cause disease, Henle believed that they should be found in infected tissue, they should be isolated from all other living matter, and when these isolates are injected into animals, the germs should cause disease. Robert Koch, who is usually given credit for the postulates he put forth to prove the germ theory of disease, drew rather heavily on Henle's proposals. This theory was not a sudden inspiration; it was the result of the contributions of a number of workers, including Leeuwenhoek, Fracastorius, and Plenciz. But until Koch had provided scientific proof through practical application of his postulates, the germ theory of disease was not accepted.

MODES OF DISEASE TRANSMISSION AND PORTALS OF ENTRY

Leaders in the field of public health frequently teach that transmissible disease can often be attributed to one of the four F's: *flies, fingers, fomites,* or *food.* To this list might be added droplet infection and direct contact with infected persons and animals.

FLIES

While flies and other insects are generally considered to be passive agents in the transfer of many microorganisms, recent evidence indicates that if insects ingest a large enough dose of specific enteric organisms, a spillover point may be reached, and the insects may serve as active cultures with multiplication of the pathogens in their digestive tract and excretion of the microorganisms in their feces over an extended period. This point will be discussed further in a later chapter devoted to insects and disease transmission.

FINGERS

From the previous discussion of food poisoning it should be clear that fingers and hands may be extremely important in the spread of food poisoning and food infection. Clean hands and short, clean finger nails are imperative if the spreading of disease is to be minimized by food handlers.

FOMITES

The importance of inanimate objects, called FOMITES, in the dissemination of disease is not well understood, and ignorance has undoubtedly resulted in over-emphasis of fomites. But certain inanimate objects, such as the public drinking cup, can be instrumental in spreading microbes since it is virtually a direct contact with infected individuals. The common Communion Cup, while it is still used in some churches, has largely been replaced by individual glasses. In spite of any oligodynamic action that a shiny silver chalice may afford, the relatively short time period between human contacts with the lip of the cup does not allow much, if any, disinfecting action to take place.

FOOD

Since food was discussed in the previous chapter, further elaboration here seems unnecessary other than to repeat that much human suffering can be traced to careless handling of plant and

animal materials to be consumed as food. Lack of proper sanitation and improper refrigeration are the principal causes of food poisoning.

DROPLET INFECTION

Any person suffering from an upper respiratory infection is a potential hazard to others with whom he may come in contact. Whether this disease is spread to others depends upon the virulence of the organism, the susceptibility of the contact, the time factor after the droplets were expelled, the number of organisms involved, and a host of other inter-dependent forces.

DIRECT CONTACT

If pathogens are transferred directly from one individual to another through direct contact of kissing, etc., little opportunity is afforded for the organism to be weakened by drying, exposure to sunlight, and other factors. If the new host provides favorable conditions for the multiplication of the bacteria, another link in the chain reaction has been provided, and the disease continues to spread.

TYPES OF DISEASE

CHRONIC DISEASE

When a disease progresses slowly and lasts for extended periods of time, it is called a CHRONIC DISEASE. Rheumatism, arthritis, leprosy, and some forms of tuberculosis fall into this category.

ACUTE DISEASE

If a disease reaches a point of greatest intensity in a relatively short space of time and recovery is not prolonged for months or years, we term it an ACUTE DISEASE. Pneumonia, typhoid fever, and diphtheria are examples of acute diseases. There is no sharp dividing line, however, between acute and chronic illnesses.

ENDEMIC DISEASE

An ENDEMIC DISEASE (*en,* in: *demos,* people) is one which is usually present in small numbers in any given community. Some-

one always seems to be suffering from a common cold, or from measles or mumps.

EPIDEMIC DISEASE

If endemic diseases increase in number very quickly, they become EPIDEMIC (*epi,* upon; *demos,* people) in character. Childhood diseases have a way of appearing in epidemic waves among school children. These diseases seem to disappear just as rapidly as they came, even though there are still a number of children who apparently were equally exposed yet resisted infection. Their immunity, in other words, protected them from particular attacks, but subsequent epidemics, perhaps caused by more virulent organisms, may well affect those children who escaped the first wave of a particular infection.

PANDEMIC

When epidemics "go wild," that is, spread over vast areas—whole continents or over many continents—they are termed PANDEMICS (*pas,* all; *demos,* people). The plague of the middle ages and the influenza pandemic after World War I are classed in this group.

PRIMARY INFECTION

A full-blown case of the common cold is probably caused not by one but by a number of microorganisms. The *primary* cause appears to be a virus, but unless the infection progresses, a typical common cold will not result.

SECONDARY INFECTION

After the initial groundwork has been accomplished by the primary invaders, *secondary* opportunists take over in the common cold, and much of the misery of this affliction can be attributed to these secondary infecting organisms. The use of antihistamines might be considered to be an attempt to slip a wedge between the primary and the secondary infections of a common cold. Sometimes it appears to work, while at other times, the disease takes its usual course. The conclusion of an extensive study in England on the causes and possible cures for the common cold are of interest.

The investigators reported that a treated cold lasted just about seven days, while an untreated cold lasted a week!

Bacteremia

There are times when bacteria gain entrance to the blood stream of man and of lower animals. When biological supply houses are interested in drawing quantities of blood from horses used in the production of antitoxins, food is withheld from these animals for a number of hours before the bleeding operation. Experience has shown that shortly after eating, particularly after a meal of dry hay or grain, it is not uncommon to find bacteria in the bloodstream of these animals. Such a condition is called a BACTEREMIA. To prevent contamination of the blood and also to insure that the blood serum will be clear rather than turbid from circulating food, this withholding of food prior to bleeding is necessary.

Septicemia

It is only when bacteria get into the bloodstream and multiply at the expense of the blood in spite of the body defenses that the term SEPTICEMIA is applied to designate a true blood poisoning which might be fatal unless corrective measures are instituted. It is not uncommon to hear the term *bacteremia* being used synonymously with the term *septicemia.*

TOXEMIA

Bacterial poisons, either pre-formed or generated after entering the body, cause a condition known as TOXEMIA. The destructive power of *Clostridium tetani* and *Corynebacterium diphtheriae* is the result of true toxins excreted by the organisms which themselves generally remain localized in the body. Bacterial toxins have affinities for specific organs or tissues, and these poisons wreak their havoc at sites sometimes far removed from the focus of the original infection. In the terminal stages of diphtheria, however, it is not uncommon to isolate the living organisms from many sites other than the original localized infection.

MECHANICAL AND PHYSIOLOGICAL BARRIERS OF MAN

Unless we were endowed with powerful protective armor, the hordes of microorganisms on us and around us would make our life expectancy very brief indeed. Just as a military strategist prepares for any eventuality to the best of his ability with the weapons at his command, nature has provided us with two major lines of defense against possible invading microorganisms. The physical structure of our body is the first protective barrier. Internal physiological forces step in when the first line of defense has been breached. If the combination of these weapons is insufficient to cope with the situation, and if medicine does not have the proper last ditch stand weapons to destroy the pathogens, the death of the animal is the expected consequence.

EXTERNAL PROTECTIVE FACTORS

The Skin

Contrary to popular belief, the human skin is not tissue paper thin; it is composed of many layers of cells suitably interlocked to prevent the entrance of microorganisms under normal conditions. Because of the minute size of microbes, however, a large opening is not required to invite penetration by bacteria. Since micrococci found as normal skin inhabitants measure less than 1/25,000 of an inch in diameter, it is easy to see that even though there are no breaks in the skin visible to the unaided eye, bacteria can penetrate what appears to be unbroken skin, and such infections as pimples may result.

Not only is skin a resistant physical barrier, it also contains germicidal substances which are capable of destroying many transient organisms which may lodge on the skin.

The Stomach

Considerable disagreement exists as to the germicidal action of the stomach contents, but the physical barrier which the stomach tissue affords is not questioned. The relatively high concentration

of acid found in gastric juice probably is detrimental to some organisms, and some of the enzymes undoubtedly affect bacteria if food remains in the stomach long enough for such enzyme activity to take place. Liquids tend to pass through the stomach into the intestines rather quickly, and organisms are carried along with the tide of fluid which, incidentally, temporarily dilutes the acid in the stomach.

Mucous Membranes

The moist membranes lining the nose, mouth, and nasopharynx serve a useful purpose as part of our defense mechanism. With the aid of cilia which sweep trapped material up toward the mouth, and the protective sieve afforded by the hairs of the nose, microorganisms have a relatively difficult time getting into the deeper respiratory passages. Since people do contract pneumonia, however, it is evident that the more aggressive organisms can overcome the body defenses, particularly when the resistance of the host is reduced.

The Lungs

These organs are constantly bathed with body fluids from their extensive blood supply. The many barriers between the hairy moist surface of the nose and the rather distantly removed lungs, help to minimize the number of organisms which find their way to the lungs. Once bacteria do arrive at the lungs, various physiological forces help to destroy them.

The Intestines

After food leaves the stomach and enters the upper intestines, a *p*H reversal occurs, and at the point where bile flows into the intestines, marked changes in the bacterial flora take place. Practically a pure culture of *Escherichia coli* can be found just below this bile inlet. Enteric pathogens, including the typhoid and paratyphoids, must be able to survive the action of stomach juices, bile, and enzyme activity in the intestines in order to cause disease in the digestive tract.

INTERNAL RESISTANCE FACTORS

The Blood

In addition to the usual cellular elements and fluids found in the blood, there are protein elements called antibodies which have been manufactured by selected tissues of the body and have spilled over into the blood and other body fluids. Since these antibodies are specific and work against the bacteria, or ANTIGENS, which stimulated their production, individuals who have had microbial diseases, or who have been injected with vaccines containing weakened or dead organisms, will possess protective antibodies to combat the specific bacteria. This is a form of IMMUNITY, and this topic will be discussed later in the chapter.

The Lymph

This fluid is similar to blood in its composition but it lacks the cellular elements found in blood. Lymph seeps out of the finer blood vessels (capillaries) and bathes the surrounding tissue spaces, eventually gathering at large drainage points from where it is returned to the blood vessels.

The Tissues

The liver and the spleen serve as filtering devices for the removal of circulating microorganisms. These organs are included in the sites generally accepted for antibody production, and hence they may be better able to cope with the microbes which find their way into these organs.

FACTORS AFFECTING RESISTANCE TO DISEASE

HEREDITY AND CONGENITAL FACTORS

When a geneticist speaks of heredity he usually means that the individual acquired certain characteristics directly through the genes. Microbial diseases, therefore, are not inherited. After fertilization of an egg takes place, the embryo or fetus may be

acted upon by microbes, and the child or animal may be born with microbial diseases if it survives. This, however, is a *congenital* not an *inherited* disease. Without considering all of the factors, the layman observes that infants may suffer from the same microbial diseases, such as tuberculosis, that one or both parents may have. It seems logical to them to assume that the child inherited the condition, whereas the disease was congenital or was acquired after birth through direct contact with the infected parent.

AGE

There are such things as childhood diseases: measles, mumps, chicken pox, whooping cough, and the like. Since these diseases are contagious, the initial contact that a young child has with them may be sufficient to cause infection of the child who may have little or no resistance, or immunity, to the infectious agent unless he has been previously vaccinated. By the time that people reach adulthood they either have had these diseases as children, or, having escaped early contact, they may have come in contact with subclinical doses of the organisms and built up a degree of immunity to the disease. While the number of adults who contract so-called childhood diseases in their later adult life is not statistically great, there are plenty of such cases on record. In general, adults are affected more severely than they would have been had they contracted these diseases as children. Mumps is particularly severe in males after the age of puberty, since the virus of mumps has an affinity for the sex organs of males and may cause sterility of the individuals concerned.

MENTAL STATE

While it is impossible to point with assurance to mental state as a factor influencing our resistance to disease, it does appear that a happy, care-free individual is less likely to be ill, or at least to feel ill, than a person who is constantly worrying about himself or his unfortunate lot in life. This is certainly true with regard to ulcers, which is a disease primarily of worriers. People who are always

feeling sorry for themselves begin to imagine that they have all kinds of illnesses. The feeling reaches such proportions that they may exhibit definite signs of organic disturbances. Yet it is their mental state, not organic dysfunction, which accounts for their misery. Psychosomatic medicine is an attempt to treat such conditions, and the success of some of these treatments probably justifies the existence of such a theory. Just exactly how mental state can affect your health is not clearly understood, but if being optimistic, happy, and care-free helps to maintain your health, it seems worth a try.

LIVING CONDITIONS

Crowding is not to be encouraged if people are to live happily and healthfully. Certain diseases, once gaining a foothold in crowded areas, can spread quickly and cause a disproportionate number of cases of disease when individuals are living in close contact with one another. But again, the exact effect of crowding cannot be pinpointed without considering the many other forces operating at the same time. Persons who are well fed, for example, would have a better opportunity of resisting disease than a malnourished group of individuals crowded together in close quarters. The widespread epidemics so prevalent among refugees herded together in compounds during war time are accentuated by the malnutrition and unsanitary conditions so apt to be present at the same time.

FATIGUE

A run-down person is more susceptible to disease than an individual who has had plenty of rest. When the tissues of the body are over-worked without adequate periods of rest to allow them to repair and rebuild themselves, it is logical to assume that the fighting power of such tissues will be less than that of well-rested tissues. Growing children who are active so much of the time need plenty of rest. The afternoon nap prescribed for young children is not only a device for getting the child out from under foot of the mother who also needs rest, but it serves to break up the periods of intense activity while the body recuperates. It is

true all persons do not require the same amount of sleep. Some can get by with less than what is considered normal and would find themselves sluggish with what others would consider to be normal sleep requirements.

OCCUPATION

Because of the hazards involved in certain occupations, Departments or Bureaus of Industrial Hygiene have sprung up on the State and the National level. By determining the particular health hazard involved, such Bureaus are able to institute corrective measures as an important phase of preventive medicine. Stone cutters, for example, can breathe in a sufficient number of sharp silica particles during a given time period to cause SILICOSIS and to predispose the individual to pulmonary infections. So-called wet-grinding and the use of protective goggles and face masks have been responsible for marked reductions in silicosis.

SPECIES, RACE, AND NATIONALITY DIFFERENCES

A living thing as complex as a higher animal possesses so many factors which influence its resistance that it is difficult to single them all out. Because a horse is a horse, he does not contract measles. Did you ever see a mouse with mumps? Because horses and mice are members of a given species, they are not susceptible to some diseases to which man falls heir, and likewise we humans do not contract some diseases of lower animals. There are specific diseases, however, to which both man and lower animals are susceptible, including tuberculosis, to mention but one disease.

Being a member of a given race may have its advantages and disadvantages with respect to susceptibility to disease. The Negro appears to be highly susceptible to tuberculosis, at the same time he is more resistant to malaria and to yellow fever than persons of other races. American Indians have a disproportionately high rate of tuberculosis.

There is some evidence that nationality differences exist, but the literature is conflicting in its conclusions.

TEMPERATURE

Some rather interesting studies have been conducted by C.-E. A. Winslow and his co-workers on the relationship of temperature to disease. They found that a temperature of between 84° F. and 89° F. resulted in a marked decrease in at least one antibody, HEMOLYSIN, about which more will be written later. Such studies indicate a possible explanation of some differences recorded in the resistance of individuals in temperate climates as compared with persons in tropical or in sub-tropical areas. The presence of specific disease vectors must also be considered before hasty conclusions are drawn relative to a single factor like temperature.

It is known that certain diseases have a seasonal incidence which may bear a direct or an indirect relationship to temperature. The higher rate of upper respiratory infections during the colder months of the year, for example, may be related to greater indoor living in heated homes with an atmosphere low in humidity. The drying action of such air on mucous membranes may be more important than the effect of outside temperature on the respiratory tract.

IMMUNITY

The term IMMUNITY is derived from the Latin word *immunis*, which means exempt. The word today has various meanings depending upon the specific use to which it is put. From our point of view immunity might be considered to be the tendency to resist infectious disease. This definition implies that there are degrees of resistance, and this happens to be the case. Immunity to some virus-induced diseases, for example, may be virtually absolute for varying periods of time; or there may be decreasing degrees of immunity with other viruses and other microbes, until the degree of resistance induced is so weak as to be ineffectual.

THEORIES OF IMMUNITY

It was only natural for keen observers eventually to notice that individuals who contracted certain diseases rarely became ill with that same disease a second time. Still other diseases, however,

tended to affect the same person repeatedly. Once the germ theory of disease had been established, a number of attempts to explain these differences in resistance were proposed, but the following four theories received more than passing interest at one time or another.

Pasteur's Exhaustion Theory

The more one studies the history of microbiology, the more he is impressed with the diversity of topics with which Louis Pasteur concerned himself. He was a brilliant scholar, and while subsequent research in the light of new knowledge disproved some of Pasteur's ideas, his fertile mind proposed lines of investigation faster than people were able to study them. With respect to immunity, he felt that the host probably harbored some substance vital to the growth of given organisms. Once the microbes had exhausted the supply of this required substance, the invading microorganisms died or left the host. Since this same species of microorganism could not survive in the absence of this essential substance, further infection was impossible in that particular host. In other words, he was immune. While interesting historically, this theory of immunity has no scientific foundation.

Metchnikoff's Cellular Theory

As early as 1870 it had been observed by several investigators that white blood cells (leucocytes) sometimes harbored bacteria, but it was not for nearly fifteen years after this observation that Elie Metchnikoff put forth his concept of immunity based upon the reaction which is called PHAGOCYTOSIS. Phagocytes, which means devouring cells, are certain types of white blood cells which are capable of ingesting and destroying bacteria, but this devouring ability is increased in the presence of an antibody called OPSONIN, which prepares the bacteria for engulfment by the leucocytes. When PUS is examined under a microscope it is found to consist of bacteria engulfed by white blood cells, serous fluid, and debris. While many invading bacteria may meet their doom as the result

Fig. 51. Elie Metchnikoff (1845–1916). (*By permission from* Introduction to the Bacteria *by C. E. Clifton. Copyright, 1950. McGraw-Hill Book Company, Inc.*)

of phagocytic activity, this mechanism is not the only explanation we have to account for immunity.

The Bacteriophage Theory of d'Herelle

As minute as bacteria are, they are still subject to parasitic action by sub-microscopic entities called BACTERIOPHAGE, or PHAGE for short. The term literally means "bacteria eater," and it is regarded as being a virus specific for bacteria. After the phage has invaded a susceptible bacterial cell, active multiplication of the virus occurs with an eventual bursting of the host cell and a spilling out of the bacteriophage. This dissolving action is called LYSIS. The lytic action of phage was first reported in 1915 by Twort who was working with a staphylococcus culture growing as a contaminant in a cowpox vaccine. This original observation was substantiated by d'Herelle in 1917 when he was studying a dysentery culture to which had been added a bacteria-free filtrate of feces obtained from individuals suffering from bacillary dysentery

The peculiar dissolving properties of this agent have since been called the "Twort-d'Herelle Phenomenon."

Phage resembles viruses in size (range of from 10 to 75 millimicrons), in requiring a living host cell for growth, in its extreme specificity, and in other properties.

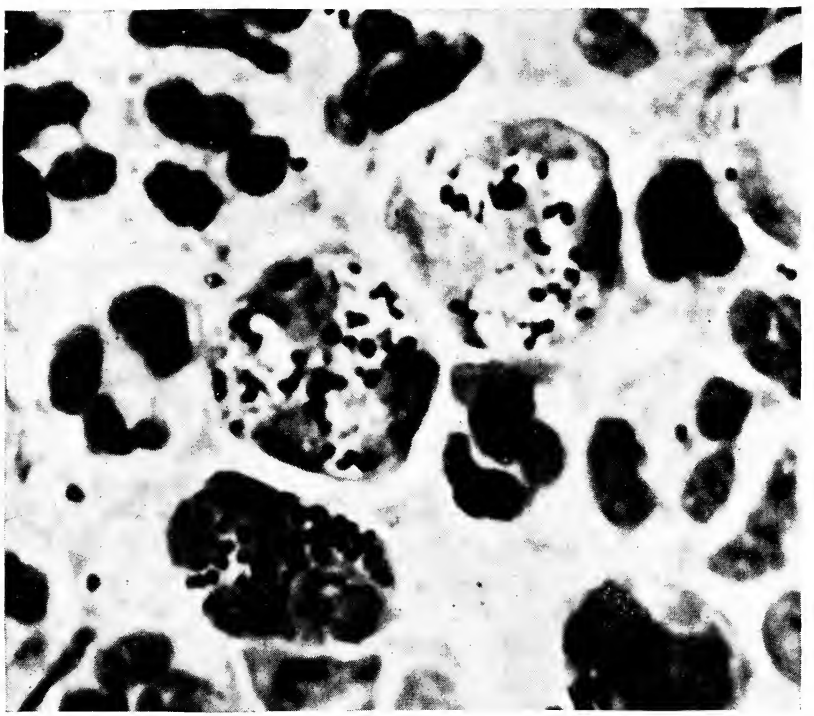


Fig. 52. Photomicrograph of pus showing phagocytosis of diplococci by white blood cells. (*From* Microbiology, *W. B. Sarles, W. C. Frazier, J. B. Wilson, and S. D. Knight. Copyright 1951, Harper and Brothers, New York.*)

It was d'Herelle's belief that as soon as bacteriophage had been sufficiently activated within the animal body, the virus could destroy the bacteria for which they were specific, and the patient recovered. The accuracy of this theory has never been substantiated.

The Humoral Theory of Immunity

Toward the end of the nineteenth century Buchner, von Behring, and others reported that the blood serum of persons who had been vaccinated, or who had recently recovered from certain diseases, contained protein fractions called ANTIBODIES. Such humoral (fluid) immunity appeared to be the clue for which research workers had been looking. Our present beliefs with respect to immunity are largely dependent upon these antibodies as our explanation of degrees of resistance to disease. The types of these antibodies will receive more attention in later discussions in this chapter.

TYPES OF IMMUNITY

Whenever a person or a lower animal plays an active part in building up his own resistance to microbial diseases, it is termed ACTIVE IMMUNITY. But if antibodies produced in other persons or in lower animals are injected into an individual who has played no active role in the manufacture of these protective substances, this is PASSIVE IMMUNITY. Active immunity is longer lasting than passive immunity.

Having been born the particular species of animal that we are endows us with a *natural* resistance to certain diseases and natural susceptibility to other diseases. Man is apparently the only animal susceptible to typhoid fever. Dogs can contract distemper, but man is refractory. While immunity is a relative thing, natural resistance to certain microbes because we are human beings is a complete immunity.

Acquired immunity can be induced either by recovering from a disease, or by injecting the microbial agent or its products. Therefore there is both a NATURAL ACQUIRED IMMUNITY and an ARTIFICIAL ACQUIRED IMMUNITY.

When we are born we acquire for relatively short periods of time an immunity to at least some of the diseases for which our mothers have antibodies. Even though the cellular constituents of the mother's blood are not common to the circulation of the

fetus during the gestation period, the fluid portions and the soluble protein fractions are transferred to the developing fetus. Since the fetus has done nothing to build up these antibodies, it is a NATURAL, PASSIVE, ACQUIRED IMMUNITY. If antibodies built up in other animals are injected into a person, this is ARTIFICIAL, PASSIVE, ACQUIRED IMMUNITY.

ANTIGENS AND ANTIBODIES

An ANTIGEN is any substance, usually protein or protein-like, which when introduced into the body by some route other than through the digestive tract, will induce the production of ANTIBODIES which are capable of reacting specifically with the antigen which caused their stimulation. It is the chemical structure of the antigen which determines specificity, and the corresponding antibody might be looked upon as a lock and key type of relationship. Individual keys rather than master keys are the rule in antigen-antibody reactions. In order to serve as an antigen, the material must be foreign to the animal into which it is being introduced. Different portions of a single bacterial cell may function as individual antigens. The flagella, for example, are chemically distinct from the rest of the cell, and therefore the flagella will stimulate production of specific antibodies which are distinguishable from the cellular (*somatic*) antibodies.

Some materials while not antigenic in themselves will, when linked with proteins, impart to the host protein a specificity different from that of the underlying protein. These substances, sometimes carbohydrates and other times fats, are termed HAPTENES, or partial antigens. The more than seventy types of pneumococci are differentiated from each other on the basis of such carbohydrate haptene fractions found in the capsule layer. The underlying pneumococci are very similar, if not identical with each other, once the capsule has been stripped away.

It is difficult to know how far to go with this rather complex yet fascinating topic of antigens and antibodies. Volumes have been written on the subject, but a few of the fundamentals will be

presented here merely to acquaint the reader with some of the terminology and concepts of immunity as expressed through antigens and antibodies.

An *antibody* is defined in terms of antigens. That is, antibodies are the substances produced as the consequence of specific stimulation of certain body tissues by the agents we term antigens. Antibodies are protective substances which can be found in the blood and in other body fluids and tissues after a suitable lapse of time following antigenic injection. The time period depends upon the particular antigen and upon the serological response of the animal injected. Many antigens will produce measurable quantities of antibody in a week or less, with a maximum antibody level (called *titer*) appearing in from ten to fifteen days after primary antigen injection.

Considerable controversy still exists relative to the nature of antibodies, and as to whether one species of organism produces many different antibodies which we detect by different tests, or whether there is but one antibody—the unitarian hypothesis. A few of the recognized types of antibodies will be discussed.

Agglutinins

If whole cells are injected into an animal for which they are a foreign substance, the blood serum can in time be shown to contain a substance capable of causing the cells employed as antigen to clump together, or to agglutinate. This antibody is called an AGGLUTININ, and the antigenic cells are called AGGLUTINOGENS (make agglutinins). Cells in suspension tend to repel each other because of physical phenomena including electrical charges, but when the cells have been acted upon by agglutinins, something happens at the cell surface which encourages cells to stick together, or to agglutinate.

This type of serological reaction is useful in the identification of bacteria which are divisible according to their antigenic components. When known antibodies react with given organisms, the antigenic structure of those bacteria can be determined. Agglu-

tination reactions with red blood cells, including O-A-B blood typing and Rh grouping, will be considered in detail later.

Precipitins

If disintegrated cells or any proteins in virtual solution are employed as antigens, an antibody termed a PRECIPITIN is formed by the body of the injected animal. This antibody when put in contact with the colloidal antigen will result in the precipitation of the dissolved cellular material. Such an antigen is a PRECIPITINOGEN.

One of the intriguing uses of the precipitin reaction is in medico-legal work, especially in the identification of blood stains on clothing or on weapons used in assault and murder cases. The exact technic of performing these tests is left for advanced textbooks in the field.

Opsonins

In Metchnikoff's cellular theory of immunity, discussed earlier in this chapter, it was pointed out that before cells could be phagocytized (or phagocyted) they had to be prepared for this migration through the walls of leucocytes. The agent which prepares organisms for such engulfment is the antibody OPSONIN, which literally means "to prepare food for." Even in the apparent absence of antibody, white blood cells ingest a certain number of organisms, but in the presence of opsonins which are formed as the animal builds up immunity to the invaders, a marked increase in phagocytic activity occurs. The difference between the normal number of organisms ingested and the number taken in by leucocytes in the presence of opsonin is used in calculating the so-called OPSONIC INDEX, which has some diagnostic significance in such diseases as undulant fever and tularemia.

Lysins

These are antibodies which lyse (dissolve) cells. But in addition to these antibodies, a second non-specific thermo-labile (de-

stroyed by heat) substance called COMPLEMENT, found in the blood of warm-blooded animals, must also be present before dissolution of the cells can occur. If the antibody dissolves blood cells, it is called HEMOLYSIN, and if it lyses bacteria, it is termed BACTERIOLYSIN. The Wasserman test for syphilis employs hemolysin as one step in this rather involved serological reaction.

Antitoxins

When sub-lethal doses of exotoxins are injected into animals, the host responds by producing ANTITOXINS which are capable of neutralizing the specific toxins. Such an active immunity requires a matter of days before the antitoxin titer builds up to levels which are required to protect individuals from otherwise lethal doses of toxin. If a person has been diagnosed as suffering from a disease in which exotoxins are the destructive agents, a passive immunity should be conferred on the patient by injecting antitoxins produced in other persons or in lower animals. The injected antitoxin will neutralize the effects of the circulating poisons, and if the disease has not progressed too far, this passive transfer of antibodies will help to protect the patient until his own antitoxin-producing mechanism can confer active immunity upon him.

Neutralizing Antibodies

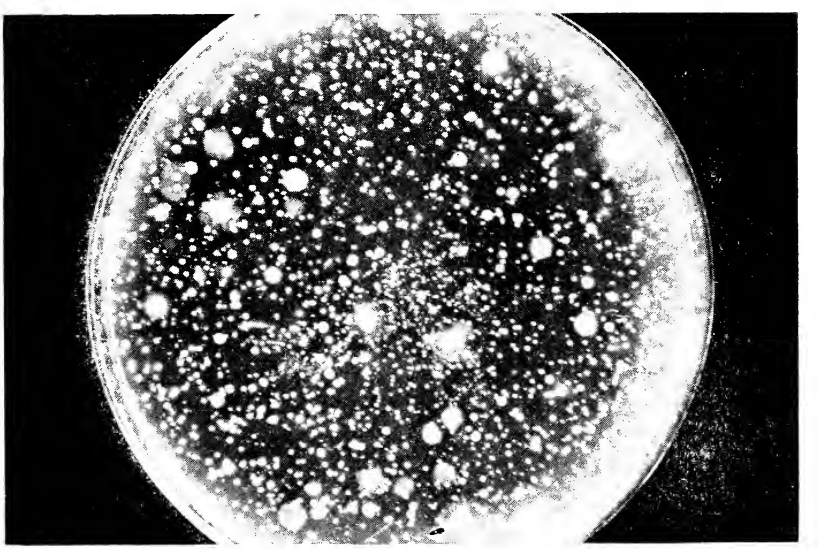
When dealing with virus diseases, the antibody which reacts with the pathogenic virus is capable of inhibiting the virus action, and this antibody is termed a NEUTRALIZING ANTIBODY. The nature of this reaction is not clear, but presumably it involves a physicochemical union of antibody molecules with virus particles.

PREVENTIVE MEASURES IN DISEASE

QUARANTINE

The term *quarantine* has its origin in the French word *quarantaine*, meaning forty. As early as 1403 quarantine stations were set up in Venice to control the spread of disease from passengers

A



B

Fig. 53. (A) An unstifled sneeze directed into a culture dish containing nutrient agar. (B) Colonies of bacteria which have developed on the medium exposed to the sneeze. (*Courtesy of M. W. Jennison, S.A.B. Nos. 17 and 18.*)

on incoming ships to susceptible individuals on shore. A period of forty days was established as a safe interval during which any infected persons on shipboard would come down with clinical symptoms of any diseases harbored on the ship. The United States still devotes considerable time and effort to minimize chances for diseases to be spread from persons arriving in the country by land, sea, or air. While our technics are quite different from those employed five centuries ago, the results have justified the effort.

Persons suffering from some of the more serious contagious diseases, including diphtheria and scarlet fever, are usually cared for in isolation wards of our modern hospitals, and every precaution must be exercised to control the spread of the infectious agents to other patients and to hospital personnel. The body discharges of such patients must be handled in a special way to insure that they do not serve as a source of material for other infections.

Not too many years ago hospitals caring for patients suffering from contagious diseases were located on the outskirts of cities to minimize the chance for microbes to find their way from the sick rooms to the healthy people of the city. While this may have seemed a reasonable measure in the early days of microbiology, we know that such precautions about locating hospitals are unwarranted, and hospitals today are placed where they can most effectively serve the people in the community.

USE OF DIAGNOSTIC TESTS

The Schick Test

This reaction is based upon the INTRADERMAL (into the skin) injection of a carefully measured amount of diphtheria toxin into the forearm of individuals to test their susceptibility to diphtheria. The test was devised in 1913 by Bela Schick. If the person is immune to diphtheria, he has sufficient antitoxin circulating in his blood stream to neutralize the small amount of injected toxin. Lacking such antitoxin, a person exhibits a reddened, swollen, inflammatory reaction called a positive Schick Test, and such a response indicates susceptibility to the disease.

The Dick Test

This test was devised by George and Gladys Dick in 1924 as a means of determining susceptibility of the individuals to scarlet fever. The underlying principle is the same as that in the Schick test, and mass surveys can be conducted during impending epidemics to determine susceptible and resistant individuals in a given population.

The Tuberculin Test

In contrast to the Schick and the Dick tests the tuberculin reaction is not an index of susceptibility. It is an allergic response to specific proteins produced by *Mycobacterium tuberculosis.* ALLERGY means altered reactivity, and the word is more or less synonymous with hypersensitivity. Microbial antigens injected into animals usually initiate the production of specific antibodies which help to protect the recipient from the specific microbe or its products. The introduction of some proteins, however, may cause the animal to become sensitive (or hypersensitive) to that protein, and upon subsequent injection of this antigenic material, an allergic response may cause reactions severe enough to cause death in some instances. When hypersensitivity is induced in animals other than man, it is termed ANAPHYLACTIC SHOCK or anaphylaxis —"against protection."

Robert Koch prepared the first tuberculin in 1890, and this crude material is called *old tuberculin,* in contrast to a modern preparation known as *purified protein derivative* (PPD). By injecting, or by rubbing into the skin, a minute amount of tuberculin, which is an extract produced from old cultures of *Mycobacterium tuberculosis,* a localized hypersensitivity manifests itself by a reddening and swelling at the site of the injection, if the animal is tuberculin positive.

If a person has ever been infected with tuberculosis organisms, he is allergic to the tuberculo-protein; even an old walled-off lesion is capable of causing a positive skin reaction. Positive reactors, particularly in the adolescent age group, should follow up this

finding with other diagnostic tests, including X-ray examination to determine the nature and the extent of the focus of infection, microscopic examination of sputum for the presence of acid-fast organisms, and guinea pig inoculation with sputum or other test material if clinical symptoms warrant such tests.

The extensive application of tuberculin testing of cattle has paid rich dividends, since a positive reaction in cattle is good evidence that the animal has an active case of the disease and should be slaughtered to prevent spread of the infection to healthy cattle or to humans.

VACCINES

Smallpox Vaccine

When Edward Jenner demonstrated the effectiveness of smallpox vaccine in 1796, a new attack on disease was available to medicine. The word VACCINE is derived from the Latin *vacca,* which means cow, and the technic of vaccination had its origin in

Fig. 54. Edward Jenner (1749–1823). (*By permission from* Introduction to the Bacteria *by C. E. Clifton. Copyright, 1950. McGraw-Hill Book Company, Inc.*)

Jenner's use of cowpox virus as an immunizing agent against smallpox. He observed that milkmaids who contracted cowpox appeared to be immune to dreaded smallpox. By rubbing or scratching cowpox vaccine into the skin of humans, they became immune to smallpox.

Fig. 55. The first vaccination to prevent smallpox. (*From the Fisher Collection of Alchemical and Historical Pictures.*)

Such vaccine is presently prepared by rubbing living cowpox virus into scratches made on the shaved and disinfected abdominal surface of calves. When typical eruptions develop in about a week's time, the crusts are scraped off and the material in the underlying lesions is harvested and suspended in 50% glycerol.

After storage in a refrigerator to permit any contaminating bacteria to be killed by the mildly antiseptic glycerol, the material is tested for purity and suitability before the virus is dispensed in capillary tubes for individual vaccination doses. Proper refrigeration of these tubes is important if the virus is to be stored for any period prior to use.

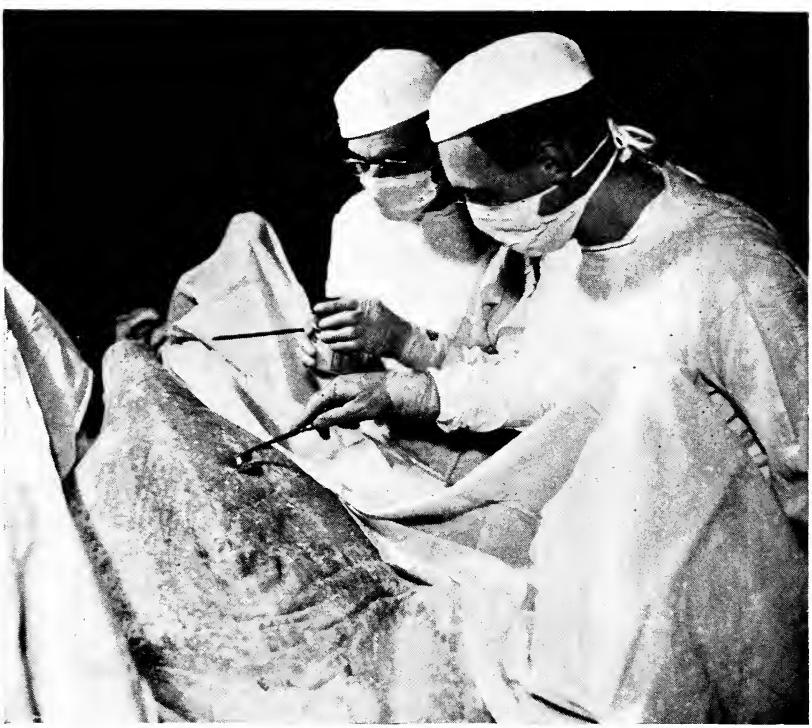


Fig. 56. Harvesting smallpox vaccine. (*Reproduced by courtesy of Parke, Davis and Company's* Therapeutic Notes.)

Smallpox vaccination usually lasts for about five years. It is customary to vaccinate children at about the age of one year, and again at age six. This usually confers life-long immunity, although during impending smallpox epidemics, mass vaccination of all persons is recommended. This disfiguring and killing disease has become a rarity in countries where smallpox vaccination is required.

Rabies Vaccine

When one visits Paris and sees the statues and monuments, he gets the impression that Louis Pasteur's development of a treatment for the prevention of rabies is the contribution most memorialized by his countrymen.

Because of the relatively long incubation period for rabies, up to eight weeks or more, active immunization can be employed in persons bitten by rabid animals, or by animals suspected of having rabies. The most common technic is the use of Semple vaccine, which is a phenolized preparation of infected rabbit spinal cord. Daily subcutaneous injections of vaccine for a period of fourteen days after exposure will produce active immunity and will usually prevent the development of the disease in humans.

Rabies is caused by one of the larger viruses, measuring up to 250 millimicrons. The etiological agent is transmitted to man through the saliva by bites of dogs, wolves, and vampire bats. Other animals have also been known to carry the virus. The organism has a strong affinity for cells of the central nervous system, and the incubation period before clinical manifestations appear is related to the distance from the brain that the bite is inflicted. Bites around the face generally have a shorter incubation period than bites on the legs or on the arms.

When an animal suspected of being rabid is sent to the laboratory for examination to confirm the suspicion, stained sections of the animal's brain are examined under the microscope for characteristic granules within certain brain cells. The presence of these *Negri bodies* confirms the diagnosis of rabies. Once the clinical symptoms appear in humans, the chances for recovery drop off very sharply.

Bacterins

A bacterin is a killed culture of bacteria, and when this vaccine is injected into susceptible animals it induces an active immunity. After cultivating the organisms under suitable growth conditions, which sometimes involves growing them in animals (so-called

animal passage), the bacteria are killed by subjecting them to a temperature of between 56 and 60° C. for from forty-five to sixty minutes. Temperatures above 60° C. often destroy certain antigenic properties of the bacterin. Inactivation of the organisms may also be brought about by the addition of chemicals, such as formalin, or by exposing them to ultra-violet radiation or to supersonic vibrations.

After adjusting the concentration of organisms with physiological saline as a diluent, sterility checks are conducted to make certain that neither aerobes nor anaerobes are alive in the bacterin after the physical or the chemical treatment. Merthiolate in a bacteriostatic concentration is frequently added to serve as a preservative.

Bacterins have proven valuable in prevention of typhoid fever (*Salmonella typhosa*), whooping cough (*Hemophilus pertussis*), staphylococcus infections (*Micrococcus pyogenes*), and other diseases.

Autogenous Vaccines

Chronic infections, including various skin pustules, boils, etc., are sometimes treated by injecting killed suspensions of the organisms causing the infection. Since the patient is the source of the bacteria used in the preparation of the vaccine, the suspension is called an AUTOGENOUS VACCINE (*auto* means self, and *genic* means to create). An antigen made from organisms freshly isolated from the patient for whom it is to be used, usually yields more satisfactory results than a vaccine prepared from old stock laboratory strains of the same species. Minor strain differences between organisms spell the difference between an excellent or a mediocre response in clearing up the infection. Antigens are highly specific, as are antibodies, and the more specific treatment it is possible to give the patient, the better, in general, are the results.

B.C.G. Vaccine

These letters are an abbreviation for *Bacille Calmette-Guerin,* in honor of the two French scientists who developed the particular

technic for treating *Mycobacterium tuberculosis* to make the organisms avirulent. Vaccines made from dead bacteria fail to provide protection against tuberculosis. B.C.G. vaccine is prepared by growing the tuberculosis organisms on special media at temperatures above optimum for the bacteria. This attenuates their virulence, and living *Mycobacterium tuberculosis* organisms can be introduced as vaccines to stimulate active immunity to the disease.

Extensive field tests have been underway in France for a number of years, but it will require several generations before the full interpretation of the results can be made. Those who are working on the project, however, are encouraged by what they have seen to date, and this has prompted public health authorities in the United States to institute similar programs in areas where tuberculosis rates are high.

Since tuberculosis is a major health problem during periods of war and famine, B.C.G. vaccine is being put to use in many far-flung parts of the world. Again, it is too early to draw conclusions as to the merits of the program.

Toxoids

A TOXOID is a toxin that has been chemically treated, usually with formalin, to remove its toxic properties without interfering with its antigenic qualities. Toxoids, therefore, can induce an active immuntiy and serve a useful function in protecting individuals for varying periods of time. An active case of a disease caused by toxins, however, must be treated with antitoxin to afford immediate protection; this is passive immunity and does not endure for more than a short time. Diphtheria, tetanus, and gas gangrene are three diseases for which effective toxoids are available.

Gamma Globulin

Adults who have been vaccinated against certain diseases, or who have actually had clinical symptoms of the disease, possess antibodies which are found in the protein portion of the blood known as GLOBULIN. Globulin, in turn, can be further broken down into smaller fractions, one of which is called GAMMA

GLOBULIN. Since antibodies are normally found in the gamma globulin fraction of the blood, passive immunization with this globulin fraction is of value.

In recent years it has been found that children who have been exposed to measles can be protected from this disease by the in-

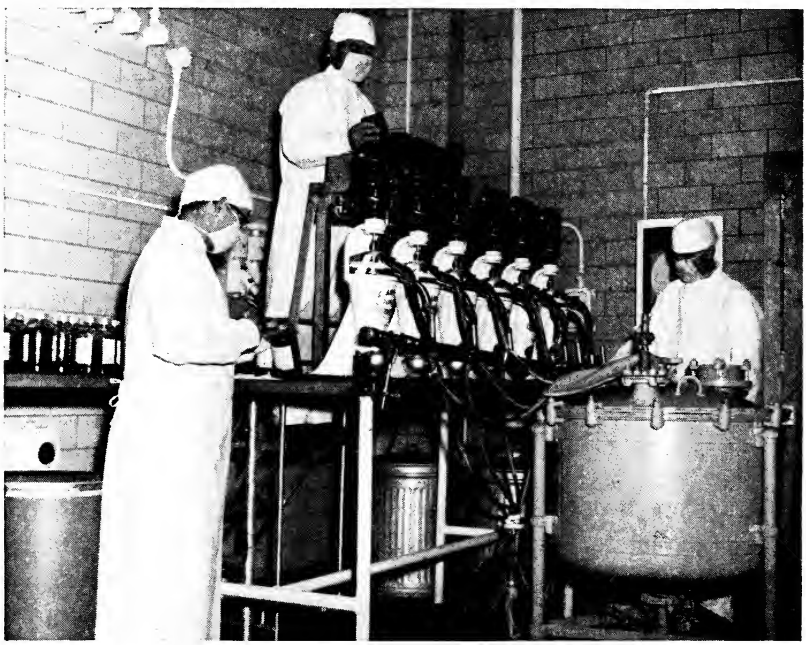


Fig. 57. Human blood fractionating laboratory. Poliomyelitis-Immune Globulin as well as Serum Albumin for the prevention of shock are separated from the blood after centrifuging. (*Courtesy of Armour and Company, Chicago, Illinois.*)

jection of gamma globulin immediately after exposure. If several days elapse before the injection of gamma globulin, there is a good possibility that the child will contract measles but the disease will be milder in character. Some physicians feel that a case of measles modified by the use of gamma globulin is better than not contracting the disease at all, since the patient will build up his own immunity if he has the disease, and this will protect him from possible

future attacks which might be more severe at an older age. The principal source of this protective material is the blood donated by individuals through the Red Cross Blood Donor Programs.

Gamma globulin is also showing some promise in preventing or attenuating the symptoms of poliomyelitis (infantile paralysis). Such protection is of short duration, since it is an example of passive immunity, but during impending polio epidemics the use of gamma globulin is one of our more encouraging prophylactic measures. In general, one attack of measles or polio confers a life-long immunity to the disease.

CHAPTER 17

Pathogenic Bacteria

THE GRAM POSITIVE COCCI
- Pneumococci
- Streptococci
- Micrococci

THE GRAM NEGATIVE COCCI
- *Neisseria gonorrheae*
- *Neisseria meningitidis*

THE GRAM POSITIVE RODS
- *Corynebacterium diphtheriae*
- *Mycobacterium tuberculosis*
- *Bacillus anthracis*
- *Clostridium tetani*
- *Clostridium botulinum*

THE GRAM NEGATIVE RODS
- *Salmonella typhosa*
- *Shigella dysenteriae*

THE SPIRALS
- *Treponema pallidum*

Although some disease-producing bacteria have been mentioned by weaving their names into the text of the book, it is appropriate to devote one chapter to a brief discussion of some prominent pathogenic bacteria. Of necessity, many important organisms must be omitted, and the finer points relative to the bacteria that are discussed will be left out.

THE GRAM POSITIVE COCCI

PNEUMOCOCCI

Non-motile oval or spherical forms about 0.5 to 1.25 microns in size, typically in pairs but occasionally in short chains, with the

distal end of each pair of cocci pointed or lance-shaped. Gram positive and encapsulated. Optimum growth temperature of 37° C. Facultative aerobes. Habitat is the respiratory tract of man and animals. Most common cause of lobar pneumonia.

Whenever one speaks of pneumonia, the condition refers to any inflammation of the lungs in which an exudate accumulates in the alveoli (spaces of the lungs). More than 90% of the cases of LOBAR PNEUMONIA are caused by *Diplococcus pneumoniae*, and this same organism may be the etiological agent in OTITIS MEDIA (middle ear infection), MENINGITIS (infection of the membrane covering the brain and spinal cord), PERITONITIS (inflammation of the membrane lining the abdomen), and SEPTICEMIA (blood poisoning.) The pneumococcus is not the only cause of lobar pneumonia; any one of a number of other bacteria, rickettsiae, and viruses might be involved.

At least seventy-five different types of pneumococci have been described, based solely upon serological differences demonstrable in the capsular material of the organisms. The underlying diplococci are antigenically similar. Pneumococci are pathogenic for mice and for rabbits, and if the bacteria are introduced into the trachea of dogs and monkeys, these two animals contract a disease resembling clinical pneumonia in human beings.

Many persons are healthy carriers of pneumococci, and the carrier rate increases during the colder months of the year when respiratory infections are most prevalent. Transmission of the disease appears primarily to be by droplet infection and by intimate contact with carriers or infected individuals. Fatigue, undue exposure, and nutritional deficiencies seem to predispose persons to pneumonia, a disease which frequently follows a common cold that has not been properly cared for.

The capsular material of pneumococci is composed of a complex sugar, called a POLYSACCHARIDE, and because of the antigenic specificity and the solubility of this capsular material, it is called SOLUBLE SPECIFIC SUBSTANCE (SSS).

Before the advent of sulfanilamide drugs and antibiotics for the treatment of pneumonia, it was necessary for physicians to treat

patients with antiserum that was specific for their particular type of pneumococcus infection. If a patient had type I pneumonia, he had to be administered type I antiserum if beneficial results were to be expected. Cross benefits between these antisera do not exist. Antisera are prepared by the injection of rabbits or horses with killed suspension of the specific pneumococci. These animals build up antibodies against particular pneumococcus types, and the blood serum of the rabbits or horses contains antibodies that will act specifically on the organisms against which they are prepared.

When type specific antiserum is placed in contact with the HOMOLOGOUS (same) organisms, a marked swelling of the bacterial capsule can be demonstrated under the microscope. This is called the *Neufeld capsular swelling reaction* or the *Quellung reaction,* and this result is the basis for classifying pneumococci into types. Attempting to determine the exact type of pneumococcus involved in a given infection is time consuming. If a bacteriologist encountered difficulty in typing the organisms found in the patient's sputum, precious time was lost, and this often spelled the difference between life and death for the patient. Modern treatment with antibiotics can be initiated without regard to the type of pneumococcus involved, and the time saved by this immediate therapy is reflected both in a lower mortality rate and in a shorter convalescence for the patient.

STREPTOCOCCI

Non-motile cells, spherical or ovoid, occurring in pairs, short or long chains. Gram positive, usually facultative aerobes. Capsules not regularly formed. Optimum temperature varies with the species. Found in the mouth and intestines of man and other animals, in dairy products, and in fermenting plant juices. Some species are highly pathogenic.

While not all streptococci are pathogenic, the virulent members of the group are of great importance in PYOGENIC (pus-producing) infections. They are generally hemolytic, and they have a growth temperature range of from 10° C. to 45° C. When streptococci

are grown on blood agar media, three different reactions are produced by various species with respect to hemolysis:

Alpha hemolysis—A greenish discoloration of the medium adjacent to the colony, called a viridans type reaction.

Beta hemolysis—A punched-out appearance on blood plates due to complete lysis of red blood cells around the colony.

Gamma reaction—No visible change noted in blood agar.

Streptococci are killed by a few minutes exposure to boiling temperature, and pasteurization destroys them. Their resistance to drying, however, aids in their spread from infected persons to susceptible individuals, since the organisms can remain viable in dust for long periods of time. Selected antibiotics, penicillin in particular, are effective in combatting streptococcal infections.

In addition to their reactions on blood-containing media, streptococci are classified on the basis of serological reactions, particularly the precipitin test. Lancefield classified these organisms according to their antigenic components into the following groups:

	SOURCE
Group A	Human infections.
Group B	Bovine infections.
Group C	Generally infections of animal origin but occasionally associated with human infections.
Group D	The intestines.
Group E	Dairy products.
Group F	Normal flora of human throat.
Group G	Human origin; fibrinolytic.

The usual reactions accompanying infections with streptococci can be traced to the products of metabolism of these organisms: HEMOLYSIN, FIBRINOLYSINS, SPREADING FACTOR, SKIN NECROTIZING TOXIN, etc. Antitoxins can be produced to combat exotoxins, but injections of such antibodies will act only against the true toxins, not against the bacteria themselves. Antibiotics and sulfa drugs must be employed to combat the bacteria, but the passive protection afforded by antitoxins is necessary to neutralize the circulating poisons.

RHEUMATIC FEVER may develop in children following repeated infections of the respiratory tract caused by streptococci. Fever, pain in the joints, and involvement of the heart valves are common symptoms of rheumatic fever. While streptococci are not usually isolated from these foci of disease, evidence is strong that the products of streptococcus metabolism are the direct cause of the symptoms.

SEPTIC SORE THROAT is an acute infection involving the tonsils and lymph nodes, with an accompanying fever and prostration. Epidemics of this disease have been traced to raw milk contaminated by an infected handler, although droplet infection may also initiate epidemics.

Septicemia may result from improper treatment of flesh wounds and if the blood poisoning is allowed to progress, death may follow.

Other diseases of streptococcal origin include SUBACUTE BACTERIAL ENDOCARDITIS (infection of the heart valves), ERYSIPELAS (an acute specific inflammation of the skin), PUERPERAL FEVER (child-bed fever), and SCARLET FEVER.

MICROCOCCI

Spherical cells, usually less than 1.0 micron in diameter, generally gram positive in young cultures. Occur in singles, pairs, tetrads, packets, or irregular masses. Motility rare. Preferably aerobic. Facultative parasites and saprophytes with an optimum growth temperature between 22°–37° C. Frequently live on the skin, in skin glands or skin gland secretions of vertebrates (animal having a spinal column). Cause of many localized infections.

Pasteur recognized these spherical bacteria in 1880, and the organisms were first isolated from an abscess by Ogsten in 1881. Rosenbach described differences in pigmentation of colonies of these cocci which today are designated *albus* (white), *aureus* (golden), *citreus* (lemon yellow), and *lutea* (yellow).

Micrococcus pyogenes var. *albus* (formerly known as *Staphylococcus albus*), and *Micrococcus pyogenes* var. *aureus* (formerly *Staphylococcus aureus*) are the most common micrococci im-

plicated in localized infections. The *aureus* species appears to be the more virulent of the two, although *albus* species have been known to cause serious infections and food poisonings. A number of toxins and metabolic products are produced by these bacteria including a LETHAL TOXIN, a SKIN-DESTROYING TOXIN, LEUCOCIDIN (white cell-destroying poison), HEMOLYSIN, and COAGULASE. Some strains produce an ENTEROTOXIN which causes food poisoning symptoms when it acts in the intestines. This latter toxin is thermostable and resists boiling for extended periods of time. Although man is the most susceptible animal, enterotoxins can affect monkeys and young kittens. Cramps, vomiting, diarrhea, pain, and prostration are the usual symptoms of food poisoning, but the mortality rate is practically nil.

Unfortunately, food that is contaminated with micrococci does not usually exhibit signs of spoilage, and the preformed toxins are able to cause food poisoning symptoms in from three to twelve hours after ingestion. Custards, cream-filled pastries, potato salad, gravy, poultry dressing, and meats are the usual foods implicated in such outbreaks of staphylococcus poisoning.

Micrococci are more resistant to heating than are most other non spore-forming bacteria. Ten minutes boiling will usually kill these bacteria, but some strains have been found to withstand 80° C. for thirty minutes.

Although some pathogenic micrococci produce coagulase which might conceivably aid in localizing skin infections caused by these bacteria, other factors are probably more important in this localization response. There is evidence that metabolic products of the micrococci attract large numbers of leucocytes to the area, and the white blood cells wall off the focus of infection before it has an opportunity to become generalized. PIMPLES, BOILS (furuncles), CARBUNCLES, CYSTITIS (bladder infection), PYELITIS (kidney infection), OSTEOMYELITIS (bone infection), PUERPERAL FEVER, SINUSITIS, MASTITIS (mammary gland infection), and SEPTICEMIA all may have a micrococcus etiology.

The noxious agents produced by micrococci have been suggested as possible agents for dissemination in public drinking water

supplies in biological warfare attacks. Since the toxin is not an agent that is communicable, invading armies would not have to fear the spread of this substance to their own troops.

THE GRAM NEGATIVE COCCI

Paired, gram negative cocci with adjacent sides flattened. Aerobic and anaerobic species. Non-motile. Some grow poorly or not at all without mammalian body fluids. Optimum temperature of 37° C. Parasites of mammals. Genus *Veillonella* occurs in masses, rarely in pairs, and is anaerobic.

The two important species in the genus *Neisseria* are *Neisseria gonorrhea*, the cause of GONORRHEA, and *Neisseria meningitidis*, the etiological agent of EPIDEMIC MENINGITIS. Albert Neisser discovered the microbes responsible for gonorrhea while examining pus from an infected individual in 1879, and the genus has been named in his honor.

Neisseria gonorrheae

These organisms are spheres measuring between 0.6 and 1.0 micron, occurring singly but more usually in pairs, and where the two cells come together, the sides are flattened, giving a coffee bean appearance. Primary cultivation is enhanced by growing the cultures under an atmosphere containing about 10% of carbon dioxide.

Man appears to be the only animal for which the gonococcus is a natural pathogen. Gonorrhea is one of the most common of the venereal (from *Venus*, the goddess of love) diseases, and the infection is contracted almost exclusively by sexual contact with infected individuals, particularly prostitutes. The extreme sensitivity of these pathogens to drying and to minor temperature changes on either side of 37° C. minimizes the opportunity for their spread by means of fomites, including toilet seats. There is some evidence that infected bed linen, night clothes, and towels have been responsible for a small number of cases of the disease when the contact with the viable discharges was almost immediate.

Many persons consider gonorrhea "no worse than a bad

common cold," and this unfortunate notion is partly responsible for the more than one million new cases of this disease reported annually in the United States. The unreported cases must be added to this figure. While the disease is normally an infection of the genito-urinary tract, the organisms can leave these areas and become established in other parts of the body, leading to gonorrheal rheumatism (an arthritis-like affliction) and to blindness if the organisms are transferred to the eyes as a result of unhygienic habits of infected individuals. Damage to the heart valves is not uncommon. Untreated cases of gonorrhea can lead to sterility, both in males and in females.

The disease is usually easy to diagnose in its primary stages. The microbes affect the mucous surfaces of the reproductive organs of men and women, with a subsequent discharge of white pus (leukorrhea) in from three to five days after exposure. The presence in the pus of gram negative, intracellular diplococci morphologically resembling gonococci is laboratory confirmation of a clinical case of the disease. Not all discharges from the genito-urinary tract, however, are the result of gonorrhea; other causes may be responsible.

A few startling statistics should help to emphasize the magnitude of this venereal disease in the United States, where the population is supposedly the best informed in the world. Nearly 6% of all males examined for military service are found to be infected with gonorrhea. The mean age for acquiring the infection is twenty-nine years for white males, twenty-four years for negroes, and twenty-four years for white females. Almost 250,000 potential mothers acquire the disease annually. In 1910 24% of 351 persons admitted to schools for the blind had lost their sight as the result of gonorrheal infection acquired from infected mothers. The highest disease incidence is found in cities having populations ranging between 50,000 and 500,000. The rate is lowest in metropolitan areas and in rural communities. The need for sex education and the moral issues involved in venereal disease control will be left for discussion in other books. The statistics speak for themselves.

Neisseria meningitidis

Neisseria meningitidis (formerly called *Neisseria intracellularis*) was discovered in 1887 by Weichselbaum. The organisms are spheres measuring about 0.6 to 0.8 micron in diameter, and morphologically the bacteria closely resemble the gonococci. These microbes were found originally in cerebrospinal fluid, but they can be isolated during an epidemic from the nasopharynx, blood, conjunctiva, joints, and petechiae (minor hemorrhages under the skin) of infected individuals.

About 15% of persons are healthy carriers of these organisms in their nasopharynx, and during outbreaks of the disease the carrier rate may rise to over 50%. The onset of meningitis is usually preceded by a respiratory infection, and this is followed by a stiffness of the neck, severe fever, and sometimes coma. Fresh virulent strains of these *Neisseria* will kill mice if the organisms are injected. Four different types have been described on the basis of serological reactions. Before the use of antibiotics, fatality rates as high as 50% were reported, but with modern treatment the mortality rate has dropped to less than 10%.

Meningitis is a condition that can be caused by any microbe, including the tuberculosis organisms, streptococci, staphylococci, influenzae, and others, but the epidemic form of the disease is caused only by *Neisseria meningitidis*.

GRAM POSITIVE RODS

Diseases of human beings caused by gram positive rods may be considered in groups based upon the oxygen requirements of the bacteria and the ability of the organisms to produce spores.

CORYNEBACTERIUM DIPHTHERIAE

Rods, varying greatly in dimensions, 0.3 to 0.8 by 1.0 to 8.0 microns, occurring singly. The rods are straight or slightly curved, frequently swollen at one or both ends. Do not stain uniformly with methylene blue, and have granules best shown by special stains. Non-motile. Gram positive in young cultures. Aerobic and facultative, with an optimum growth temperature of from 34–36° C. Source is from membranes in the pharynx,

larynx, and trachea in human cases. The cause of diphtheria in man, but also pathogenic for guinea pigs, kittens, and rabbits.

Diphtheria is potentially one of the most dangerous of the childhood diseases. It begins as a sore throat and the organisms tend to remain localized while the powerful exotoxin is carried through the bloodstream to the peripheral nervous system and to the heart, kidneys, and adrenals. Because the poison is a true toxin, effective antitoxins are available to combat the toxin. The diphtheria organisms themselves, however, are not affected by injections of antitoxins. Active immunity can be provided by the injection of toxoid.

This disease is spread chiefly through the agency of droplets expelled from the mouth and nose of carriers and active cases. About 1% of the population is estimated to harbor diphtheria organisms in their respiratory tract without showing any clinical symptoms of the disease. By the time that persons reach the age of fifteen, most of them have had subclinical attacks of this disease, and measurable amounts of antitoxin in their blood circulation can be demonstrated by employing the *Schick test.* This test is conducted by injecting a minute amount of diphtheria toxin intradermally (into the skin) on the forearm. If the person has sufficient antitoxin circulating in his system to neutralize this injected poison, no marked reaction at the site will be apparent. In the absence of such circulating antibody, however, the injected toxin will produce a reddened, swollen, inflammatory condition known as a Schick positive reaction. During impending epidemics all Schick positive persons should be immunized.

Different strains of *Corynebacterium diphtheriae* have been found to exhibit degrees of virulence, and a classification scheme is based upon these findings, coupled with differences in cultural and physiological reactions. The three groups of organisms are designated *gravis* (most severe), *mitis* (least severe), and *intermedius* (those in between the most and the least virulent strains). A number of diphtheria-like saprophytic organisms closely resemble pathogenic corynebacteria both morphologically and biochemically, but they are non-virulent when subjected to the usual guinea

pig or rabbit virulence tests. Bacteriophage has been found to alter non-virulent strains of otherwise typical diphtheria organisms in such a way as to make them suddenly virulent.

MYCOBACTERIUM TUBERCULOSIS

Non-motile rods, ranging in size from 0.3 to 0.6 by 0.5 to 4.0 microns, straight or slightly curved, occurring singly and occasionally in threads. Sometimes branched. Stain uniformly or irregularly, with banded or beaded forms. Acid-fast. Gram positive. Growth in all media is slow, requiring several weeks for development. Optimum temperature 37° C. Facultative. Produce tuberculosis in man, monkey, dog, and parrot. Can infect guinea pigs, but not rabbits.

Most tuberculosis in human beings is pulmonary, and infection is acquired principally through droplet infection or by direct contact with infected individuals. Inhalation of dust containing dried sputum should not be overlooked as a potential source of infection. Bovine tuberculosis may be contracted by drinking raw milk from infected cows. The death rate from tuberculosis in the United States has had a dramatic drop in the last fifty years from well over two hundred deaths per one hundred thousand population to less than thirty deaths per hundred thousand at the present time. This progress can be attributed to decreased consumption of raw milk, better diagnostic technics, improved treatment of recognized cases, and increased facilities for keeping infected persons isolated from healthy individuals. The disease is still one of tremendous public health significance, however, as evidenced by the thirty-three thousand deaths annually in the United States. Nearly one-half million persons are suffering from tuberculosis in our country, and this is a challenge to public health authorities who know that tuberculosis is a preventable disease.

Pulmonary tuberculosis is almost always due to the human tubercle organisms contracted from infected persons or their discharges, while non-pulmonary tuberculosis (bones, joints, viscera) appears to be food-borne.

Detection of infected individuals is difficult in the early stages of the disease, since clinical symptoms may be absent for months.

Early case detection can do a great deal to lower the number of new infections encountered each year, and mass X-ray programs are playing an important part in this type of preventive medicine. The *tuberculin test* when found to be positive indicates that tubercle organisms are present in the body. It does not necessarily mean that the person has an active case of tuberculosis. Because of the body's remarkable ability to wall-off foci of infection, much tuberculosis does not progress past this primary stage. Practically all adults have come in contact with *Mycobacterium tuberculosis* sometime during their lifetime, but unless the body is unable to cope with the initial infection, active disease does not develop. A negative tuberculin test, therefore, has more diagnostic significance than does a positive reaction.

The use of B.C.G. vaccine, described in the previous chapter, is showing promise as a means of preventing tuberculosis, but a longer range study is required before conclusive statements can be made about the exact effectiveness of this vaccine. Some recent advances in drug therapy offer hope for those persons who are suffering from active cases of this disease.

Other diseases caused by mycobacteria include LEPROSY (*Mycobacterium leprae*), and JOHNE'S DISEASE (*Mycobacterium paratuberculosis*), a chronic diarrhea in cattle and in sheep.

BACILLUS ANTHRACIS

Rod-shaped, spore-forming, non-motile, gram positive, facultative organisms, measuring from 1.0–1.3 by 3.0–10.0 microns, and having square or concave ends. Occur in long chains. The cause of anthrax in man, sheep, and swine.

The word ANTHRAX comes from the Greek and means boil or carbuncle, which is the typical type of lesion seen when man acquires a skin infection with *Bacillus anthracis.* A malignant pustule forms and develops a black crust which covers a focus of infection that is teeming with organisms. The infection may remain localized or it may become generalized. When growing in the body the anthrax bacillus produces no spores but capsules are developed. No exotoxin has been demonstrated. It has been

suggested that since the organisms occlude blood vessels, the death of the individual may be caused by sheer numbers of the microbes present in the infected host.

Anthrax spores are able to resist 140° C. for three hours of dry heat, and 100° C. live steam for five to ten minutes. Improperly sterilized shaving brushes have caused a number of cases of anthrax of the face. Inhalation of the spores from wool can cause fatal ANTHRAX PNEUMONIA (wool-sorter's disease). About 80% of infected animals succumb to the disease. Vaccines prepared from killed cultures are ineffective in preventing anthrax in animals, but by growing the bacilli in broth at temperatures of 42–43° C. for prolonged periods, the organisms are attenuated and fail to produce spores. Injection of these modified cultures provides active immunization. This technic was developed by Pasteur, and it is still the accepted method for preparing such vaccines.

CLOSTRIDIUM TETANI

Rods measuring 0.4–0.6 by 4.0–8.0 microns, with rounded ends, found in singles or pairs, and sometimes in chains. Gram positive. Spherical terminal spores. Form potent exotoxin. Normal habitat in soils, human and horse intestines, and in feces. Anaerobes. Optimum growth temperature 37° C. Cause of tetanus (lockjaw).

These anaerobic rods were discovered in 1889 by Kitasato from a case of tetanus, and he reported that the microbes produced a powerful exotoxin. Together with von Behring, he developed an effective antiserum to neutralize the destructive action of the true toxin liberated by *Clostridium tetani* during its anaerobic growth. The poison is called TETANOSPASMIN, and it causes a contraction of the skeletal muscles. In pure form tetanus toxin will kill a man when as small a dose as 0.00025 gram is injected, but this same toxin is non-poisonous when taken by mouth.

Owing to the anaerobic requirements of these bacteria, deep, dirty, puncture wounds are particularly favorable for the growth of *Clostridium tetani,* which remain localized and give off their powerful poison which is carried to other parts of the body where

it unites with susceptible nerve tissue. The muscles of the jaw involved in mastication are affected by the destructive action of the poison on the nerves controlling these muscles. Hence, the popular term LOCKJAW is used to indicate this clinical symptom of the disease.

Specific antitoxin is very effective in neutralizing the exotoxins produced by *Clostridium tetani* if the toxins have not already acted upon the nerve tissue. Tetanus toxoid proved highly effective in preventing tetanus during World War II, and this toxoid can be combined with diphtheria toxoid to give "one shot" double protection to young children as part of their immunization series. Tetanus toxoid will stimulate production of measurable antitoxin in the bloodstream within two weeks after administration, and the active immunity lasts for about one year before a "booster" dose is required to raise the antitoxin titer of the blood.

CLOSTRIDIUM BOTULINUM

Rods 0.5–0.8 by 3.0–8.0 microns, in singles, pairs, and chains. Motile. Oval spores located centrally or subterminally, but terminal at maturation. Rods slightly swollen. Gram positive. Optimum temperature for growth 20–30° C., but 28° C. best for toxin production. Anaerobic. Habitat appears to be the soil. Cause of botulism.

The word BOTULISM comes from the Latin and means *sausage.* This food was the cause of some of the first recognized cases of botulism in Europe, and the work of van Ermengem (1896) showed that *Clostridium botulinum* was the cause of this highly fatal disease. Certain foods that are improperly processed and are stored in sealed containers under anaerobic conditions are the usual source of botulinus poisoning. This is the most powerful biological poison known to man, and because of certain characteristics of the toxin, it has been suggested as a possible weapon in biological warfare. Poisoning of water supplies is probably feasible, and while the toxin may not live up to all the fantastic claims put forth in popular magazine articles pertaining to biological warfare, botulinus toxin has definite wartime possibilities.

Gastro-intestinal symptoms are practically non-existent with this poison. Weakness, double vision, difficulty in swallowing, and finally death from respiratory failure can be expected for about 60 to 70% of the victims. Several immunological types of the poison are recognized, and specific antitoxins must be given to patients at the earliest possible moment after the condition has been diagnosed. Polyvalent antitoxins are available for administration to save time in treating afflicted persons. A prophylactic dose is 5,000 units of antitoxin, and a therapeutic dose is 50,000 units. Persons engaged in handling these organisms or their products should be actively immunized with toxoid.

The toxin is thermolabile, and ten minutes boiling will inactivate it. Steam under pressure, however, is the only safe method of processing high protein, neutral foods (peas, beans, corn) for canning if the spores of *Clostridium botulinum* (found in the soil in varying concentrations) are to be destroyed, and if the processed food is to be made safe for consumption without fear of botulinus poisoning.

GRAM NEGATIVE RODS

SALMONELLA TYPHOSA

Rods 0.6–0.7 by 2.0–3.0 microns, occurring singly, in pairs, occasionally in short chains. Motile. Optimum temperature 37° C. Gram negative. Facultative. Source is feces of infected human beings. Cause of typhoid fever.

The genus *Salmonella* includes more than 150 species, all of which are believed to be pathogenic. *Salmonella typhosa* is the etiological agent of typhoid fever, and these bacteria may be spread through the agency of feces, flies, fomites, food, and fingers. Water and milk are particularly important in the dissemination of typhoid fever.

Once ingested, the typhoid organisms localize in the small intestine, and they eventually invade the blood stream. The bacteria may then set up new foci of infection in the spleen, bone marrow, gall bladder, and other organs. Slightly less than 5% of infected persons remain as typhoid carriers for indefinite periods,

sometimes for the rest of their lives, and it is of the utmost public importance that such carriers be excluded as food handlers. An arbitrary designation of three months has been set as the dividing line between CONVALESCENT CARRIERS and CHRONIC CARRIERS. Viable typhoid organisms are excreted by these carriers in their feces, and strict attention must be paid to sanitary practices if the spread of this disease is to be avoided.

A classic case in medicine is that of Mary Mallon, a cook who was a typhoid carrier. She refused to heed warnings of public health authorities and continued to spread virulent typhoid to unsuspecting persons for whom she prepared meals. The New York Health Department was finally forced to take "Typhoid Mary" into custody, and she remained in detention for three years. The courts upheld the action of the Health Department in this unique case. After promising to give up her occupation as a cook, Mary Mallon was released from detention, and nothing further was heard of her for about five years. When typhoid broke out in a New York City hospital, it was discovered that Mary was the cause of the epidemic. She had found her way back in a kitchen and was spreading her virulent microbes once more. She was taken into custody for a second time, and she finally died after causing over fifty cases of typhoid fever in ten known outbreaks. There is no way of knowing how many other cases she was indirectly responsible for spreading.

Diagnosis of typhoid depends upon the isolation of the causative organisms from the patient. Blood cultures are generally positive during the first week or ten days of the disease, and stool cultures and urine cultures are positive after ten days. Typhoid agglutinins can be detected in the blood serum by means of the *Widal test* after about the tenth day of the disease. Disappearance of the microbes from the bloodstream is apparently correlated with the increase in typhoid agglutinin titer. Carriers may retain a high titer indefinitely, and this may sometimes aid in detecting these individuals when repeated stool culture examinations prove negative for *Salmonella typhosa*.

Typhoid vaccination induces active immunity. Such vaccines

are killed suspensions of bacteria in a concentration of about one billion cells per milliliter. A combination of typhoid, paratyphoid A, and paratyphoid B organisms (TAB vaccine) is customarily administered to provide protection against all three related organisms. Vaccination does not provide iron-clad protection from infection; this immunity is one of degree. Ingestion of large numbers of *Salmonella typhosa* will result in disease, but the vaccination will provide protection against minimal doses of ingested organisms. The symptoms appear to be moderated if a vaccinated person contracts the disease.

SHIGELLA DYSENTERIAE

Non-motile rods, 0.4–0.6 by 1.0–3.0 microns, occurring in singles. Aerobic and facultative. Optimum growth temperature 37° C. Cause of dysentery in man and in monkeys.

In contrast to most salmonellae, *Shigella* species are non-motile and produce no gas when they ferment carbohydrates. The genus is named for the Japanese, Shiga, who discovered the organisms in 1896. Other species were discovered in rapid succession. The disease ranges from mild diarrhea to ulceration of the large bowel, but bloodstream invasion does not normally take place. The fatality rate is rather high in some epidemics, but by prompt treatment with some of the newer drugs, the death rate has been reduced. Vaccination is unsatisfactory. Even though *Shigella dysenteriae* produces a true toxin, antitoxin appears to have little or no useful effect in patients.

This disease is extremely important as a cause of sickness in tropical countries. The combination of poor sanitary facilities and lack of proper personal cleanliness and hygiene is largely responsible for the spread of bacillary dysentery, with an assist from flies, cockroaches, and mice.

Other diseases caused by gram negative rods and their etiological agents include TULAREMIA (*Pasteurella tularensis*), BUBONIC PLAGUE (*Pasteurella pestis*), WHOOPING COUGH (*Hemophilus pertussis*), and UNDULANT FEVER (*Brucella* species).

SPIRALS

TREPONEMA PALLIDUM

Very fine, motile spirals, 0.25–0.30 by 6–14 microns. Stain with difficulty except with Giemsa's stain. Appear black with silver impregnation. Have a stiffly flexible motion. Possible cultivation under anaerobic conditions in ascitic fluid with addition of fresh rabbit kidney. Optimum temperature about 37° C. The cause of syphilis in man; can be experimentally transmitted to anthropoid apes and to rabbits.

Like gonorrhea, the statistical figures for the case rates of syphilis are unbelievably high. About 10% of the population in the United States will yield a positive serological test for this venereal disease during their lifetime, and between 1 and 2% of children have congenital syphilis.

The disease gets its name from *Syphilus* an infected hero in a poem written by Fracastorius in 1530, and it has been recognized in Europe since the latter part of the fifteenth century. History suggests that syphilis was unknown in that part of the world until Columbus's crew returned from their 1492 voyage to the new world.

Treponema pallidum was first recognized as the etiological agent of syphilis by Schaudinn and Hoffmann in 1905, and the generic and specific names when literally translated mean "pale thread." The organism is probably the least resistant of all pathogenic bacteria; even short exposures to drying or to water will kill the anaerobic spirals.

Because of their poor staining qualities, *Treponema* are usually examined under a dark-field microscope. As the words indicate, the field of the microscope is dark, and the organisms appear as unstained spirals. The characteristic movement of these bacteria aids in recognizing them when fluids obtained from primary syphilitic lesions are examined under darkfield illumination.

Syphilis allowed to go untreated passes through three stages: *primary, secondary,* and *tertiary.* About four weeks after exposure, a primary CHANCRE appears, and another six weeks usually elapses before secondary generalized symptoms put in their appearance.

The tertiary stage, in which the central nervous system is affected with various degrees of paralysis, may occur at any time, even years, after the secondary symptoms. Non-venereal syphilis is practically non-existent. Sexual promiscuity, particularly with infected prostitutes, accounts for a major percentage of cases of this disease.

Fig. 58. *Treponema pallidum* in dark field. (*Courtesy of the American Optical Company, Instrument Division, Buffalo, New York.*)

Treponema pallidum can migrate through the placenta and infect a developing fetus, causing congenital syphilis. It is unfortunate that it is considered "bad taste" to publish pictures showing what advanced syphilis can do to the human body, especially to innocent children, because a few pictures could convey more than many pages of printed words on the subject. Too many persons must learn through bitter experience, and the total damage to themselves and to others is difficult to evaluate.

The first effective treatment for syphilis was Paul Ehrlich's arsenic-containing compound "606." This "magic bullet," as it was called, is capable of killing *Treponema pallidum,* but the course of treatment is long and painful. Penicillin has given us the most encouraging treatment yet devised to fight this disease, but with the passage of time, the syphilis microbe has been found

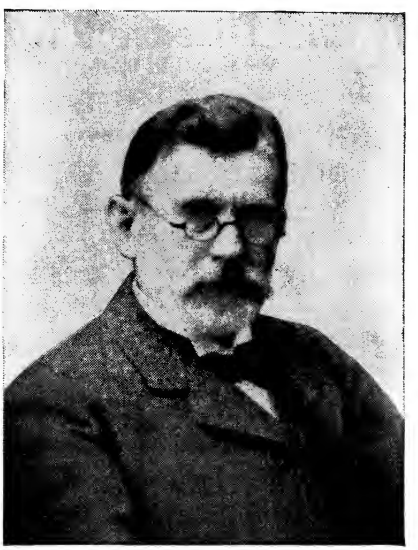


Fig. 59. Paul Ehrlich (1854–1915). (*By permission from* Introduction to the Bacteria *by C. E. Clifton. Copyright, 1950. McGraw-Hill Book Company, Inc.*)

to build up a resistance to the antibiotic in some cases, and newer antibiotics or a return to some of the older technics for treating the disease must be used to combat these resistant infections. Syphilis is called the great imitator, since it presents clinical symptoms resembling many other diseases.

Another disease caused by curved rods is ASIATIC CHOLERA (*Vibrio comma*), and *Borellia vincenti* is associated with a fusiform (rod with pointed ends) organism in the human mouth in cases of VINCENT'S ANGINA, popularly known as trench mouth.

CHAPTER 18

Arthropods and Disease Transmission

WHAT IS AN INSECT?

Zoologists consider insects as any member of the class *Insecta,* and true insects are six-legged arthropods, which are animals with articulated bodies and jointed limbs. The more popular concept of an insect is any one of numerous small invertebrate animals belonging to a group including beetles, bugs, bees, flies, and mosquitoes, and to allied groups of arthropods including spiders, mites, ticks, and lice.

HISTORY OF INSECT-BORNE DISEASES

Edward A. Steinhaus (1947) in his book *Insect Microbiology* has presented an extensive review of the literature dealing with

insects and insect-transmitted diseases. Interested students are referred to this work for additional background material, but a few historical highlights will be presented here.

One of the earliest proposals that insects might be instrumental in the dissemination of disease was made in 1577 when Mercurialis expressed the idea that bubonic plague could be transmitted by flies which had been in contact with the corpses of persons who had succumbed from this disease. Relatively few significant confirmatory studies were conducted for about three centuries, but a sudden impetus was provided to this field of science by a series of fundamental discoveries near the close of the nineteenth century. The finding in 1893 by Smith and Kilbourne that the cattle tick *Boophilus annulatus* transmits *Babesia bigemina,* the etiological protozoan in Texas cattle fever, is a milestone in insect microbiology. These workers also discovered that the protozoan is transmissible through the egg of the tick to succeeding generations.

Other significant discoveries during this same period included the finding by David Bruce (1895) that the tsetse fly transmits the trypanosome of sleeping sickness; the discovery by Ronald Ross in 1897 that malaria is carried by the *Anopheles* mosquito; Simond's report in 1898 that plague organisms are transmitted to rats by infected fleas; and that yellow fever virus is spread by the mosquito *Aedes aegypti* (Finlay 1881; Reed 1900).

Each of these discoveries opened up new fields of attack for man in his fight to control debilitating and killing diseases. Research aimed at the destruction of the vectors and the development of therapeutic measures once a patient has contracted the disease has paid rich dividends during the last fifty years. These diseases have not been eradicated, but their control is better today than ever before in man's history.

IMPORTANT INSECT VECTORS

FLIES

Insects have long been considered to be undesirable as far as human habitation is concerned. In addition to their being a nuisance, these arthropods may also serve as passive or as active

carriers of microorganisms—both pathogens and non-pathogens. The first serious attention paid to flies was in 1898 during the Spanish-American war, when it was discovered that lime sprinkled on open pit latrines was finding its way back to the kitchens in which food was being prepared for the troops. The white tracks left by the flies were identified as lime and indicated that these insects were capable of transferring fecal matter and possibly enteric pathogens from open latrines to food located at some distance from the latrines.

Intestinal diseases have always presented serious problems whenever large numbers of persons were obliged to live under crowded conditions with inadequate sanitary facilities. As a direct outcome of the studies conducted during the Spanish-American war, better methods for the disposal of human wastes have resulted, and increased attention has been focused on flies as vectors in disease transmission.

The common housefly, *Musca domestica,* has no biting or sucking mouth parts, and its food must therefore be lapped up. Its nutritional requirements include proteins for growth and for production of eggs, and sweets are needed for energy. Flies dissolve sugar with their own saliva, and when the sugar is in solution, the insects ingest the sweetened liquid.

Examination of a common fly with a simple hand lens will reveal the hairy nature which permits these insects to carry such large numbers of organisms on the outside of their bodies. The use of the low power of a microscope will make this point still more apparent. The filthy living habits of a fly, which prefers manure as a place in which to lay as many as one thousand eggs during its lifetime, points out the potential danger that lurks when flies gain access to uncovered food, especially food that is allowed to stand at room temperature for long periods of time. The few hundred or few thousand bacteria deposited on the food by a single fly can, if the medium is favorable, increase to millions or billions of organisms when the temperature is favorable for their development.

In spite of the usual statements found in textbooks that flies are filthy insects and can spread disease, relatively little carefully

controlled fundamental experimental work has been conducted with these insects to prove that they are active transmitters of intestinal disease. Hawley, *et al.* (1951), by mounting individual *Musca domestica* and feeding them a controlled diet and a known number of *Salmonella schottmulleri, Escherichia coli,* or *Shigella dysenteriae,* found that unless at least one thousand viable organisms were fed to the flies, recovery in the feces of the fed bacteria was not possible. But when the number of ingested organisms increased above one thousand per feeding, decided multiplication occurred within the insects as evidenced by the excretion of significantly greater numbers of the bacteria than were fed. More such controlled investigations need to be conducted to indicate more clearly what the true nature of the problem is with respect to insect transmission of disease.

While it is conceivable that a fly might ingest large enough numbers of pathogenic bacteria to allow active multiplication to occur within the digestive tract of the insect with a subsequent spill-over into the excreta, it would appear that mechanical transfer of organisms on the outside of the insect is probably more often the case. Survival of bacteria on the hairy surface of the insect's body will depend upon a number of factors, but studies indicate that survival may extend for as long as several days, and a fly can contaminate many objects during this period of time.

The number of bacteria it is possible to wash from the outer surface of a fly will naturally vary with the living habits of the specific insect, but studies have shown that millions of organisms per fly is a common finding. In general the number of bacteria found within the digestive tract of flies is many times that found on the outer surface of the insect. The honeybee, on the other hand, harbors few or no bacteria within the body. Still other insects may have certain segments of the digestive tract that are free of viable microorganisms, while other segments are heavily loaded with bacteria. Over 250 species of microbes have been found associated with insects.

As early as the sixteenth century Paré reported that severe wounds in which blowflies had deposited their eggs healed more

rapidly than might normally be expected had the eggs been absent. Surgeons in the army of Napoleon confirmed this early report. Further support was given this finding during World War I when men who had been left on the battlefield for several days without medical attention were found to harbor maggots in their wounds. Oddly enough, however, many of these individuals had no fever and their dressed wounds healed very quickly. Pus and debris were absent in the wounds, and the underlying tissues were pink and healthy in appearance. Subsequent investigations conducted on maggots have revealed the presence of a thermostable bactericidal substance in their excretions. This substance is particularly active against staphylococci, streptococci, and the organisms (*Clostridium perfringens*) responsible for gas gangrene. After the first World War cases of OSTEOMYELITIS (a bone disease) were commonly treated with maggots which devoured the dead tissue, destroyed the bacteria, and kept the wounds clean. With the advent of the sulfa drugs and later the antibiotics, the use of maggots has been discontinued.

COCKROACHES

The cockroach has long been suspected of harboring and disseminating disease organisms. Relatively little work, however, has been done to implicate these insects as definite vectors of disease. The filthy habits and cursorial nature of roaches makes them ideally suited for the mechanical spread of certain pathogenic organisms by contamination of foodstuffs and fomites through contact with the infected appendages of the roach.

A number of reports have been published, including those of Mackerras and Mackerras (1948) and Bitter and Williams (1949), indicating that enteric pathogens belonging to the genus *Salmonella* may be excreted by roaches under certain circumstances. Should these roaches gain access to food to be consumed by humans, infections with these enteric pathogens might well take place. Janssen *et al.* in 1952 reported that feeding massive doses of *Salmonella typhosa* to the roach *Blattella germanica* resulted in no excretion of these organisms after a twenty-four-hour period. In fact, it was impossible to isolate the typhoid organism from the

alimentary canal of most roaches fifteen hours after feeding them untold millions of viable cells. This indicated that passive transfer of typhoid organisms on the outer surfaces of these roaches is more significant than the excretion of these bacteria by *Blattella germanica.* It is this type of finding that indicates the importance of carefully controlled studies before sweeping statements are made concerning roaches as vectors of disease.

MOSQUITOES

The discovery of Sir Ronald Ross in 1897 that malaria is a mosquito-transmitted disease, and the finding of Walter Reed in 1900 that yellow fever virus is spread by the mosquito *Aedes aegypti* have provided us with technics for attacking the problem of control of these diseases. If breeding grounds are destroyed, the mosquito population will drop. Smaller numbers of infected persons will furnish fewer possible sources of infectious material for the biting mosquitoes. It is the female of the species, incidentally, that does the biting.

Other diseases which may be transmitted through the bite of various mosquitoes include the virus of DENGUE FEVER (also called breakbone fever), and the endemic disease FILARIASIS found in tropical and in semi-tropical countries. Filariasis is caused by a threadworm which occludes capillaries and lymphatic vessels causing a condition known as *elephantiasis.*

TICKS

Since ticks have eight legs, they are not considered to be true insects. Ticks are found on many animals, including dogs, and a walk through the underbrush in many areas will result in a person picking up ticks on his clothing. Not all ticks harbor pathogenic organisms, but care should be exercised in removing ticks which have embedded themselves in the skin. The lighted end of a cigarette or a lighted match when applied to the posterior end of an imbedded tick will usually convince the arthropod that he should back out. A little chloroform applied on the tick will usually accomplish the same results. It is inadvisable to try to pick

out imbedded ticks. Some part, usually the head or mouthpart, may remain in the skin and set up a severe irritation which may become infected.

Ticks are important in the spread of the rickettsial disease ROCKY MOUNTAIN SPOTTED FEVER, the spirochete disease RELAPSING FEVER, and the bacterial disease TULAREMIA. Ticks are also important from the standpoint of their bites which are slow to heal and cause intense local irritation. These arthropods are widespread throughout the tropics, subtropics, and the temperate zones.

FLEAS

Fleas are small insects without wings, but they are endowed with great jumping ability. They thrive in dark and damp (not wet) places. Fleas are of medical importance since some species can transmit BUBONIC PLAGUE, which is caused by a gram negative bacterium, *Pasteurella pestis,* and ENDEMIC TYPHUS which is caused by *Rickettsia mooseri* (*Rickettsia typhi*). The most important species is the tropical rat flea, *Xenopsylla cheopis,* which, although primarily a parasite of the rat, during epidemics can transmit plague from rat to rat, from rat to man, and from man to man. Endemic typhus and plague may also be maintained in the temperate zone rat flea, *Nosopsyllus fasciatus,* which is found in the United States. Fortunately, the dog and the cat fleas are not of general medical importance.

MITES

Mites in general are extremely small, almost invisible to the naked eye. *Sarcoptes scabiei* is the common itch mite which causes the skin disease SCABIES, usually between the fingers. Itching is severe and scratching frequently results in secondary bacterial infections.

The larvae of mites are parasites on various animals. American chiggers or redbugs bite into the skin not to obtain blood but to obtain lymph. A highly fatal disease called TSUTSUGAMUSHI DISEASE is caused by *Rickettsia tsutsugamushi,* and it is transmitted by a larval mite belonging to the genus *Trombicula.* In the Philippines

and in other parts of the southwest Pacific this same disease goes under the name of *scrub typhus, tropical typhus,* and *mite-borne typhus.* Rodents serve as the principal reservoir of these mites.

LICE

Man is affected by two species of lice, namely, the crab louse (*Phthirus pubis*) and the head and body louse (*Pediculus humanus*). The crab louse, which localizes chiefly in the hairs of the pubic region, does not transmit disease. Head and body lice may be instrumental in spreading TYPHUS, TRENCH FEVER, and at least one form of RELAPSING FEVER. *Rickettsia prowazeki* is the cause of European or epidemic typhus. When the skin has been penetrated by the louse, infection of the person may be caused by a deposition of louse feces on the site, and the natural tendency to scratch the itching area will drive the contaminated feces into the skin opening. Crushing the lice may also cause infection, and there is some evidence that the bite of the louse in itself might be sufficient to result in typhus.

Trench fever is probably of rickettsial etiology, and relapsing fever is caused by the spirochete *Borrelia recurrentis* transmitted by lice when they are crushed on the skin.

In general, bacterial diseases are mechanically transmitted by arthropods, while virus and rickettsial diseases are biologically transmitted. That is, the viruses and rickettsiae must live within the arthropod host part of the time.

There are many arthropods which are vectors, or potential vectors, of disease, but a complete discussion of their activity is not warranted here. The more important vectors and the more serious diseases have been mentioned.

PREVENTION OF THE SPREAD OF ARTHROPOD-BORNE DISEASES

Diseases transmitted by arthropods cause more human misery from the standpoint of numbers of cases than is generally realized. Prevention of these diseases is aimed primarily at destroying the breeding grounds of the vectors or breaking a link in the chain of

events required by some organisms which must pass through various stages in a life cycle.

As our knowledge about these diseases has increased, prophylactic and therapeutic drugs have been developed. The use of

Fig. 60. Typhus control by dusting Russian prisoners of war in Germany with DDT. (*Courtesy of the Armed Forces Institute of Pathology, Washington 25, D.C. Negative #B-814-0.*)

vaccines in the prevention of typhus has produced extremely encouraging results, and when typhus vaccination is coupled with direct attacks on the vectors of this disease through such measures as treating individuals with DDT, Chloradane, or Lindane, typhus can be controlled.

Understanding the breeding habits of mosquitoes has made effective control of these insects possible. *Aedes aegypti* is domesticated and breeds in tin cans, water buckets, shallow pools, etc., and it is usually more easily controlled than are the *Anopheles* species which breed in almost any water, whether it be shallow or deep, fast-moving or sluggish. Draining of swamps, oiling, and dusting likely breeding sites can materially reduce mosquito populations, as has been demonstrated so amply in such areas as the Panama Canal Zone.

Fly control principally involves proper disposal of wastes—a sanitary problem. While screening, spraying, and swatting the adult flies helps to control their numbers to some extent, these attacks on the adults are not nearly as effective as is the destruction of their breeding grounds.

Bed bugs are best removed by fumigation with hydrogen cyanide, by spraying with insecticides or aerosol bombs; heat applied directly to the springs and bedsteads may also be employed.

Head and body lice can be controlled by strict attention to body cleanliness. DDT and other compounds are effective in destroying lice once they have become established on the body.

CHAPTER 19

The Fungi—Molds

Fungi are an ever-present threat as contaminants in bacterial cultures, and students engaged in growing microorganisms should be able to recognize molds as such when they find them growing in their cultures. This chapter will briefly outline the general characteristics of molds, and a few genera and species will be considered in a limited way.

The science of fungi is termed MYCOLOGY, and fungi include molds, yeasts, smuts, rusts, blights, mildews, and mushrooms. The word MOLD has no taxonomic significance, but it is commonly associated with those loose thread-like plants found on decomposing matter and in such foods as cheeses to which molds impart characteristic flavors.

CLASSIFICATION OF MOLDS

The following abbreviated classification indicates the position occupied in the plant kingdom of these chlorophyll-free, multicellular plants.

KINGDOM: *Plant*
PHYLUM: *Thallophytes* (Lack roots, stems, and leaves)
I. *Eumycetes* (True fungi)
CLASS: 1. Phycomycetes (Algae-like fungi)
GENUS: A. *Rhizopus*
B. *Mucor*
2. Ascomycetes (Sac fungi)
A. *Aspergillus*
B. *Penicillium*
3. Basidiomycetes (Club fungi)
Includes puffballs, mushrooms, toadstools, smuts, and rusts.
4. Fungi Imperfecti (Heterogeneous group in which sexual reproduction has not been demonstrated)
Includes certain mildews, plant pathogens, and food spoilage fungi, and it may also include organisms given the same generic names as in other classes, just as if they had sexual reproductive methods. *Ex. Aspergillus* species (Fungi Imperfecti). The *Geotrichum* genus is classified as a member of the Fungi Imperfecti.
II. *Pseudomycetes* (False fungi)
CLASS: Myxomycetes (Slime molds)
Molds resembling protozoa.

MORPHOLOGY

Molds are identified and classified largely on the basis of morphology, with physiological reactions assuming secondary importance. The cell walls on fungi are thick and rigid and are composed principally of chitin (the horny-like substance found on insects). Just as is true with bacterial cells, molds have a cytoplasmic membrane which limits the cytoplasm within the cell. As the plant matures, vacuoles form within the cells, and these vacuoles consist of glycogen (a sugar related to starch and dextrin), fat, and volutin (chiefly a chemical called ribonucleic acid).

Molds develop into thread-like structures termed HYPHAE (singular *hypha*), and a mass of hyphae is called a MYCELIUM. Microscopic examination of these threads will reveal that some of them are sectioned into individual cells by means of cross walls, or SEPTA

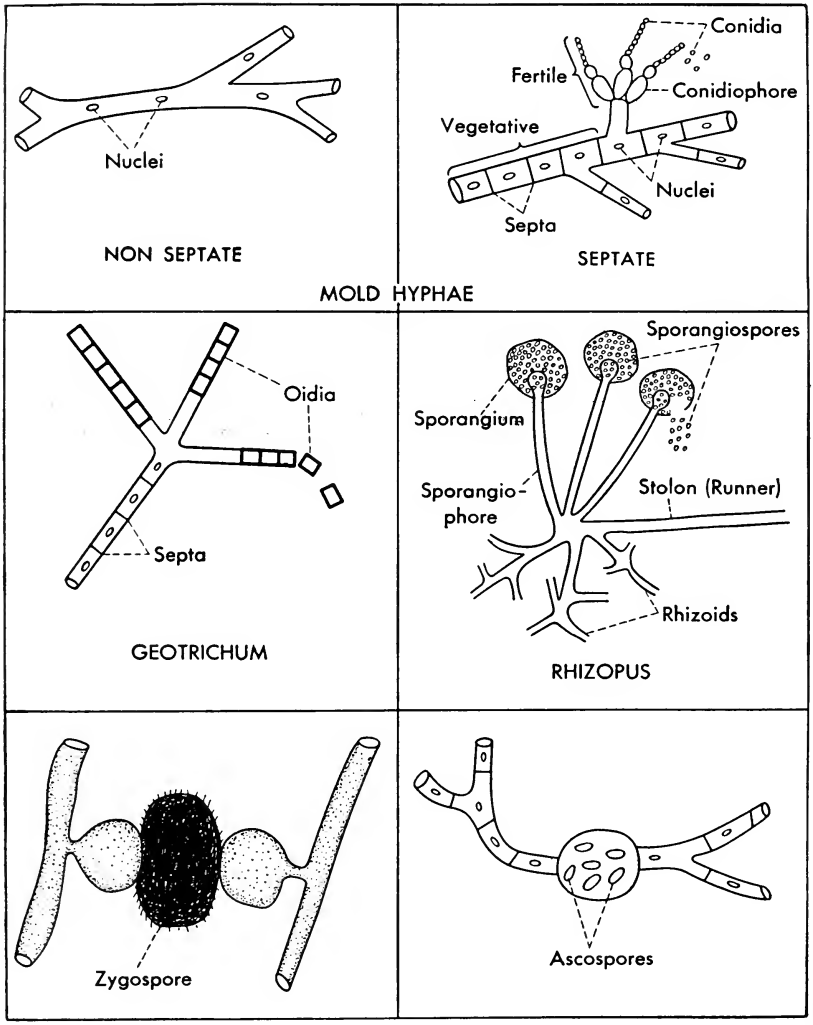


Fig. 61. Mold structures.

(singular *septum*), and such molds are referred to as SEPTATE in contrast to the NON-SEPTATE molds which lack these cross-walls. Specialized cells, concerned primarily with obtaining nutrients for the plant, are called VEGETATIVE HYPHAE, and reproductive structures formed on aerial hyphae are known as FERTILE HYPHAE. Each

cell in a septate plant has one or more NUCLEI, while in non-septate molds the nuclei are seen spaced at regular intervals within the hyphae, and the protoplasm appears to be continuous with no cross-wall effect. Both sexual and asexual reproduction are demonstrable in molds, with the exception of members in the class of Fungi Imperfecti.

PHYCOMYCETES

Phycomycetes are often called the lower fungi since they closely resemble the green algae in morphology. Members of this class of fungi are non-septate. Asexual multiplication is by the production of spores borne within a structure called a SPORANGIUM, and the stalk upon which this fruiting body develops is called a *sporangiophore.* Phycomycetes also possess so-called plus and minus types of hyphae which conjugate and form a spore designated as a ZYGOSPORE, the product of sexual reproduction. The *Rhizopus* and *Mucor* genera belong in this group of molds.

ASCOMYCETES

These fungi are septate, and they multiply asexually by the pinching of the tips of the fertile hyphae to form structures called CONIDIA. Sexual reproduction of members of the ascomycetes is by a process in which spores are formed within a sac (*ascus*) as the result of the fusion of two hyphae which coil together.

BASIDIOMYCETES

Basidiomycetes are septate molds in which sexual multiplication occurs by the formation of spores borne externally on a club-shaped stalk called a BASIDIUM; the spores are called BASIDIOSPORES. The difference between the sexual and the asexual types of reproduction of basidiomycetes is difficult to distinguish.

FUNGI IMPERFECTI

These fungi derive their name from the fact that no sexual spores are demonstrable, and since asexual reproduction is considered to be an imperfect stage, these molds have been designated *Imperfecti.* It has been suggested that these plants might possibly

belong to the Ascomycetes, but that the ascus stage has not been demonstrated. Fungi Imperfecti can be considered to be a waste-basket group from which fungi graduate to one of the other classes as research is able to discover sexual types of reproduction. Exceptions to this are certain apparently degraded fungi, including

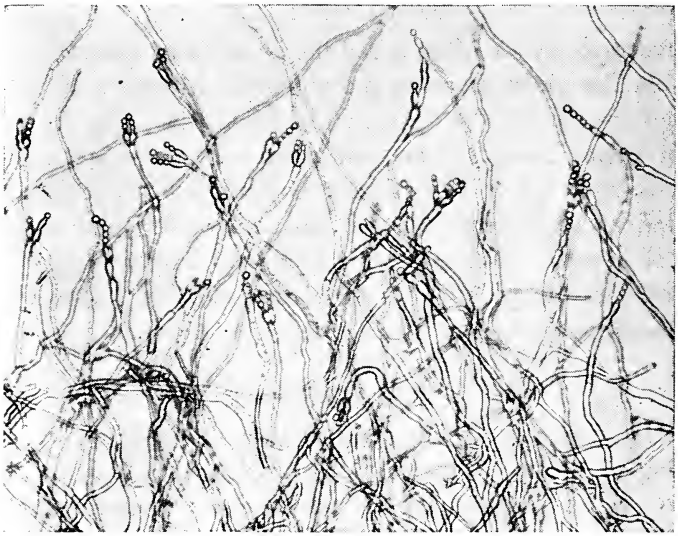


Fig. 62. Photomicrograph of *Penicillium notatum* showing conidia and septate hyphae. This was one of the first molds used for the production of penicillin. (*Courtesy of the Abbott Laboratories, North Chicago, Illinois.*)

Candida and *Geotrichum*. The genus *Geotrichum* forms OIDIA when the tip of the fertile hyphae breaks up into individual cells, usually rectangular in shape.

MYXOMYCETES

During the vegetative stage of their growth myxomycetes exhibit a protozoa-like migration on the substrate, since their cells are amoeboid rather than rod-like. In other stages of their development, these slime molds are fungus-like and sessile. Zoologists refer to myxomycetes as mycetozoa, which means fungus animals.

DISTRIBUTION IN NATURE

Molds are widely distributed in soil, water, air, and in more limited numbers in plant and animal tissues. Their light reproductive spores may be carried by air currents to points some distance from where the mold plant produced them. If conditions are favorable, these spores germinate and produce a new plant, which in turn forms many additional fruiting bodies. While these spores are usually not as resistant as the spores produced by bacteria, they are, nevertheless, capable of remaining dormant for extended periods of time under conditions relatively unfavorable for most vegetative cells. In time, however, mold spores will die unless they have an opportunity to germinate.

ECONOMIC IMPORTANCE

Molds influence their environment both physically and chemically. A few of these considerations will be briefly mentioned.

SOIL FERTILITY

There are certain preprequisites which must be fulfilled before a medium can be considered as favorable for the growth and multiplication of molds. While their demands generally are not as stringent as those required for bacteria, the factors of *p*H, moisture, available foods, etc., are still of importance. The optimum *p*H for the cultivation of molds lies within the range of 4.5 to 5.5, but when attempting to cultivate molds on laboratory media to the exclusion of bacteria and other microorganisms, the medium may be adjusted to a *p*H as low as 4.0. As the acidity decreases (the *p*H increases), bacteria and actinomyces increase in numbers more rapidly than fungi. Moisture requirements are much less for fungi than for bacteria.

Molds help to stabilize the soil both chemically and physically. Mycelial threads tend to entangle soil particles and to form stable aggregates of soil substance. Free nitrates in soils may be assimilated by fungi, and when these plants die, the nitrates become available for use by higher plants and by other microorganisms

from these mycelial storehouses. The importance of molds in the decomposition of cellulose, that relatively undigestible material of which plants are composed, has recently been reaffirmed. By adding cellulose to soils, a prompt increase in the number of fungi will be registered. Spoilage of cellulose-containing materials by fungi was amply demonstrated during World War II, especially in tropical and sub-tropical countries. Lignin, which is an essential part of woody tissue, is also attacked by molds. Unless cellulose, lignin, and starch are decomposed by biological action, the chemical elements found in these complex materials would be bound up and unavailable for use by other plants and by animals.

SPOILAGE

While most bacteria tend to spoil foods which are neutral or nearly neutral in reaction, molds thrive in environments where the *p*H is too low for usual bacterial activity. Organic acids which bacteria in general cannot tolerate, may be metabolized by molds as a source of carbon and energy, and these acids may be oxidized to carbon dioxide and water. As the acids oxidize, the *p*H may rise to the point where bacterial growth may become possible.

The low food and moisture requirements of molds will allow them to grow on optical glass, which they are capable of etching. Such glass stored in moist areas has been known to be ruined by the growth of fungi. Mildew damage to tentage that is stored without adequate drying is a constant problem with the armed forces and with circuses. Warm damp weather encourages mold growth on the leather of shoes, on paper, and on cloth. Nylon and rayon, however, are resistant to mold action.

High osmotic pressure is little deterrent to fungi, as evidenced by mold growth on the surface of jellies and jams containing a high sugar content. As long as air is excluded, however, molds cannot grow on these products. Spoilage of bread and other starchy foods by *Rhizopus* species is a common problem during the summer months, and the green fuzz seen on decaying fruits is usually a member of the *Penicillium* genus.

INDUSTRIAL APPLICATIONS

Man has capitalized upon the abilities of fungi to attack complex materials, and many industries are founded upon the production of end products by the action of molds and other fungi. The ancient process of retting flax and hemp to free the fibers of the binding substance, pectin, is a hydrolytic action brought about by *Mucor* species and by the enzymatic activity of certain anaerobic bacteria. *Aspergillus niger* is the mold that is instrumental in making gallic acid, which in turn is employed in manufacturing ink. Starch is acted upon by *Aspergillus oryzae,* and diastase is recovered in the process. The flavor of roquefort cheese comes from the formation of caproic acid through the breakdown of butter fat by *Penicillium roqueforti.* When casein is attacked by *Penicillium camemberti,* the characteristic flavor of camembert cheese is the result. *Aspergillus niger* is employed in the manufacture of citric acid from cane sugar. The antibiotic, penicillin, is produced through the action of *Penicillium notatum.* This is only a brief list of some of the products manufactured through the biological activities of molds. It should become apparent, therefore, that controlled spoilage by fungi is a valuable thing from the standpoint of both industry and medicine.

FUNGUS DISEASES

Unfortunately for plants, lower animals, and man fungus diseases are widespread and sometimes are difficult to control. Once a fungus disease becomes firmly established it may be relatively resistant to the therapeutic measures usually employed. With the widespread use of antibiotics in combatting bacterial diseases, fungi are better able to establish themselves, and they flourish when bacterial competition diminishes. Fungus diseases are called MYCOSES, and many of the etiological agents fall under the class of Fungi Imperfecti. Some mycoses occur only in restricted parts of the world, while others are geographically widespread.

In naming fungus diseases the suffix *mycosis* is added to the

name of the tissue or organ affected. For example, a skin infection is called a DERMATOMYCOSIS, while an OTOMYCOSIS is an ear infection, and a PNEUMONOMYCOSIS is a fungus invasion of the lungs. In other instances the suffix mycosis is added to the name of the etiological agent, as in COCCIDIOIDOMYCOSIS.

DERMATOMYCOSIS

The most common fungus diseases are DERMATOMYCOSES, popularly referred to as "ringworm." So-called "athlete's foot" is undoubtedly the most prevalent fungus disease in the United States, and it is caused by the genus *Trichophyton*, although the genus

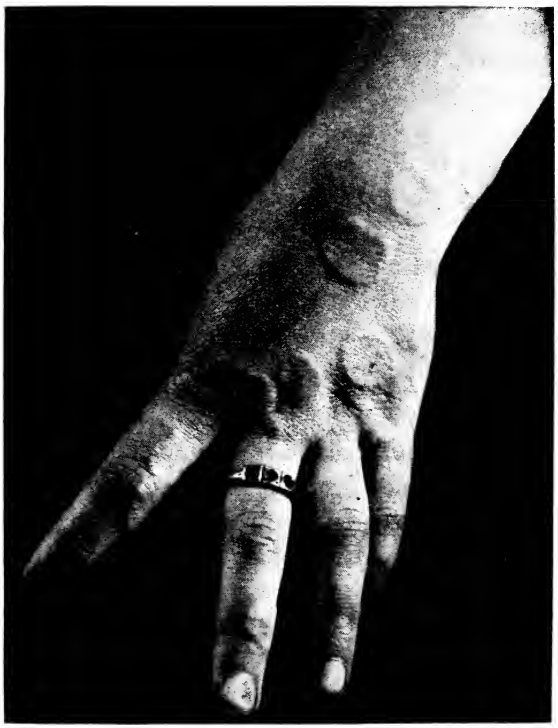


Fig. 63. Lesions of ringworm, a fungus disease. (*From* The Biology of Bacteria, *A. T. Henrici and E. J. Ordal, 3rd ed. Copyright 1948, D. C. Heath and Company, New York.*)

Epidermophyton may also be involved. Skin, hair, and nails may be infected by the dermatophyte, *Microsporum.*

The fungus of "athlete's foot" thrives during warm weather, particularly in such localities as locker rooms and indoor swimming pools where individuals walk around in their bare feet. The infection usually becomes established between the toes and initially appears as small raised blisters. In time the blisters break, and the dried skin peels off, exposing the underlying tissue. Itching may become severe, and the skin may crack. The condition may become severe enough to require medical attention, particularly if pyogenic cocci, such as *Micrococcus pyogenes,* become localized in the open wounds and cause secondary bacterial infections.

ASPERGILLOSIS

Aspergillosis of birds may occur when they ingest *Aspergillus fumigatus,* a common green mold found on moldy grain and silage. The fungus localizes in the air sac of the birds and frequently causes fatal pneumonia. This disease is rare in man, but it has been known to occur as a secondary infection following pulmonary tuberculosis.

COCCIDIOIDOMYCOSIS

This fungus derives its name from its resemblance in tissues to the protozoan genus *Coccidium* in that a number of spores are produced within the cell. *Coccidioides immitis* is the fungus causing the disease variously named coccidioidomycosis, valley fever, and coccidioidal granuloma. In the United States the disease appears to be transmitted almost exclusively by the inhalation of dust in the semi-arid regions of California and the southwest. It is relatively widespread, but it is not generally a severe disease. When it does become a generalized infection, however, it is highly fatal.

When cultivated on laboratory media this fungus develops an abundant mycelium, and in about eight days spores are borne on fertile hyphae. In lesions of the body, however, the large spores (which sometimes attain a size of from 60 to 80 microns) fill up with many small spores which eventually are liberated and

infect adjacent tissues. Hyphae are not formed when this fungus is growing *in vivo*.

A test similar to the tuberculin test has been developed to detect persons who have the disease or who have had the infection. In extensive tests conducted in the southwestern part of the United States it has been found that a high percentage of residents in these areas react with a positive coccidioidin reaction.

CHAPTER 20

The Fungi—Yeasts

DEFINITION AND BRIEF DESCRIPTION

A completely satisfactory definition of a yeast is difficult to formulate if it is to include all of the considerations involved in describing these particular fungi. The word YEAST like the word MOLD has no taxonomic significance. According to Henrici, yeasts are fungi that permanently maintain a unicellular growth form, not developing mycelia. Many fungi may temporarily fulfill this description, but since the condition is not permanent, these organisms are excluded as yeasts. Yeasts are generally considered to be those unicellular, chlorophyll-free fungi which possess a demonstrable nucleus, which multiply by budding and ferment simple sugars with the formation of carbon dioxide and ethyl alcohol. Not all fungi classified as yeasts exactly fit this definition or the one proposed by Henrici.

Yeasts are non-motile and are facultative with respect to their oxygen requirements. Because of their relatively large size (range of 3 to 10 μ wide and 3 to 100 μ long), more of the structures of yeasts can be seen under the oil immersion objective than is true of most bacteria. In spite of their unicellular make-up, yeasts are considered to be a little higher in the scale of plant life than bacteria—perhaps between the bacteria and the molds. In general, the technics employed for the cultivation of bacteria are the same for yeasts.

It was Leeuwenhoek in 1680 who first described yeasts as living things, but it was not until the fundamental work of Pasteur in 1865 that the role of these fungi in fermentation was established. In 1881 Hansen explained how yeast cells, found as part of the normal flora on ripening grapes, spent the winter in the soil, probably in the spore stage. He felt that these spores were blown about on dust particles with the aid of wind currents, and the following growing season they were transported to the grapes by this same means. Qualitative bacteriological studies conducted on the digestive tract of insects will frequently disclose the presence of yeasts, and with insects serving as incubators during the cold winter months, fungi may survive and eventually be deposited on grapes and other fruits by the activity of these insects.

CLASSIFICATION OF YEASTS

It is to be hoped that the changes and the counter-changes in the naming and the classifying of yeasts will eventually settle down and become standardized and accepted by microbiologists. A single species masquerading under several different names is very confusing, particularly to those persons who are approaching the study of yeasts for the first time.

For the sake of simplicity yeasts may be considered as existing in just two major groups: the family *Endomycetaceae*, which includes those cells which produce ascospores and are known as TRUE YEASTS, and the family *Torulaceae* which do not form ascospores and are known as PSEUDO (false) YEASTS. Subgroups in these families are based principally upon morphology—the size and

form of the cells, the types of spores formed, and the mode of multiplication of the organisms. The character of growth on laboratory media and the fermenting abilities of the cells constitute the most important cultural and physiological characteristics employed in classification schemes. Yeasts may be classified either as Ascomycetes or as Fungi Imperfecti.

MORPHOLOGY AND REPRODUCTION

Yeasts may be round, egg-shaped, or elongated, and the morphology is rather constant for a given species grown under standardized conditions. The thick walls of yeasts are not difficult to demonstrate by ordinary staining methods. A considerable space within the cell is occupied by a distinct nucleus which divides during multiplication of the cell, with part of the nucleus entering each of the offspring. Granules of various sizes are particularly evident in older yeast cells. Vacuoles contain material, the complete chemical nature of which has not been determined, but fat and glycogen have been demonstrated. These vacuoles serve as reserve food supplies that may be drawn upon during periods when extracellular food substances are not available. Cytoplasm comprises the remainder of the cell substance.

Reproduction of yeasts takes place asexually by BUDDING and BINARY FISSION, sexually by ASCOSPORE FORMATION, and by fusion of two yeast cells to form ZYGOSPORES.

The most common method of reproduction is budding. In this process a blister-like formation makes its appearance on the parent cell. As the bud, or daughter cell, becomes larger, a strand of protoplasm connects the mother and the daughter. Part of the parent nucleus breaks away and enters the bud, and when the new cell reaches a predetermined size, it breaks away and begins to produce buds of its own. Daughter cells have been observed to produce buds while still attached to the parent cell, resulting in the formation of clumps of yeast cells. Under ideal growth conditions cell division may take place once in about every twenty minutes.

Yeasts belonging to the *Schizosaccharomyces* (fission sugar fungi) divide by transverse fission in which the two cells thus

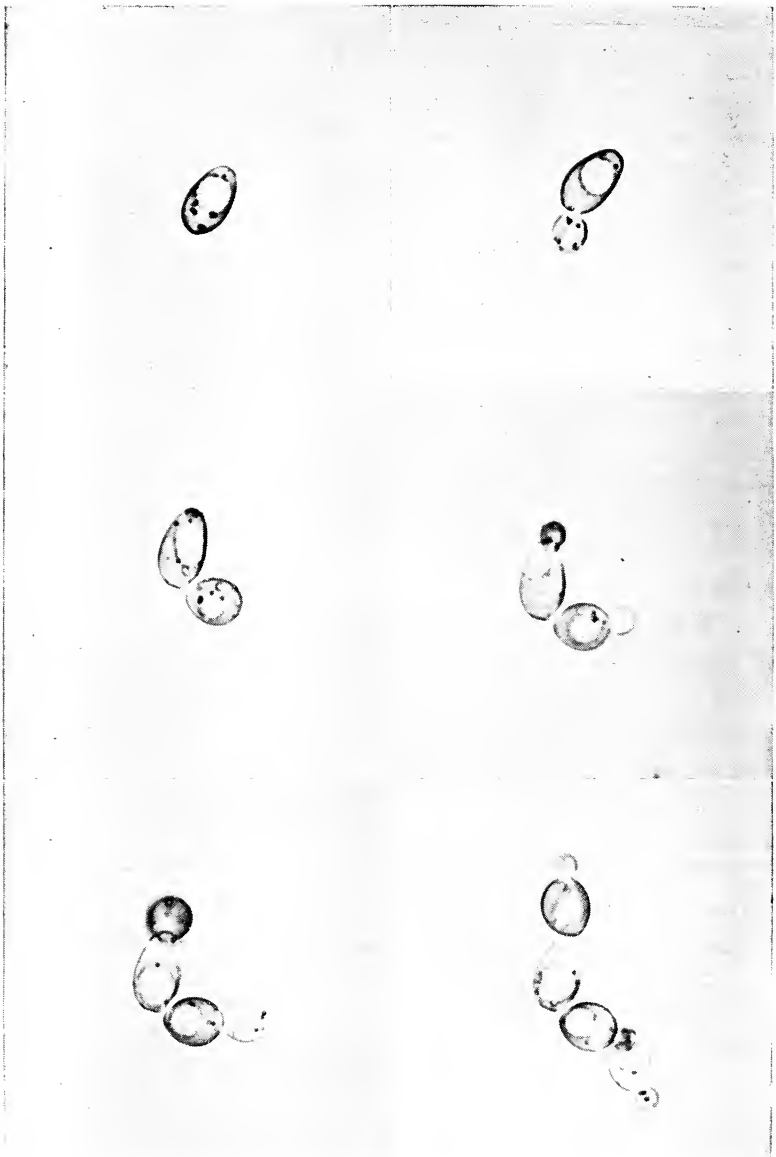


Fig. 64. Photomicrographs showing the sequence of budding of *Saccharomyces cereviseae.* (*Courtesy of the Fleischmann Laboratories of Standard Brands, Inc., New York.*)

formed are approximately equal in size. This type of reproduction is the same as that found with the bacteria.

True yeasts are characterized by their ability to produce ascospores—spores within a sac, generally found in pairs or in multiples of two. Each individual spore is capable of producing a new

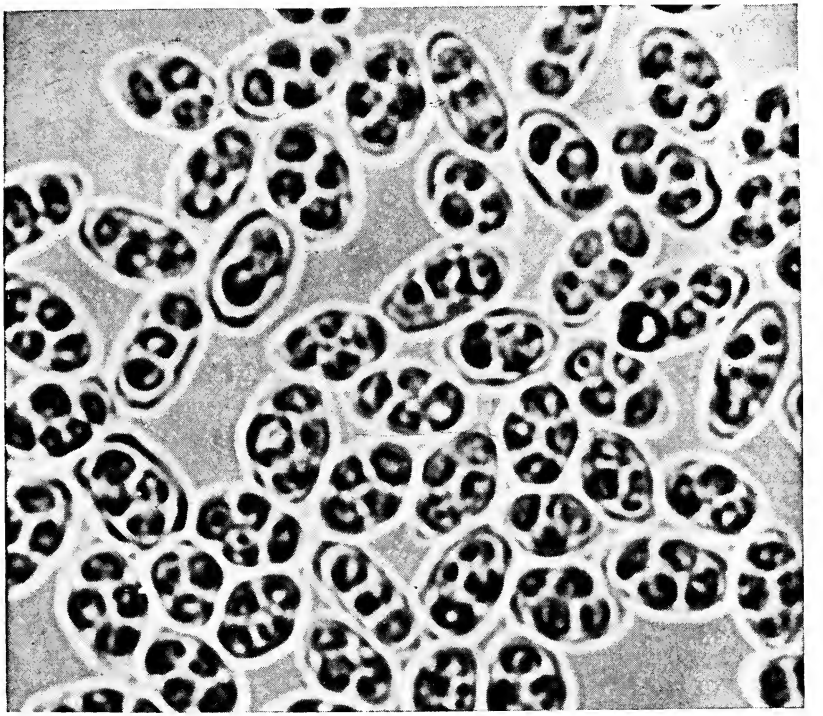


Fig. 65. Asci of *Saccharomyces cereviseae.* (*Courtesy of the Fleischmann Laboratories of Standard Brands, Inc., New York.*)

vegetative cell if allowed to grow under ideal conditions. Bacteria usually produce but one spore which cannot be considered as a reproductive body in the same sense as an ascospore. The shape and the number of ascospores produced in a cell aid in the identification of the yeast species. Sexual reproduction by the fusion of two cells has been demonstrated in *Schizosaccharomyces.*

Vegetative yeasts are generally killed by an exposure to 60° C.

for just a few minutes, and their ascospores are only slightly more resistant. This is in contrast to bacterial spores, some of which may be boiled for an hour without being inactivated.

DISTRIBUTION IN NATURE

Yeasts are probably not as abundant in nature as molds because of differences in nutritional requirements. Yeasts may be found in places where sugar is available, and even though the substrate contains high concentrations of sugar, as in jam, jelly, and syrup, the osmotic pressure does not materially affect the growth of these cells. Soil is not the natural habitat of yeasts the way it is for bacteria and for molds. Yeasts find their way into the ground when they are washed or blown from the surface of fruits, particularly grapes. Insects might be considered to be reservoirs of yeasts, and these arthropods are undoubtedly important in the dissemination of fungi of all kinds. Healthy persons may serve as carriers of yeasts. The nectar of flowers and the exuding sap of trees and plants may contain large numbers of yeasts which have been transported to these localities by the action of wind or by insects.

ECONOMIC IMPORTANCE

INDUSTRIAL APPLICATIONS

Because of their high fermentative abilities members of the genus *Saccharomyces* are employed extensively in the baking and in the brewing industries, with carbon dioxide and alcohol the chief end products of their action. The yeast cake that you buy at the grocery store is composed of about 95% true yeast cells (*Saccharomyces*) and about 5% starch which serves as a binding agent to help maintain the shape of the yeast cakes and the viability of yeast cells. As the enzymes liberated by the yeasts attack the fermentable constituents of dough, the carbon dioxide gas released in the process makes the dough "rise." The yeast, therefore, serves as a leavening agent.

Yeasts are particularly rich in vitamin B_1 (thiamine) and B_2 (riboflavine), and by irradiating cells with ultra-violet light, they become fortified with "sunshine vitamin" D. When the diet of

cows is supplemented with yeasts, the milk produced by these animals contains a high amount of these vitamins.

Some individuals eat yeast as part of their daily diet. These microorganisms have been recommended as an aid in clearing up skin blemishes and preventing constipation. While yeasts may contain as much as 50% protein and readily available sources of the B complex vitamins, ingestion of large numbers of yeasts as part of the daily diet is not recommended except upon the advice of a qualified physician, because undesirable changes in the intestinal flora of the individual may be brought about by too many of these organisms.

Brewer's yeasts are usually classified as "top" and "bottom." Top yeasts are active fermenters, and the large amount of carbon dioxide gas which they produce drives the cells to the surface of the vats where the yeasts form a scum. Ales are made with top yeasts, while beers are generally prepared by the slower-fermenting bottom yeasts. Distillery yeasts are high alcohol-producing strains of *Saccharomyces.* When the alcohol concentration reaches about 10%, yeast activity is markedly diminished, and when the strength of alcohol reaches 15%, further production of alcohol virtually ceases. Hard liquors, therefore, must be distilled to concentrate the alcohol produced in fermentation vats. When grains are the substrate employed for the cultivation of yeasts, the enzyme *diastase* found in the sprouting grain converts the starch into sugar which in turn is acted upon by yeasts specifically selected for their pronounced ability to ferment such a substrate. Molasses, potatoes, sugar beets, and rice may also be employed to produce alcohol.

Wines are made by the fermentation of fruit juices or sugar through the action of particular strains of yeasts, usually *Saccharomyces ellipsoideus* (elliptical-shaped cells). When home-made wines are prepared, it is usually the action of bacteria and yeasts found as the natural flora on the fruit which results in the production of alcohol. Pure cultures of yeasts may or may not be added to initiate the fermentation.

Another important product which is produced by the enzymatic action of yeasts is glycerin. This compound is used as a sweeten-

ing agent, as a solvent, as a compound of anti-freeze, and as a constituent of medicines, inks, adhesives, and gunpowder.

The high fat content of some strains of yeasts provides a source of this substance for certain commercial applications. During World War I when the Germans were faced with a shortage of gunpowder, it is reported that they employed the false yeast *Torula lipofera,* which is exceptionally well suited to converting simple compounds into fat, for the manufacture of fat which in turn was converted into nitroglycerine. As much as 20% dry weight of these yeast cells may be composed of fat.

SPOILAGE

The principal yeast offenders in the spoilage of food are members of the family *Torulaceae,* which produce pigments and undesirable chemical end products during their metabolism. So-called "bloody sauerkraut" may be caused by pigmented yeasts, and the colors sometimes found in milk and dairy products are frequently traced to the growth of false yeasts. Many foods not usually attacked by bacteria because of the low *p*H are readily employed as a substrate by yeasts which can tolerate and thrive at *p*H levels not conducive to bacterial growth. Members of the *Rhodotorulaceae* (red false yeasts) may be particularly troublesome in places where oysters are shucked, and in butcher shops.

DISEASE

A few yeasts are pathogenic for man and for lower animals. One such disease is caused by *Candida albicans* which affects the throat, mouth, and other parts of the body. This yeast, according to Diddens and Loder, has eighty-eight synonyms. The disease is called MONILIASIS, and when it causes ulceration of the mouth and throat, it is termed "thrush." If the organisms invade the stomach and intestines, they may cause an ulcerative condition accompanied by diarrhea; this disease is "sprue."

In cases of thrush characteristic patches of creamy, membranous-like material are found, and stained smears prepared from material taken directly from the lesions reveal an abundance of the causative

organisms. When cultivated on laboratory media, *Candida albicans* produces a mycelium but it does not form ascospores.

Another fungus disease of both man and the lower animals is BLASTOMYCOSIS, or Gilchrist's disease, caused by *Blastomyces dermatitidis.* This organism affects the skin, the deeper tissues, the lungs, bones, and the spleen. Its filamentous-like nature when cultivated on Sabouraud's medium may lead some persons to believe that the organism is a mold. Its typical yeast-like appearance in affected tissues, however, warrants the organism being classified as a yeast. In the tropics this disease is widespread as a primary skin infection, and lung invasion is not uncommon with death from pneumonia the usual prognosis.

Torulopsis neoformans is an asporogenous yeast which causes a highly fatal disease of the nervous system, the lungs, or of other internal organs. This organism is also known under the generic name of *Cryptococcus.* Like many fungi which have several different names, this disease is also known as torula meningitis, cryptococcosis, American torulosis, and European blastomycosis.

CHAPTER 21

The Rickettsiae

HISTORICAL INTRODUCTION

It was in 1909 that Howard T. Ricketts found small, spherical and coccoid bacteria-like organisms in wood ticks. These microbes are called *rickettsiae* in honor of their discoverer. Rickettsiae resemble bacteria in that they may be observed under the oil immersion objective of an ordinary compound microscope, but they are

virus-like in that they require living cells for growth. They are normally found only inside other living cells. The original studies of Ricketts were concerned with the cause of Rocky Mountain spotted fever, and a year later in Mexico he discovered the etiological agent in typhus fever, also a rickettsial disease. He found short, rod-like forms of organisms in the blood of patients suffering from typhus, and infected lice were found harboring similar organisms

Fig. 66. Howard T. Ricketts (1870–1910). (*From* Principles of Microbiology, *F. E. Colien and E. J. Odegard. Copyright 1941, C. V. Mosby Company, St. Louis, Missouri.*)

in their intestines. Unfortunately, during the course of his investigations Ricketts contracted typhus and died in 1910. Typhus fever should not be confused with typhoid fever which is a bacterial disease caused by gram negative rods reported in 1880 by Eberth, who discovered the organisms in the spleen of typhoid fever patients.

It was a Brazilian, Rocha-Lima, who demonstrated the louse-borne nature of typhus in 1916. He is also the person who named the etiological agent *Rickettsia prowazekii* in honor of Howard Ricketts and an Austrian, von Prowazek, both of whom lost their

lives as a result of infections with the typhus fever organisms during the course of their studies. Rocha-Lima contracted the disease but he was fortunate enough to recover. Since these pioneer discoveries, other rickettsial diseases have been demonstrated, and they will be discussed as the chapter progresses.

CHARACTERISTICS OF RICKETTSIAE

MORPHOLOGY

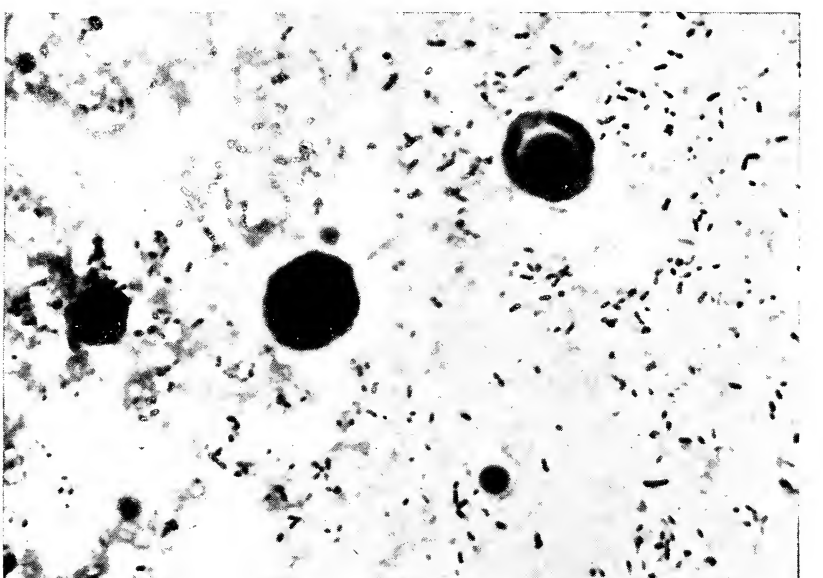
Bergey's *Manual of Determinative Bacteriology* (1948) describes rickettsiae as "small, rod-shaped, coccoid, spherical, and irregularly-shaped microorganisms which stain lightly with aniline dyes. Gram negative. Usually not filterable. Cultivated outside of the body, if at all, only in living tissue, embryonated eggs, or rarely in media containing body fluids. Parasitic organisms intimately associated with tissue cells and erythrocytes, chiefly in vertebrates and often in arthropods which act as vectors . . . . May cause diseases in man or animals, or both."

These microbes are non-motile and range in size from about 0.3 μ in diameter to as much as 2.0 μ in length, although 0.5 μ is more common for the length. With the exception of the rickettsiae causing "Q" fever, none of these organisms is filterable. That is, they are retained by the types of filters usually employed to hold back bacterial cells. When stained with Giemsa's stain rickettsiae appear as lavender, blue, or purple-stained bodies, and when Macchiavello's staining technic is employed, the basic fuchsin colors the rickettsiae red and the background material retains the color of the methylene blue.

CULTURAL REQUIREMENTS

These minute organisms have not been grown in a cell-free medium, but in contrast to viruses which require young, actively growing cells for their development, rickettsiae prefer older, slowly metabolizing cells for their cultivation. Some investigators believe that rickettsiae are forms of bacteria which have adapted themselves to intracellular life.

A



B

Fig. 67. (A) Endemic typhus rickettsiae from yolk sac smear X 1500. (B) "Q" fever rickettsiae cultivated in chick egg X 1500. (*Courtesy of the Rocky Mountain Laboratory, U.S. Public Health Service, Hamilton, Montana. Photographs by N. J. Kramis.*)

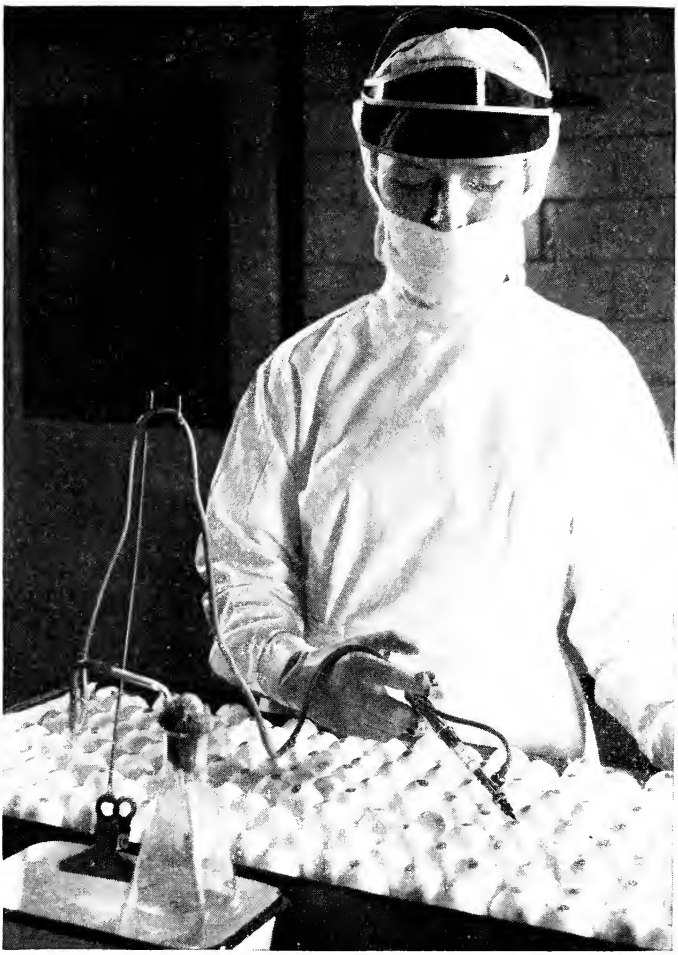


Fig. 68. Pressure-fed syringe being used to inoculate fertile eggs with suspensions of living rickettsiae. (*Courtesy of E. R. Squibb and Sons, New York.*)

Incubation temperatures about five degrees less than that of the human body (37° C.) are favorable for optimum growth of rickettsiae, and this appears to bear a direct relationship to the reduced metabolism of host cells at this lower temperature of 32° C. Raising the temperature of laboratory animals which have

been injected with virulent rickettsiae definitely affords the animals protection, since their survival rate is significantly higher than that found in groups of like animals kept at "ordinary" temperature. Any treatment or process which slows down the metabolism of cells, including the use of chemicals, irradiation of tissues, and withholding of certain vitamins, will generally favor better growth of rickettsiae. Unless some means is discovered for growing these organisms in the absence of cells, it will be necessary to continue to investigate their metabolism through indirect approaches, such as studies concerned with the acceleration and the inhibition of enzymes.

The usual technics employed for killing vegetative bacteria and other microorganisms—application of heat, use of chemicals, drying, etc.—are generally effective for the destruction of rickettsiae. Such preservatives as glycerol may keep these microbes viable for months when they are stored at 10° C.

Animals to which sulfa drugs have been administered are usually more susceptible to rickettsiae due, perhaps, to the direct action that sulfa drugs have on metabolism. The antagonist of sulfa —para-amino benzoic acid (PABA)—has been employed to combat typhus fever. It speeds up cellular respiration, and if the drug is administered early enough and in large enough doses, it appears to be effective. The B complex vitamins, vitamin B_2 (riboflavine) in particular, may be fed to animals to speed up their metabolism and to aid in combatting rickettsial infections. If this vitamin is deficient in the diet, the animals are made more susceptible. This observation may explain in part why typhus fever spreads so rapidly during wartime and in periods of famine when dietary deficiencies are prevalent.

CLASSIFICATION

Bergey's Manual provisionally places the order Rickettsiales as a supplement following the bacteria. The main discussion in this chapter deals with members of the family Rickettsiaceae. The more common vectors, genera, species, and diseases of this group may be summarized in tabular form as follows:

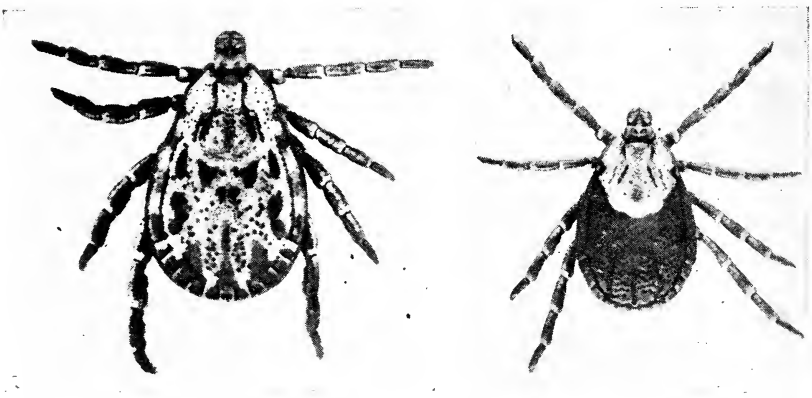
VECTORS	GENERA AND SPECIES	DISEASES
Ticks	*Rickettsia rickettsii*	Rocky Mountain spotted fever
	Rickettsia conorii	Boutonneuse fever
	Coxiella burnetii	"Q" fever
	Cowdria ruminantium	Heartwater fever
Lice	*Rickettsia prowazekii*	Epidemic typhus
Fleas	*Rickettsia typhi*	Endemic (murine) typhus
Mites	*Rickettsia tsutsugamushi*	Scrub typhus
	Rickettsia akari	Rickettsialpox

The family Bartonellaceae includes parasites of erythrocytes in man and other vertebrates, and these organisms may be insect-transmitted. The family Chlamydozoaceae contains obligate intracytoplasmic parasites, and one of its members causes the serious eye disease, trachoma.

RICKETTSIAL DISEASES AND THEIR VECTORS

ROCKY MOUNTAIN SPOTTED FEVER

As stated earlier in this chapter the field of rickettsiology had its origin in studies concerning the etiology of Rocky Mountain spotted fever. This disease was first noted in the Bitter Root Valley region of Montana and in the Snake River Valley of Idaho



A B

Fig. 69. Wood ticks. *Dermacentor andersoni.* (A) male. (B) female. (*Courtesy of the Rocky Mountain Laboratory, U.S. Public Health Service, Hamilton, Montana. Photographs by N. J. Kramis.*)

where it caused a mortality rate as high as 80%. Since these original findings, however, other sections of the United States have reported cases of this disease with mortality rates of only 5%.

The causative organism is *Rickettsia rickettsii* transmitted by the bite of infected ticks which may be carried by rodents and other animals in endemic areas. Infected ticks bite these animals and man and thus maintain a reservoir for still other ticks to acquire the disease and keep it going.

One of the diagnostic features of these particular rickettsiae is their predilection for nuclear material in the cells they invade. This intranuclear affinity is in contrast to most other rickettsiae which grow best in the cytoplasm of invaded cells (intracytoplasmic growth). The reason for these differences of localization within parasitized cells needs further investigation.

BOUTONNEUSE FEVER

This disease, caused by *Rickettsia conorii,* is tick-transmitted and is immunologically related to Rocky Mountain spotted fever. It can be distinguished from this latter disease, however, by certain serological tests. The organism is pathogenic for man and for guinea pigs, and in man it produces localized sores and an inflammatory reaction in the regional lymph nodes. There is a fever but the mortality rate is low. The disease is also known as Mediterranean fever and Marseille fever, since it is endemic in this area.

TYPHUS FEVER

Typhus fever has played an important role in world history since it flourishes during times of war. The etiological agent was discovered in 1910 by Ricketts. The disease is also commonly referred to as camp fever and jail fever, and it has a death rate varying during epidemics from 5 to 75%. Hans Zinsser, the author of *Rats, Lice and History,* has presented an excellent review of this and other arthropod-borne diseases. He reported that there is no authenticated evidence of typhus fever before the twelfth century, and that the disease was not epidemic before the sixteenth century. Other historical reviews state that typhus was recognized as long

ago as 400 B.C., if writings left by ancient historians and physicians are being properly interpreted.

Typhus fever is endemic in Russia and in sections of Poland, and it has been epidemic during times of war, as is shown by the 100,000 cases reported in Russia alone during the first year of World War I. Between 1917 and 1921 it is estimated that at least twenty-five million people suffered from this disease in territories controlled by Russia, with a mortality rate of about 10%. Over 300,000 deaths were reported in Serbia during World War I.

There are two principal forms of typhus fever: EPIDEMIC and ENDEMIC. The former is louse-borne and is caused by *Rickettsia prowazekii*, while the endemic form is borne by the rat flea *Xenopsylla cheopis* which carries the etiological organism *Rickettsia typhi*. Since rats carry the fleas, endemic typhus is commonly referred to as murine or rat typhus, which goes under the name of *tabardillo* in Mexico.

Severe headache, chills, and fever mark the onset of the disease, and about four days following the first symptoms, a macular (spotty) eruption appears on the skin and lasts as long as the fever. The acute symptoms disappear on about the twelfth day with a subsequent slow recovery. In parts of the United States where typhus is endemic, it is sometimes called *Brill's disease*.

The human body louse, *Pediculus* var. *corporis* carries the rickettsiae in its gut, and this louse is primarily responsible for epidemic typhus. The microorganisms gain entrance to the human body through apparently intact skin or through injured skin after being deposited there in the excreta of lice.

TSUTSUGAMUSHI DISEASE (SCRUB TYPHUS)

A mite-transmitted disease that is widespread in Japan, Malaya, and other parts of the southwest Pacific is caused by *Rickettsia tsutsugamushi* (meaning "of a dangerous mite"). Field mice are infested with these mites whose larvae bite animals and man. The adult mite does not transmit scrub typhus to man but does pass the organisms on to her eggs. These mites are members of the genus *Thrombicula*.

HUMAN RICKETTSIALPOX

This recently recognized disease caused by *Rickettsia akari* is transmitted by the mite *Allodermanyssus sanguineus,* and the clinical symptoms resemble those of chickenpox. The first case was discovered in New York City in 1946, and since then Connecticut and Massachusetts have also reported finding cases of this disease. Undoubtedly other parts of the United States will report additional cases when they are recognized.

The mite bites house mice (*Mus musculis*) and man, and the causative rickettsiae have been isolated from mites, mice, and infected human beings. When cultivated in embryonated eggs, the rickettsiae are found localized both intra-nuclearly and intracytoplasmically in the yolk sac cells.

Mites gather on the warm walls where heating ducts pass to the upper floors, and the cases reported to date have localized in apartment houses where rodents are a problem. Although the disease is accompanied by an inflammation of the lungs, the mortality rate is low.

"Q" FEVER

In 1936 Derrick of Queensland, Australia, was studying a febrile disease which had clinical symptoms resembling those of influenza. He injected some blood and some urine of a patient into a guinea pig and the pig became ill. After recovering from the disease, the pig displayed an immunity to subsequent injections of like material. The causative organisms were finally isolated from a mouse which had been injected with blood from an infected patient. Textbooks frequently give the impression that "Q" fever derives its name from Queensland where it was studied, but Derrick claims that it was the *questionable* nature of the disease that led him to call it "Q" fever.

This disease may be more common than is generally believed, and many cases of questionable influenza might well be "Q" fever if subjected to thorough diagnostic studies. Persons engaged in occupations which bring them in close contact with animals are most likely to contract the disease. Those who handle hides, work

in stockyards, slaughterhouses, or dairies are particularly prone to infection with *Coxiella burnetii.* Coxiellae were named for Herald R. Cox who first described the organism in guinea pigs inoculated with infected ticks collected in Montana. F. M. Burnet discovered the microbes in Australia.

Several species of ticks have been found to harbor coxiellae, but the exact mode of transmission to man is not clear. There is even some evidence that persons who drink raw milk might contract "Q" fever, and man-to-man transmission by way of the respiratory tract has been suggested by others. Coxiellae are capable of passing through bacterial filters which hold back other microorganisms classified as rickettsiae.

HEARTWATER DISEASE

The third genus listed under the family Rickettsiaceae is *Cowdria,* and the species name is *ruminantium.* They are small, pleomorphic, spherical or rod-shaped organisms found intracellularly in infected ticks. Once the ruminants are infected with these microbes, fluids accumulate in the sac around the heart (pericardial sac) of the animals, and the mortality rate is high. Cowdria differs morphologically from rickettsiae in being spherical and elliptical.

TRENCH FEVER

This was apparently a new disease first recognized during World War I. There is still enough remaining to be learned about the etiological organisms causing trench fever to warrant their being placed in an appendix to the regular classification schemes until such time as they can be properly classified. The organisms are slightly larger than those found in typhus fever, and they have been designated *Rickettsia quintana.*

These rickettsiae resist a moist temperature of 60° C. for thirty minutes and dry heat at 80° C. for twenty minutes. Drying in sunlight for at least four months has not inactivated them. Lice allowed to feed on trench fever patients have been found harboring the microorganisms, and the habitat appears to be the lining of the

gut of the body louse, *Pediculus humanus,* and the head louse, *Pediculus capitis.* Synonyms for the disease include Wolhynian fever, shin bone fever, and five-day fever.

The two other families, Bartonellaceae and Chlamydozoaceae, listed under the order Rickettsiales will not be discussed. Interested students are invited to obtain further information on these microorganisms from other textbooks in the field and from *Bergey's Manual.*

LABORATORY DIAGNOSTIC TECHNICS

While most clinical laboratories are not equipped or staffed to carry out extensive isolation and identification procedures with respect to rickettsiae, there are serological tests available which when correlated with the clinical findings will corroborate a diagnosis of disease of rickettsial origin. The diagnosis, however, is primarily a clinical one.

Weil and Felix in 1915 isolated a strain of the bacterium *Proteus vulgaris* (designated OX 19) from a patient suffering from typhus fever, and by employing a suspension of these bacterial cells as antigen, the serum from typhus patients was found capable of agglutinating the proteus cells. The so-called Weil-Felix reaction is based upon the production of antibodies (agglutinins) in patients or in animals infected with typhus fever, but agglutinins do not appear much before the tenth day of the disease.

There is some antigenic fraction held in common by rickettsiae and certain strains of proteus bacteria, although this gram negative rod is not found associated with all cases of typhus fever. Two additional strains of these same bacteria have been found to bear an antigenic relationship to rickettsiae, and these proteus strains have been named OX 2 and OX K. Differences in the agglutinability of these three bacterial strains by the blood serum of infected persons serves as a basis for separating rickettsiae as will be seen in the table which follows. Strong agglutination reactions are given ratings of four plus, with lesser degrees of clumping being indicated by fewer pluses. A plus-minus reaction means it is doubtful.

DISEASE	PROTEUS OX 19	PROTEUS OX 2	PROTEUS OX K
Typhus fever	++++	+	negative
Rocky Mountain spotted fever	++	++	
Tsutsugamushi disease	±	negative	++++
"Q" fever	negative	negative	negative
Heartwater fever	negative	negative	negative
Rickettsialpox	?	?	?

Because agglutination differences between typhus fever and Rocky Mountain spotted fever are only a matter of degree, these reactions in themselves are not enough to differentiate these diseases. Another more complicated serological test, called the COMPLEMENT FIXATION REACTION, may be employed to separate them. This test will not be described here.

METHODS FOR CONTROLLING RICKETTSIAL DISEASES

One of the most effective means for preventing rickettsial diseases is the elimination, if possible, of the vectors and the animals upon which these vectors thrive. This is usually a herculean task, but progress has been made in this direction not only in the United States but also in many endemic areas throughout the world.

Prophylactic vaccination has proven very effective in man's fight against diseases of rickettsial etiology. The dramatic drop in the number of cases of typhus during World War II as compared with the first World War is proof that the combination of effective vaccines and the liberal use of DDT and other substances to kill lice can be employed to control this disease. The Germans were reported during the closing stages of World War I to be manufacturing typhus vaccine by the intra-rectal injection of lice with living rickettsiae. The intestines of lice serve as excellent locations for the growth of these microorganisms, but such a manufacturing process is difficult and dangerous, to say nothing of the relatively small yield of vaccine per man-hours expended. Vaccines for both typhus fever and Rocky Mountain spotted fever can be prepared by employing either the yolk sac method of Cox or the chorio-allantoic membrane of the developing chick. After separating the rickettsial bodies from the egg material, the microorganisms are inactivated with phenol and preserved with formalin. Active immunity induced by injections with these vaccines lasts

for about a year with Rocky Mountain spotted fever and over a year for typhus. Vaccines for the prevention of other rickettsial diseases are being investigated at the present time.

Antibiotics have come to the rescue in man's fight against these diseases. Terramycin is highly rickettsiostatic. In experimental infections in chick embryos it appears to be one of the most effec-

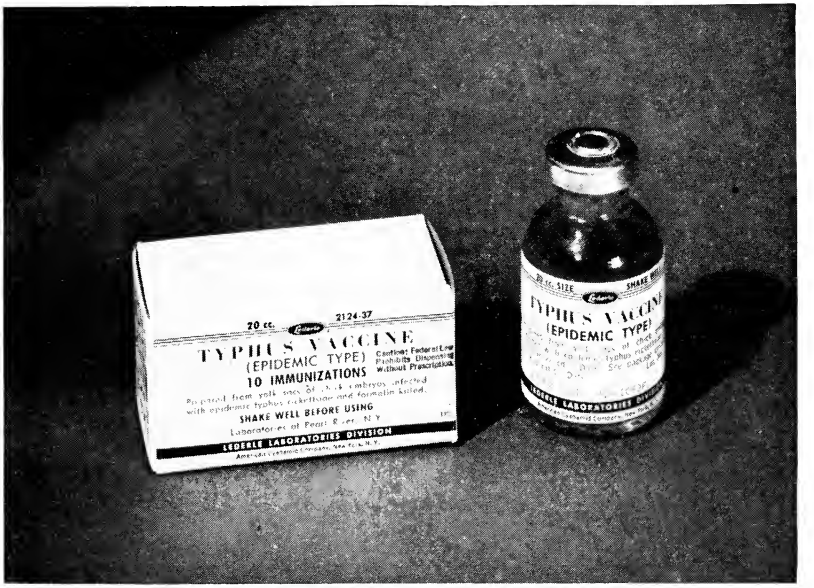


Fig. 70. Typhus vaccine. Formalin-killed preparation of epidemic typhus rickettsiae from chick embryo yolk sac cultures. (*Courtesy of Lederle Laboratories Division, American Cyanamid Company, New York.*)

tive antibiotics against the microbes of scrub typhus, Rocky Mountain spotted fever, epidemic typhus, and rickettsialpox. Clinical literature seems to bear out these experimental laboratory findings. Aureomycin and chloromycetin have also proven quite effective in combatting some of these infections.

By constant vigilance and by application of knowledge gained in war and peace, diseases of rickettsial etiology can be effectively controlled, and some of these scourges, second only to plague, should become epidemics of the past.

CHAPTER 22

Viruses

BRIEF HISTORICAL REVIEW

With the discovery of bacteria man felt that he had found the simplest forms of life—that last step between living and dead matter. But when Iwanowski in 1892 demonstrated that the sap from a diseased plant could be passed through the finest porcelain filters and the filtrate was capable of transmitting the disease to healthy susceptible plants, the concept of the smallest living thing had to be re-evaluated. The filterable agent, too small to be seen under ordinary compound microscopes, was called a *virus,* a word

derived from the Latin and meaning a slimy or poisonous liquid. Virus also means anything that poisons the mind or the soul.

It was the work of Loeffler and Froesch in 1897–1898 that first demonstrated a virus disease of animals, namely, foot and mouth disease of cattle. Three to four years later (1901) Walter Reed discovered yellow fever virus affecting man. It was not until 1915, however, that virus diseases of bacteria were announced by Twort. His work was confirmed in 1917 by d'Herelle who named this filterable agent BACTERIOPHAGE (bacteria-eater). In 1930 PHAGE, the accepted abbreviation for bacteriophage, was considered similar to viruses affecting animals and higher plants.

CLASSIFICATION

The sixth edition of *Bergey's Manual* (1948) describes these transmissible, parasitic agents as follows:

"Viruses are 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 . . . A considerable number of viruses have not 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. . . Cause diseases of bacteria, plants and animals."

CHARACTERISTICS OF VIRUSES

In addition to being too small to be seen normally under a compound microscope, ultramicroscopic viruses are characterized by their extreme dependence upon living cells for their existence and multiplication. While their mode of multiplication is not known, it is assumed that they divide in a manner similar to that employed by bacteria—BINARY FISSION. Hence, viruses are placed under the class Schizomycetes in the provisional classification scheme:

By use of electron microscopy different viruses have been found to be spherical, rod-like, oval, and tad-pole shaped, and they range in size from about 10 millimicrons to nearly 300 millimicrons.

Among the smaller viruses are those of FOOT AND MOUTH DISEASE (10 millimicrons), YELLOW FEVER (22 millimicrons), and POLIOMYELITIS (25 millimicrons). These are smaller than some of the larger protein molecules. Among the larger viruses are included those causing smallpox (250 millimicrons). For comparison, a human red blood cell is 7500 millimicrons in diameter, and as many as one million virus particles can be packed within a single bacterial cell.

All viruses studied to date have been found to consist of high molecular weight nucleoproteins. Viruses are resistant to cold, as is indicated by certain animal viruses which can withstand —76° C. for as long as one year. Pasteurization (62° C.), on the other hand, will inactivate most viruses in thirty minutes or less. Lyophilization, which is freeze-drying in a vacuum, will preserve viruses for indefinite periods, providing the oxygen is completely excluded. Phenol, tincture of iodine, formaldehyde, and exposure to ultra-violet radiation will inactivate viruses after a few minutes exposure. While some antibiotics, including aureomycin, are able to inactivate viruses, most antibiotics are not nearly as effective against these filterable agents as they are against bacteria and some of the rickettsiae.

The high degree of specificity exhibited by viruses for certain hosts—even specific tissues of specific hosts—is one of their outstanding characteristics. This tissue affinity is sometimes used as a basis for classifying virus diseases as:

1. *Dermotropic* (associated with skin): chicken pox and smallpox.
2. *Neurotropic* (associated with central nervous system): rabies and poliomyelitis.
3. *Pneumotropic* (associated with the lungs): influenza and virus pneumonia.
4. *Viscerotropic* (associated with internal organs): yellow fever.

The filterability of viruses is not attributed wholly to their minute size. Just as the type of filter, temperature, size of particles, amount of positive or negative pressure exerted on the filter, *p*H, nature of the suspending fluid, and electrical charges are im-

portant in bacterial filtration technics, these considerations also determine the filterability of viruses. But in ultra-filtration of viruses through collodion filters, called GRADOCOL MEMBRANES, the size of the pore is more important than it is with porcelain bacterial filters.

A fundamental contribution to virology was made in 1935 when Wendell Stanley crystalized the virus of tobacco mosaic disease.

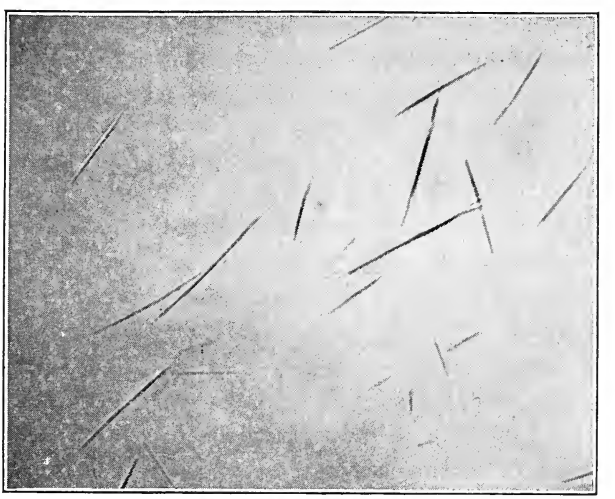


Fig. 71. Crystals of tobacco mosaic virus. (*From Stanley W.*, American Journal of Botany, *1937, 24, 59.*)

These plant virus crystals resemble those of inanimate inorganic crystals, but when the virus is placed in a suitable medium, it reproduces itself. This is further support for the concept that viruses may represent that important link between what man calls living and dead matter. Purification by crystallization is the result of rather rigorous chemical treatment which cannot be applied to animal viruses.

While most microbiologists accept the idea that viruses are living entities, since, for one thing, they can reproduce themselves, there are still plenty of characteristics of these ultramicroscopic forms which provide lively topics for debate on the living vs. the

non-living attributes of these parasitic agents. The word *life* has a man-made definition, and man recognizes degrees of complexity in living things. At the present stage of our knowledge it appears that viruses are probably the simplest forms of matter exhibiting at least some of the characteristics man assigns to living things. Future research developments may disclose that even viruses have "littler fleas to plague them," just as scientists who believed that bacteria were the smallest forms of life had to concede that viruses were still smaller.

Since there are no saprophytic viruses recognized, attempts to study viruses in pure culture completely removed from their host cells have met with failure. In some diseases of virus etiology it is possible to demonstrate granules, called ELEMENTARY BODIES, which are smaller than most bacteria but are still visible under compound microscopes. These granules aid in diagnosing some diseases, since not all viruses display this granule formation. There are strong indications that elementary bodies are aggregates of the virus itself.

Other objects called INCLUSION BODIES are found either within the nucleus or within the cytoplasm of affected cells, and these bodies vary in size and in shape with the particular virus. They probably represent colonies of the infecting virus or disintegration products of the cell, and their presence aids in the diagnosis of some virus diseases. In cases of rabies the inclusion bodies are called *Negri bodies,* in honor of their discoverer, and they are located in the cytoplasm of affected brain cells.

Viruses have been likened to genes which also are nucleoproteins. A difference between the two, however, is that genes multiply only as fast as the cell nucleus, while viruses increase at a rate independent of nuclear division.

TECHNICS FOR CULTIVATION

TISSUE CULTURE

Because of their high degree of parasitism, viruses can be cultivated only in the presence of living cells. Furthermore, they must have young, actively metabolizing cells for optimum growth.

Thomas M. Rivers proposed the use of tissue cultures in 1931 for growing microorganisms, especially viruses. This *in vitro* cultivation technic involves the use of a suspending fluid, like Tyrode's solution, which contains mineral nutrients, glucose, and sometimes blood serum; live tissue cells, including mouse or chick embryo, skin, liver, and kidney; and the inoculum containing the suspected virus.

Cells of hatched chicks are completely resistant to the action of many bacteria, rickettsiae, and viruses, while actively growing embryonic tissue in chicks developing within the egg is particularly susceptible to the action of many of these same microorganisms. The abrupt change that takes place upon hatching of the chick has not been explained satisfactorily.

FERTILE EGGS

While developing chick embryos were used by Ogston as early as 1881 for the cultivation of bacteria, it was not until fifty years later that Gerrit J. Buddingh and his colleagues employed the chorio-allantoic membrane (a membrane surrounding the chick) of the developing chick for the cultivation of viruses. Since the eggs of hens are generally free of microorganisms, nature has provided an ideal culture medium, as well as a suitable container, in which to grow pure cultures of microbes. Fertile eggs are incubated until the embryo is from four to fourteen days old. The age of the developing embryo is a critical factor in the growth of viruses, some of which will only grow during the early developmental stage of the embryo and others of which prefer older cells for their growth.

The egg shell is cut preparatory to inoculation with virus by means of a drill similar to those used by dentists. A square or triangular-shaped piece of the shell is gently removed with a pair of flamed forceps. Aseptic technic is practiced throughout this entire procedure, with tincture of iodine or other suitable disinfectants being used to treat the shell of the egg before cutting out the "window." The underlying shell membrane is kept intact until a small hole is drilled at the air sac end of the egg. By applying

slight suction to this hole, the chorio-allantoic membrane can be drawn away from the shell membrane. When the shell membrane is removed, the chorio-allantoic membrane is exposed, and the virus inoculum is deposited upon it. To exclude bacterial contamination, the hole in the egg is covered by placing a cover slip over the

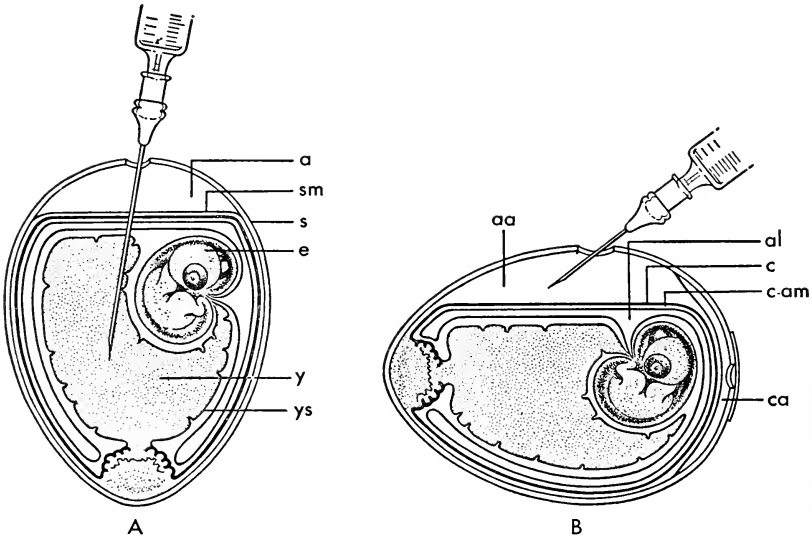


Fig. 72. Methods of inoculating eggs. (A) Inoculation of the yolk sac, a method commonly used for the cultivation of rickettsiae and also of certain viruses. a, air space; sm, shell membranes; s, shell; e, embryo; y, yolk; ys, yolk sac. (B) Inoculation of the chorio-allantoic membrane, a method used for cultivating many viruses. aa, artificial air sac; ca, collapsed air sac; al, allantoic sac; c, chorion; c-am, chorio-allantoic membrane. (*Courtesy of E. R. Squibb and Sons, New York.*)

opening. This glass window is held in place by sealing it with a vaseline-paraffin mixture. Some workers prefer to use a strip of scotch tape in place of the cover glass. After suitable incubation the virus particles develop on the membrane and produce foci of growth which might be likened to that of bacterial colonies growing on an agar culture plate.

Various modifications of the membrane inoculation technic have

been developed, including the injection of test material with a syringe and needle directly through the shell into specific cavities or directly into the yolk sac of the developing chick embryo.

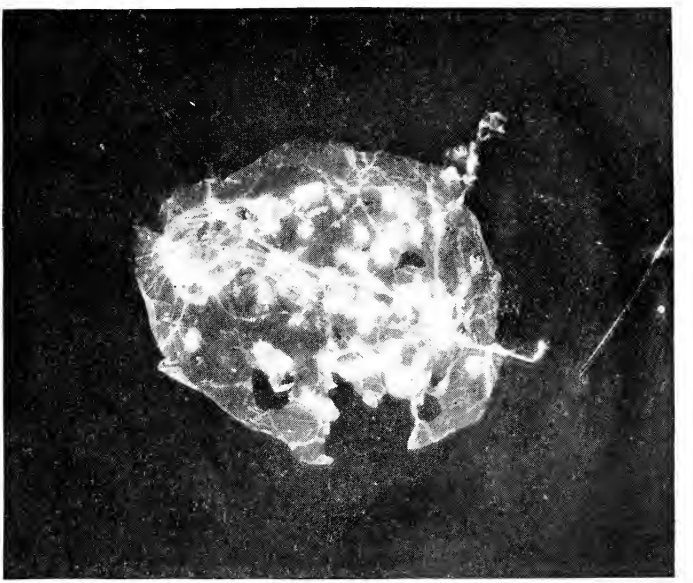


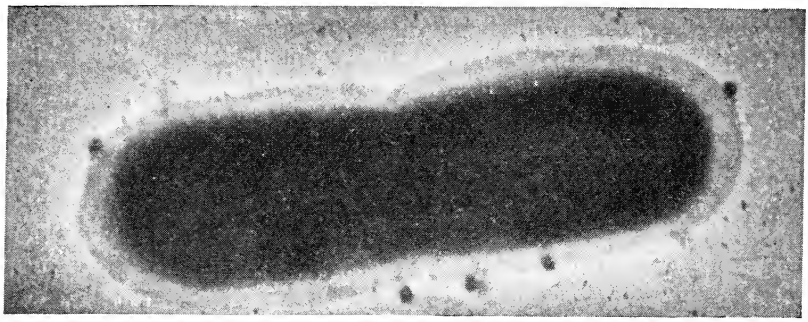
Fig. 73. Chorio-allantoic membrane infected with vaccinia virus. (*Specimen mounted in plastic by Dr. Wolcott B. Dunham, Veteran's Administration Teaching Group, Kennedy Hospital, Memphis, Tennessee.*)

Many vaccines prepared to combat rickettsial and viral diseases are being manufactured with fertile eggs serving as the culture medium.

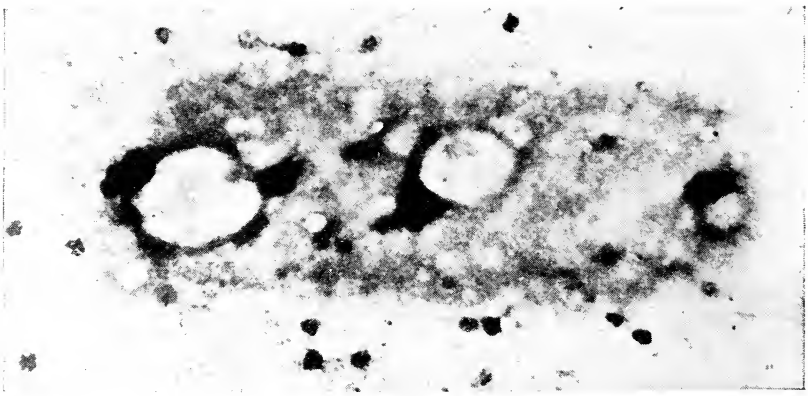
DISEASE

VIRUS DISEASES OF BACTERIA

While Twort was working with staphylococcal contaminants isolated from cowpox vaccine in 1915, he noticed that degenerative changes were taking place in some of the colonies of these bacteria growing on his culture plates. Two years later d'Herelle observed that by adding a bacteria-free filtrate, obtained from the feces of patients afflicted with bacillary dysentery, to a young broth cul-



A



B

Fig. 74. Bacteriophage action on cells of *Escherichia coli* X 25,000. (A) Phage particles adsorbed to bacteria. (B) Invasion and disintegration of bacterium after 23 minutes exposure. (*From* S. E. *Luria, M. Delbruck, and T. F. Anderson,* Journal of Bacteriology, *1943,* ***46,*** *57–58. Photographs kindly furnished by C. J. Witton.*)

ture suspension of the dysentery organisms, lysis of the bacteria occurred. He termed this transmissible agent BACTERIOPHAGE. The digestive tract of man and lower animals is the apparent natural habitat of these bacterial parasites. Bacteriophages range in size from about 10 to 75 millimicrons, and electron microscope studies reveal them to be round or tadpole shaped.

Phage has at various times through the years been considered

to be a contagious fluid, a transmissible enzyme, or nucleoprotein molecule, but today it is accepted as a virus—a living organism which has adapted itself to a completely parasitic, intracellular existence.

Phage exhibits cell specificity. That is, a phage which is capable of lysing one species of organism does not generally attack another species of microbe. Many of the cross-reactions reported in the early literature were undoubtedly due to impure cultures of the lytic agent.

To demonstrate the presence of phage, two methods are commonly employed. Using the broth culture technic of d'Herelle, young (from six to eight hours) cultures of the specific bacteria showing visible turbidity in broth are seeded with filtrates containing specific phage. Within a few hours the turbidity of the broth culture will disappear, and microscopic examination of the broth will usually reveal the absence of the host bacteria. Should the bacterial culture contain phage-resistant cells, a secondary growth and turbidity of the tube will occur after the initial lytic reaction. The strength (titer) of the phage (or the number of phage particles) can be determined by quantitative dilution procedures.

Another satisfactory method of demonstrating phage is to streak an agar culture plate with phage-susceptible bacteria, and on top of this the specific phage is streaked. Upon incubation of the plate, clear areas, called PLAQUES, will appear in the surface growth of the bacteria on the plate. Some colonies may appear "moth-eaten" where the virus has attacked the bacterial cells. Phage plaques might be considered to be the reverse in appearance of bacterial colonies. That is, bacterial colonies are visible concentrations of growth on the plate, while phage plaques are areas of no growth on heavily seeded culture plates.

The specificity of phage in its action permits what is called PHAGE TYPING to detect specific strains of given species of bacteria. An interesting epidemiological application of phage typing is in tracing the source of such disease outbreaks as typhoid fever. Not only is the phage specific for *Salmonella typhosa,* but virus

has been developed that is specific for certain *strains* of typhoid organisms.

Phage is probably responsible for the destruction of large numbers of bacteria in water polluted with human and animal sewage,

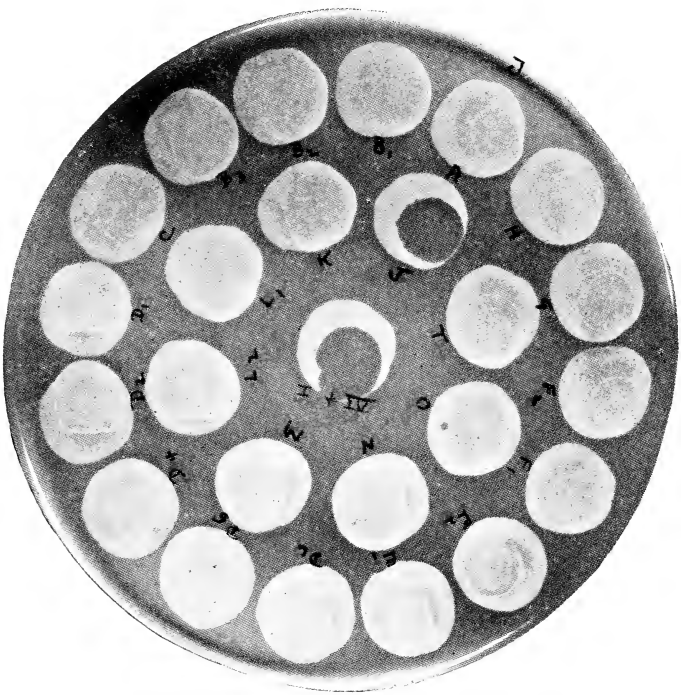


Fig. 73. Bacteriophage typing of *Salmonella typhosa.* Each spot has been inoculated with the same specific type of bacteria and with a loopful of a different phage. Clear areas indicate phage activity. (*Courtesy of P. R. Edwards and the Enteric Bacteriology Unit, Laboratory Branch, Communicable Disease Center, U.S. Public Health Service, Atlanta, Georgia.*)

and thus this lytic agent aids in the self-improvement of bodies of water with the passage of time. It was d'Herelle's belief that phage might explain the recovery of people from disease—a bacteriophage theory of immunity. He postulated that when the phage level becomes high enough, the bacteria causing the disease

are eliminated. Research has shown, however, that there is a strong tendency for phage-resistant bacterial cells to develop, and unless every cell is destroyed, the infection cannot be said to have terminated. Injection of specific phage into such localized infections as boils and carbuncles has been tried, but the results have not warranted continued use of the practice. Injection and feeding of large amounts of specific phage in cases of intestinal diseases has met with similar disappointing results.

VIRUS DISEASES OF PLANTS

In Bergey's tentative classification scheme the viruses affecting plants are classed under the suborder Phytophagineae, and there are six families listed according to the types of disease they cause in plants. These families are: Chlorogenaceae (yellow diseases), Marmoraceae (mosaics), Annulaceae (ringspots), Rugaceae (leaf-curls), Savoiaceae (leaf-savoying), and Lethaceae (spotted wilts). Although the first virus disease ever described was that of tobacco by Iwanowski in 1892, relatively little research was conducted on plant diseases until about 1920. These diseases are of extreme importance in agriculture, and methods for their control are essentially parallel with those employed for combatting bacterial diseases. By the use of various chemical sprays, insect populations can be reduced, and since insects can serve as passive transfer agents in virus as well as in bacterial diseases, insect vectors must be controlled. Keeping healthy plants away from diseased plants is another isolation control method. After handling diseased plants it is imperative that tools and gloves be adequately disinfected before handling non-diseased plants. The rotation of crops has proved effective as a control measure in some areas with certain plant diseases.

VIRUS DISEASES OF ANIMALS

The Zoophagineae are members of the third suborder of viruses, and six families are listed under this suborder. They are classified as diseases in which insects are the exclusive hosts (Borrelinaceae),

pox group diseases (Borreliotaceae), encephalitis diseases (Erronaceae), yellow fever group (Charonaceae), infectious anemia group (Trifuraceae), and the mumps group (Rabulaceae).

A discussion of all virus diseases is impractical in a book of this type, but a brief consideration of a few of the more common diseases of man will be presented.

Smallpox

This serious disease is known technically as *variola,* and it used to be the cause of much misery, disfigurement, and high death rates before the advent of mass vaccinations. The first vaccination was performed by Edward Jenner in 1798. This pioneer made the astute observation that many persons associating with cows suffering from cowpox, did not become affected during severe epidemics of smallpox. He reasoned that cowpox (vaccinia) was in some way related to smallpox. To confirm his suspicions Jenner introduced some lymph from the pustules found on the udder of an infected cow into a scratch made on the arm of a human being. The mild disease, *vaccinia,* resulted in the formation of a single pock mark at the site of the scratch on the arm. Once infected with cowpox these individuals did not contract the more serious smallpox. Cowpox is smallpox that has become modified by passage through a lower animal, and while the virus loses its lethal virulence, its vaccinating power (antigenicity) is not impaired.

The smallpox vaccine used to prevent this disease today is prepared by introducing cowpox virus into the shaved and disinfected skin surface on the abdomen of a calf. After suitable incubation the lymph formed in these pustules is harvested and tested for potency and purity before being tubed for use in vaccinating humans. Smallpox vaccine can also be prepared in test tubes as tissue cultures, and in embryonated eggs, each of which has a decided advantage over the calf method in that bacterial contamination can be more effectively controlled.

Smallpox vaccination lasts for about five years, but some persons become susceptible in shorter periods of time while others remain immune for life. Mass vaccination is advisable during im-

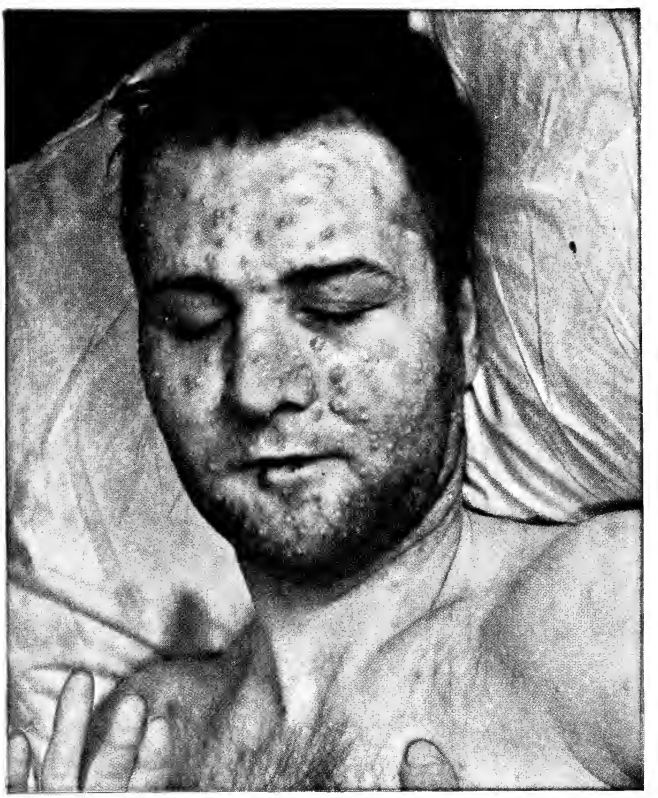


Plate II. Smallpox pustules. (*Reproduced by courtesy of Parke, Davis and Company's* Therapeutic Notes.)

pending epidemics, and such precautions have been shown to stop the further spread of the disease. The threat of a smallpox epidemic in New York City in 1942 was promptly controlled by mass inoculation. Smallpox is no longer a common disease in countries like the United States where compulsory vaccination is required before children can enter public schools. The pock-marked faces so prevalent among people just a few decades ago are now becoming a rarity, thanks to the effectiveness of smallpox vaccination.

Measles

Measles, or *rubeola*, is essentially a respiratory disease, even though the skin lesions are typical diagnostic symptoms. This infection is caused by one of the smaller viruses for which no active immunizing agent is available at the present time. By employing immune serum or globulin fractions obtained from the blood of adult persons, however, an effective passive transfer of antibodies can be effected to help prevent measles in children under the age of three. These youngsters are more prone to secondary bacterial complications which lead to permanent damage to organs, and even death, and measles can usually be prevented by the prompt injection of globulin fractions immediately after the child has been exposed to an active case of the disease. Children over the age of three years should be allowed to go for from six to ten days after exposure before immune serum or globulin is administered. In this way the disease will be contracted, but the symptoms will be modified by the immune bodies, and the children will build up an active immunity to protect themselves from subsequent attacks by this virus.

German measles, called *rubella*, is milder than "regular" measles, but this disease appears to be particularly serious in cases of pregnant women, especially during the early stages of pregnancy. There are strong indications that the virus of German measles has a particular affinity for the developing fetus, and serious deformities have been reported in such babies if they are not aborted before termination of the full nine-month gestation period.

Chicken Pox

This virus disease, known as *varicella,* produces inclusion bodies in the epithelial skin cells, and elementary bodies can be demonstrated in the fluid of the skin vesicles. The virus has not been successfully cultivated in chick embryos. There is some question as to whether "shingles," caused by *Herpes zoster,* has the same virus etiology as chicken pox.

IMMUNITY TO VIRUS DISEASES

The mode of dissemination of viruses is apparently similar to that for many bacteria: direct contact, droplet infection, and insects. Some virus diseases, such as yellow fever, are exclusively carried by insect vectors.

Immunity to virus infections varies from little or none, through moderate immunity for a number of years, to apparent lifetime immunity to still other viruses. In the case of cold sores the virus appears to remain in the tissues and provide an infection immunity. Occasionally the virus becomes aggressive and attacks skin cells, particularly in the region around the lips.

While most bacterial vaccines are suspensions of dead cells, it is found that viruses inactivated by heat or with chemicals are generally ineffective as vaccines. Any technic which kills the virus appears to alter the antigenicity of the virus protein.

DIAGNOSTIC PROCEDURES

A number of technics are available for aiding in the diagnosis of diseases of virus etiology. An experienced person can look for inclusion bodies in the tissues, but since all viruses do not produce these cell inclusions, the absence of inclusion bodies does not necessarily rule out viruses. Elementary bodies can be sought in fluids from affected tissues. Cultivation of the virus in tissue culture or in embryonated eggs can also be tried.

Some viruses, like those of influenza, have the ability to agglutinate red blood cells of selected animals. By collecting samples of a patient's blood during the acute and during the convalescent

stages of suspected influenza, it is possible to measure differences in antibody titers of the sera by an agglutination-inhibition test. That is, by taking a known concentration of influenza virus which will clump chicken red blood cells to a known titer, and by adding serum from the patient, specific antibodies for influenza will inhibit the agglutination of chicken cells when the mixture of virus and blood serum is tested against the blood cells. A marked increase in inhibition of the convalescent patient's serum over the reaction of the acute serum is indicative that the patient had influenza and has built up specific immune substances against the virus.

CHAPTER 23

Blood Grouping

HISTORICAL REVIEW

Some readers may question the inclusion of a chapter on blood grouping in a book of this nature. Since serological reactions are involved in these blood determinations, and since some courses in introductory microbiology consider this application of the agglutination reaction, a brief discussion will be presented for those who want to use this material.

About 45% of the blood in humans is comprised of cells—*leucocytes* (white cells), *erythrocytes* (red cells), and *platelets* (small discs or cell-like bodies of uncertain function). The remaining liquid portion of the blood is plasma, about nine-tenths of which is water.

An average human adult has about twelve or thirteen pints of blood in his body containing a total of about thirty trillion (30,000,000,000,000) red blood cells. Women have approximately 10% less blood than men, and persons living at high altitudes have a materially higher erythrocyte count than those individuals residing at or near sea level.

During the last decade of the nineteenth century when funda-

mental discoveries in microbiology were taking place in rapid succession, serological procedures were playing an increasingly important role in studies being conducted in the laboratories of that period.

Karl Landsteiner, an Austrian, observed in 1900–1901 that when different human blood sera were mixed with blood cells of selected human beings, the red cells of some persons clumped together, or agglutinated. Other workers had observed this reaction when the cells of one animal species were treated with the serum of a different animal species, but Landsteiner was apparently the first investigator to notice the phenomenon within a given species. The name ISO-AGGLUTINATION was applied to this reaction.

In his study of the first series of twenty-two different blood samples, Landsteiner discovered three distinct groups of blood on the basis of agglutination reactions. From these findings he postulated the presence of two antigens (agglutinogens) and two antibodies (agglutinins) in human bloods. He never found a person harboring an antibody that agglutinated his own red blood cells —a finding which might be expected by logical reasoning.

In 1902 two of his colleagues, von Decastello and Sturli, described a fourth blood group in which both agglutinogens were present in the same cells and the agglutinins were both absent from the serum. Landsteiner had missed this less prevalent type of blood since he had sampled an insufficient number of cases, and none of his twenty-two subjects happened to belong to this rare group.

This work on blood types was confirmed by Hektoen in 1907, and in that same year Jansky offered the first definite classification scheme of the four blood groups which he called, I, II, III, and IV. Two years later Moss independently devised a classification scheme in which groups I and IV of Jansky were reversed, but groups II and III remained the same. Because Moss published his findings in a more accessible publication, his scheme for classifying blood was adopted in preference to that of Jansky who had priority.

It was only natural to expect that confusion would eventually arise between those who read Jansky's article and those who read

the report published by Moss. A UNIVERSAL SYSTEM of blood group designations has since been adopted, and letters of the alphabet are assigned in place of numbers for the various blood groups. The two first letters of the Greek alphabet, *alpha* (for anti-A) and *beta* (for anti-B), are employed to designate the antibodies found in blood sera. To indicate the relationship between the Jansky, Moss, and universal systems of blood grouping, the following table is presented.

TABLE 10

SUMMARY OF BLOOD GROUPING SYSTEMS

JANSKY	MOSS	UNIVERSAL	EFFECT OF ANTISERA ON RED BLOOD CELLS
I	IV	O	Not agglutinated by either anti-A or anti-B serum.
II	II	A	Agglutinated by anti-A serum.
III	III	B	Agglutinated by anti-B serum.
IV	I	AB	Agglutinated by anti-A and anti-B sera.

The discovery of iso-agglutinins in human blood was not put to practical use until during the first world war, when many persons were dying as the result of transfusions with *heterologous* (mixed) blood. With the necessities of war serving as a stimulus, blood grouping came into its own, and the use of donors whose blood was compatible with that of the recipient resulted in successful transfusions. While the distribution of blood types varies with different races and with different locations throughout the world, the distribution in the United States is approximately 45% type O, 40% type A, 10% type B, and 5% type AB. Published figures do not agree exactly, but these percentages are representative.

BLOOD TRANSFUSIONS

In order to understand why transfusions are successful in some cases and disastrous in other patients, it is necessary to appreciate that the donor's blood should not contain erythrocytes for which the recipient possesses specific circulating antibodies. The following table should make this point clear by indicating which blood type persons are able to receive blood from other individuals.

TABLE 11

POSSIBLE RECIPIENTS IN BLOOD TRANSFUSIONS

DONOR'S CELLS (ANTIGEN)	DONOR'S SERUM (ANTIBODIES)	POSSIBLE RECIPIENTS
O	alpha and beta	O, A, B, and AB
A	beta	A and AB
B	alpha	B and AB
AB	neither alpha nor beta	AB

Persons with type O blood cells can serve as donors for all four blood types, since the injected O cells will not be agglutinated by the serum of any of the four blood types. Type O individuals, therefore, are called UNIVERSAL DONORS. Since type AB persons possess neither alpha nor beta antibodies in their blood serum, such individuals can receive blood from any of the four blood groups, and they are designated as UNIVERSAL RECIPIENTS. While it is generally advantageous to transfuse *homologous* (same type) blood into a patient, the practice of employing universal donors has proven safe and effective in tens of thousands of blood transfusions performed both in civilian and in military hospitals.

The question is often asked, is it not dangerous to inject blood containing antibodies for the recipients' cells? The relatively few isoantibodies administered in a transfusion with universal blood are diluted about twelve to one by the rapidly circulating blood of the recipient, and only minor clumping of red cells occurs. Approximately 1 or 2% of individuals administered type O blood will respond with a slight elevation in temperature—a transfusion reaction—but this is not considered serious. It is desirable to avoid these reactions in patients who are critically ill, and that is the reason for injecting homologous blood if it is available.

TYPING OF BLOOD

Preliminary to blood transfusions it is imperative that the blood be typed to determine compatibility. Two relatively simple agglutination procedures are available—the rapid slide test and the test tube technic. To perform the test one must have either A

and B serum, or anti-A and anti-B serum, and the operator must be certain of the type of sera with which he is working. Type A serum is obtained from a person who has type A blood cells, and such serum causes clumping of type B cells. But anti-A serum is produced by injecting animals, usually rabbits, with type A cells, and the injected laboratory animal builds up antibodies which cause agglutination of type A cells. The same principle holds true with type B serum and anti-B serum.

To conduct the slide test, an ordinary microscope slide is marked off with two circles about the size of a five cent piece. The left hand circle is labeled "A" and the right hand circle is marked "B." A fair-sized drop of a saline suspension of blood cells is introduced into each of the circles. Assuming that specific *antisera* are going to be employed in the typing procedure, place a drop of anti-A serum in the left circle and a drop of anti-B serum in the right circle. With the aid of a toothpick, mix the contents of the left circle thoroughly, then turn the toothpick around and mix the contents of circle B.

With a rotary motion of the slide, carefully agitate the cell suspensions for a minute or two making certain that the contents of the two circles are not allowed to come in contact with each other. Visible clumping of the erythrocytes will take place if the antiserum is specific for the cells. By employing such antisera, the following reactions will occur:

Type A blood will be clumped only by anti-A serum (left circle).

Type B blood will be clumped only by anti-B serum (right circle).

Type AB blood will be clumped by both anti-A and anti-B sera (both circles).

Type O blood will not be clumped by either antiserum (neither circle).

Macroscopic readings of these reactions should be confirmed by microscopic examination of the preparations under low power. Weak reactors may be visible only by use of the microscope.

The test tube technic is more accurate than the rapid slide test,

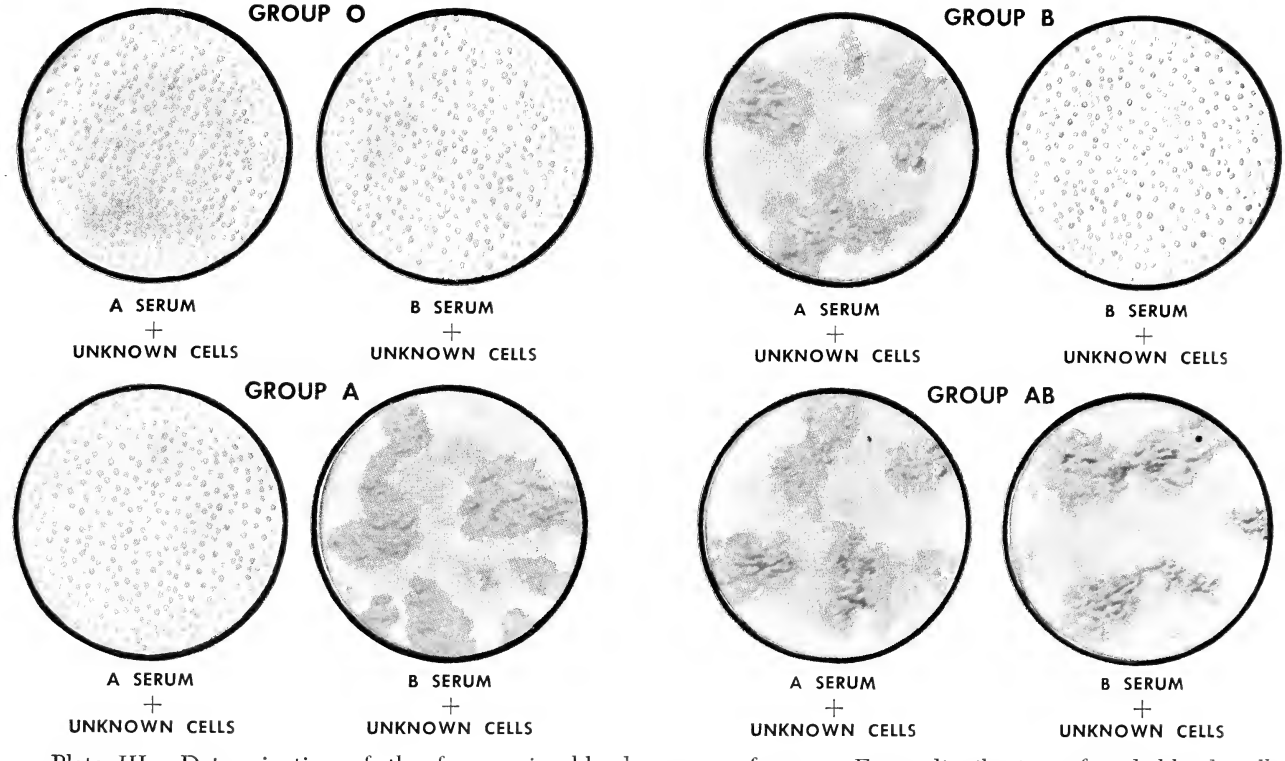


Plate III. Determination of the four major blood groups of man. Even distribution of red blood cells indicates no agglutination; clumping of red cells indicates agglutination. (*From* Seminar, *November, 1946. Courtesy of Sharp and Dohme, Inc., West Point, Pennsylvania.*)

in that the cells and antisera are shaken in the test tube and spun down with the aid of a centrifuge. Clumping of the resuspended cells after spinning down can be detected both macroscopically and microscopically, and some of the weaker reactors will be detected more readily than with the slide method.

CROSS-MATCHING

Having determined the blood type, another important operation before administering blood to a patient is to conduct a cross-match of donor's and recipient's bloods. This precaution will detect bloods that are incompatible for reasons other than differences in O-A-B types, and cross-matching serves as a double check on the accuracy of the original typing of the blood specimen.

Best results are obtained in cross-matching when undiluted samples of blood are employed. A sample of the donor's cells is mixed with the recipient's serum (the so-called *major side* of a cross-match), and a sample of the recipient's cells is mixed with some of the donor's serum (the *minor side*). To be compatible, there must be no agglutination on the major side, and only minor clumping may be permitted on the minor side. It is the injected blood cells that are important in blood transfusions; any clumping by the recipient's serum will lead to severe consequences, and possibly death of the patient. It is not a safe practice to repeatedly administer blood from the same donor to a given patient without cross-matching the bloods each time. Serological changes may have taken place since a previous transfusion, and severe reactions may result.

THE Rh FACTOR

In 1940 Landsteiner and Wiener reported that when the red cells of *Macacus rhesus* monkeys were introduced into rabbits and guinea pigs, antibodies were evoked which caused clumping not only of monkey red cells, but also of the red cells of about 85% of human beings. The antigenic factor in human erythrocytes responsible for this reaction was named *Rh* (for rhesus). This relatively simple concept of Rh-positive and Rh-negative blood

cells was soon complicated by the discovery of a number of subgroups, since the Rh antigen is not a single, homogeneous substance. But Rh-positiveness has come to mean that at least one particular antigen is present in the cells.

Expanded investigations have disclosed that the incidence of the major Rh antigen in Caucasians is close to 85%, with as low as 65% in the Basques of Argentine, and as high as 99% Rh-positives in American Indians, Chinese, and Japanese. Such studies on Rh distribution have added information to that being gathered by anthropologists interested in mass migrations of various peoples.

MULTIPLE TRANSFUSIONS

Rh-positive blood cells are antigenic for Rh-negative individuals, even though their type with respect to the "A" and "B" systems of blood groups may be identical. Introduction of such antigenic cells into an Rh-negative person will stimulate the production of antibodies which will agglutinate and hemolyze Rh-positive cells. It becomes apparent, therefore, that Rh-negative individuals must be transfused only with Rh-negative cells of the correct blood type, or subsequent transfusions with Rh-positive blood may work to the disadvantage of the recipient.

Rh AND PREGNANCY

Rh factors have considerable significance in certain pregnancies. If a woman possesses Rh-negative blood and her husband is Rh-positive, there is a strong possibility that the fetus will inherit the father's dominant Rh-positive factor. Should any of the blood from such a fetus gain entrance to the mother's blood circulation, the Rh-positive cells will serve as an antigen, and the mother will respond by producing antibodies for these Rh-positive cells. It should be remembered that even though the cellular portions of the blood of the fetus are separated from the mother's circulation by the placental barrier, antibodies can pass between mother and fetus. It is by this means that newborn babies are endowed with passive immunity to certain diseases.

It is not well understood how the cells of a fetus find their way

into the mother's circulation, but at the time of labor and delivery some mingling of bloods might occur. If the mother has had a previous transfusion with Rh-positive blood, she will exhibit antibodies. It is especially important, therefore, that Rh-negative females be transfused with only Rh-negative blood from the time of infancy through the child-bearing period.

Most Rh difficulty arises during the second or the third pregnancy when antibodies from the stimulation of the first fetus pass through the placenta and destroy the cells in the developing fetus. If the antibody titer is high, the fetus may die and be expelled before the end of the normal gestation period, but if the titer is relatively low, the child may be born alive and develop *erythroblastosis fetalis,* commonly called *hemolytic jaundice of the newborn.* The hemolytic destruction of the oxygen-carrying blood cells may be severe enough to cause oxygen starvation of the child's brain, with a mentally retarded child being the consequence. If the condition is severe, the child may die unless a complete replacement of blood is undertaken immediately after birth. Type O, Rh-negative blood is given in such transfusions.

From extensive studies involving large numbers of cases it has been concluded that not more than one in ten pregnancies in which Rh conditions are suitable for potential development of hemolytic jaundice of the newborn (that is, an Rh-positive father and an Rh-negative mother), does the condition actually occur. The reasons for this are numerous and involve such considerations as genetic inheritance of recessive characteristics, the amount and the antigenicity of the red cells finding their way into the mother's circulation, the titer of antibody produced by the mother, and many other factors not clearly understood.

QUESTIONS OF DISPUTED PARENTAGE

There are occasions when questions of disputed parentage of a child must be resolved, and the decision usually takes place in a court of law. Infants are sometimes mixed in hospital nurseries, and serological tests aid in deciding which child belongs to which set of parents. Other cases arise in which a man is accused of

being the father of a given child. By applying the principles of serology and correlating the findings with Mendelian laws of inheritance, it is possible to state with a high degree of accuracy that a given man *is not* the father of a given child. It is not yet possible to state that any one person *is* the father of a given child. Since blood types and antigenic fractions found in the blood are inherited according to predictable laws, certain genetic factors cannot arise in the offspring of certain combinations of parents. Serological tests can be employed only as exclusion technics in cases of disputed parentage.

REFERENCES

B

Bausch and Lomb. *The Use and Care of the Microscope*, Bausch and Lomb Optical Company, Rochester, New York, 1936.

Bigger, J. W. *Man Against Microbe*, The Macmillan Company, New York, New York, 1939.

Birkeland, J. *Microbiology and Man*, F. S. Crofts and Company, New York, New York, 1942.

Bitter, R. S., and Williams, O. B. "Enteric organisms from the American cockroach," *Journal of Infectious Diseases*, 1949, **85**, 87.

Bodansky, M. *Introduction to Physiological Chemistry*, 3rd. ed., John Wiley and Sons, Inc., New York, New York, 1934.

Breed, R. S., Murrary, E. G. D., and Hitchens, A. P. *Bergey's Manual of Determinative Bacteriology*, 6th ed., The Williams and Wilkins Company, Baltimore, Maryland, 1948.

Bryan, A. H., and Bryan, C. G. *Principles and Practices of Bacteriology*, 4th. ed., Barnes and Noble, Inc., New York, New York, 1951.

Buchanan, R. E., and Buchanan, E. D. *Bacteriology*, 5th. ed., The Macmillan Company, New York, New York, 1951.

Burdon, K. L. *Textbook of Microbiology*, The Macmillan Company, New York, New York, 1947.

C

Carter, C. F. *Microbiology and Pathology*, C. V. Mosby Company, St. Louis, Missouri, 1948.

Casman, E. P., and Rettger, L. F. "Limitation of bacterial growth at higher temperatures," *Journal of Bacteriology*, 1933, **26**, 77–123.

Clark, W. M. *The Determination of Hydrogen Ions*, 3rd. ed., The Williams and Wilkins Company, Baltimore, Maryland, 1928.

Clifton, C. E. *Introduction to the Bacteria*, McGraw-Hill Book Company, Inc., New York, New York, 1950.

Cohen, B. "On Leeuwenhoek's method of seeing bacteria," *Journal of Bacteriology*, 1937, **34**, 343–346.

Cohen, B. *Chronicles of the Society of American Bacteriologists 1899–1950*, The Williams and Wilkins Company, Baltimore, Maryland, 1950.

Colien, F. E. *Principles of Microbiology*, 2nd. ed., C. V. Mosby Company, St. Louis, Missouri, 1946.

Colien, F. E., and Odegard, E. J. *Principles of Microbiology,* C. V. Mosby Company, St. Louis, Missouri, 1941.

Conn, H. J. "The most abundant groups of bacteria in soil," *Bacteriological Reviews,* 1948, **12,** 257–273.

D

Dack, G. M. *Food Poisoning,* 2nd. ed., The University of Chicago Press, Chicago, Illinois, 1949.

DeKruif, P. *Microbe Hunters,* Harcourt, Brace and Company, New York, New York, 1926.

F

Frobisher, M. *Fundamentals of Bacteriology,* 4th. ed., W. B. Saunders Company, Philadelphia, Pennsylvania, 1949.

——— *Fundamentals of Microbiology,* 5th. ed., W. B. Saunders Company, Philadelphia, Pennsylvania, 1953.

G

Gage, S. H. *The Microscope,* The Comstock Publishing Company, Ithaca, New York, 1920.

Grant, M. P. *Microbiology and Human Progress,* Rinehart and Company, Inc., New York, New York, 1953.

Greaves, J. E., and Greaves, E. O. *Elementary Bacteriology,* 5th. ed., W. B. Saunders Company, Philadelphia, Pennsylvania, 1946.

H

Haberman, S., and Ellsworth, L. D. "Lethal and dissociative effects of X-rays on bacteria," *Journal of Bacteriology,* 1940, **40,** 483–503.

Hawley, J. E., Penner, L. R., Wedberg, S. E., and Kulp, W. L. "The role of the house fly, *Musca domestica,* in the multiplication of certain enteric bacteria," *American Journal of Tropical Medicine,* 1951, **31,** 572–582.

Hegner, R. *Big Fleas Have Little Fleas,* The Williams and Wilkins Company, Baltimore, Maryland, 1938.

Henrici, A. T. *Molds, Yeasts, and Actinomyces,* John Wiley and Sons, Inc., New York, New York, 1930.

Henrici, A. T., and Ordal, E. J. *The Biology of Bacteria,* 3rd. ed., D. C. Heath and Company, New York, New York, 1948.

Hill, J. *Germs and the Man,* G. P. Putnam's Sons, New York, New York, 1940.

Hilliard, C. M. *A Textbook of Bacteriology and its Applications,* Ginn and Company, Boston, Massachusetts, 1945.

Hitchens, A. P., and Leikind, M. C. "The introduction of agar-agar into bacteriology," *Journal of Bacteriology,* 1939, **37,** 485–493.

J

James, L. H., Rettger, L. F., and Thom, C. "Microbial thermogenesis. II. Heat production in moist organic materials with special reference to the part played by microorganisms." *Journal of Bacteriology,* 1928, **15,** 117–141.

Janssen, W. A., and Wedberg, S. E. "The common house roach, *Blattella germanica* Linn., as a potential vector of *Salmonella typhimurium* and *Salmonella typhosa,*" *American Journal of Tropical Medicine and Hygiene,* 1952, **1,** 337–343.

Jordan, E. O. *Food Poisoning and Food-Borne Infection,* The University of Chicago Press, Chicago, Illinois, 1931.

Jordan, E. O., and Burrows, W. *Textbook of Bacteriology,* 14th. ed. revised, W. B. Saunders Company, Philadelphia, Pennsylvania, 1945.

Jung, R. C., and Shaffer, M. F. "Survival of ingested *Salmonella* in the cockroach *Periplaneta americana,*" *American Journal of Tropical Medicine and Hygiene,* 1952, **1,** 990–998.

K

Kelly, F. C., and Hite, K. E. *Microbiology,* Appleton-Century-Crofts, Inc., New York, New York, 1949.

Knaysi, G. *Elements of Bacterial Cytology,* Comstock Publishing Company, Ithaca, New York, 1944.

Koch, R. *The Aetiology of Tuberculosis,* National Tuberculosis Association, New York, New York, 1932. (Translation of Koch's original paper read before the Physiological Society of Berlin in 1882.)

Krueger, W. W. *Principles of Microbiology,* W. B. Saunders Company, Philadelphia, Pennsylvania, 1953.

Kulp, W. L. *Laboratory Manual for the Fundamentals of Microbiology,* 1951. (Unpublished manual used at the University of Connecticut.)

L

Locy, W. A. *Biology and Its Makers,* Henry Holt and Company, New York, New York, 1915.

M

Mackerras, M. J., and Mackerras, I. M. "*Salmonella* infections in Australian cockroaches," *Australian Journal of Science,* 1948, **10,** 115.

Marshall, M. S. *Applied Medical Bacteriology,* Lea and Febiger, Philadelphia, Pennsylvania, 1949.

Marton, L. "The electron microscope. A new tool for bacteriological research," *Journal of Bacteriology,* 1941, **41,** 397–413.

Meier, F. C., and Lindbergh, C. A. "Collecting micro-organisms from the arctic atmosphere," *Scientific Monthly,* 1935, **40,** 5–20.

Metropolitan Life Insurance Company. "A century of progress in longevity," *Statistical Bulletin,* 1949, **30** (10).

——— "Life conservation and life insurance," *Statistical Bulletin,* 1952, **33** (3).

——— "Longevity of industrial population at all-time high," *Statistical Bulletin,* 1952, **33** (9).

Mitchell, D. F., Helman, E. Z., and Chernausek, D. S. "Ammoniated dentifrices and hamster caries.: further studies on the effects of ingestion," *Science,* 1952, **116,** 537–538.

Mudd, S., and Thalhimer, W. *Blood Substitutes and Blood Transfusion,* Charles C. Thomas, Springfield, Illinois, 1942.

N

Neter, E. *Medical Microbiology for Nurses,* F. A. Davis Company, Philadelphia, Pennsylvania, 1949.

Newman, H. H. *Outlines of General Zoology,* The Macmillan Company, New York, New York, 1925.

O

Olson, T. A., and Rueger, M. E. "Experimental transmission of *Salmonella oranienburg* through cockroaches," *Public Health Reports,* 1950, **65,** 531–540.

P

Paget, S. *Pasteur and After Pasteur,* Adam and Charles Black, London, England, 1914.

Porter, J. R. *Bacterial Chemistry and Physiology,* John Wiley and Sons, Inc., New York, New York, 1946.

R

Raffel, S. *Immunity, Hypersensitivity, Serology,* Appleton-Century-Crofts, Inc., New York, New York, 1953.

Rahn, O. *Physiology of Bacteria,* P. Blakiston's Son and Company, Inc., New York, New York, 1932.

Ratcliff, J. D. *Yellow Magic. The Story of Penicillin,* Random House, New York, New York, 1945.

Reed, W. "The etiology of yellow fever," *Philadelphia Medical Journal,* 1900, **6,** 790–796.

Reuhle, G. L. A., and Kulp, W. L. "Germ content of stable air and its effect upon the germ content of milk," *New York Agricultural Experiment Station Bulletin 409,* 1915.

Reyniers, J. A. *Germ Free Life Studies,* University of Notre Dame, Notre Dame, Indiana, 1949.

Rhodes, A. J., and van Rooyen, C. E. *Textbook of Virology,* 2nd. ed., Williams and Wilkins Company, Baltimore, Maryland, 1953.

Rice, T. B., and Coy, F. *Microbiology and Pathology,* The Macmillan Company, New York, New York, 1942.

Ross, R. "On some peculiar pigmented cells found in two mosquitoes fed on malarial blood," *British Medical Journal,* December 18, 1897.

S

Sarles, W. B., Frazier, W. C., Wilson, J. B., and Knight, S. D. *Microbiology,* Harper and Brothers, New York, New York, 1951.

Schaub, I. G., and Foley, M. K. *Methods For Diagnostic Bacteriology,* C. V. Mosby Company, St. Louis, Missouri, 1943.

Scaletti, J. V., Cleverdon, R. C., and Newcomer, E. H. "Orcein stain for bacterial chromatinic material," *Journal of Bacteriology,* 1952, **64,** 183–186.

Shakespeare, W. *Hamlet, Prince of Denmark,* Charles E. Merrill Company, New York, New York, 1915.

Sigerist, H. E. *The Great Doctors,* W. W. Norton and Company, New York, New York, 1933.

Simond, P. L. "La propagation de la peste," *Annales de L'Institute Pasteur,* 1898, **12,** 625.

Smith, T., and Kilbourne, R. E. "Investigations into the nature, causation, and prevention of Texas or southern cattle fever," *U. S. Department of Agriculture Bureau of Animal Industries Bulletin I,* 1893.

Snyder, L. H. *Blood Grouping in Relation to Clinical and Legal Medi-*

cine, The Williams and Wilkins Company, Baltimore, Maryland, 1929.

Society of American Bacteriologists—Directory and Constitution, 1952.

Standard Methods for the Examination of Water and Sewage, 9th. ed., American Public Health Association, New York, New York, 1948.

Stein, G. J., Hilton, K. G., Gelsing, H. P., and Mason, R. P. "Rh and ABO blood group distribution in Japanese and Ethiopians," *Science*, 1953, **117**, 100–102.

Steinhaus, E. A. *Insect Microbiology*, Comstock Publishing Company, Ithaca, New York, 1947.

Swingle, D. B., and Walter, W. G. *General Bacteriology*, 2nd. ed., D. Van Nostrand Company, Inc., New York, New York, 1947.

T

Tanner, F. W., and Tanner, F. W., Jr. *Bacteriology*, 4th. ed., John Wiley and Sons, Inc., New York, New York, 1948.

Thomas, S., and Grainger, T. H. *Bacteria*, The Blakiston Company, New York, New York, 1952.

Thompson, L. R. *Introduction to Microorganisms*, 2nd. ed., W. B. Saunders Company, Philadelphia, Pennsylvania, 1950.

Todd, J. C., and Sanford, A. H. *Clinical Diagnosis by Laboratory Methods*, 11th. ed., W. B. Saunders Company, Philadelphia, Pennsylvania, 1948.

U

Underwood, W. B. *A Textbook of Sterilization*, American Sterilizer Company, Lakeside Press, Chicago, Illinois, 1934.

V

Vallery-Radot, R. *The Life of Pasteur*, Garden City Publishing Company, Garden City, New York, 1923.

W

Waksman, S. A. *Soil Microbiology*, John Wiley and Sons, Inc., New York, New York, 1952.

War Department. *Methods For Laboratory Technicians* (TM 8-227), United States Government Printing Office, Washington, D. C., 1946.

Wedberg, S. E., and Rettger, L. F. "Factors influencing microbial thermogenesis," *Journal of Bacteriology*, 1941, **41**, 725–743.

Witton, C. J. *Microbiology With Applications to Nursing*, McGraw-Hill Book Company, Inc., New York, New York, 1950.

Winslow, C.-E. A. *The Conquest of Epidemic Disease*, Princeton University Press, Princeton, New Jersey, 1944.

Z

Zinsser, H. *Rats, Lice and History*, Blue Ribbon Books, New York, New York, 1935.

Zinsser, H., and Bayne-Jones, S. *A Textbook of Bacteriology*, D. Appleton-Century Company, Inc., New York, New York, 1939.

Zworykin, V. K., and Hillier, J. "Electron microscopy," *Scientific Monthly*, 1944, **59**, 165–179.

Index